Molecular Regulation of Gluconeogenesis by the Nuclear Receptors GR and LXRβ

By

Rucha Patel

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Pharmaceutical Sciences
University of Toronto

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Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
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2015

Abstract

The long-term use of immunosuppressive glucocorticoid (GC) drugs is limited by undesirable side effects including osteoporosis, obesity, and type 2 diabetes. The potent induction of hepatic glucose production by GCs is known to significantly contribute to the development of type 2 diabetes. Understanding the molecular mediators contributing to the metabolic effects of GCs in the liver will provide a basis from which to generate novel therapeutics to treat this disease.

At the cellular level, GCs exert both therapeutic and adverse effects through the activation of the glucocorticoid receptor (GR). Herein, we showed that the gluconeogenic and immune suppressive effects of GC administration can be separated by the liver X receptor β (LXRβ) in mice. Notably, using ChIP assays we demonstrated that either genomic knockdown or antagonism of LXRβ decreases GC-mediated GR recruitment to the GRE of Pepck, a key gluconeogenic gene in mouse liver. This causes decreased expression of Pepck and loss of glucose production following GC-
administration in mouse liver (*Chapter 2 and 3*). We also demonstrated that LXRβ is dispensable for the GC-mediated immune suppression in mice. Gene expression and glucose production studies in mouse primary macrophages and hepatocytes demonstrated that during GC administration, the beneficial effect of either LXRβ knockdown or LXRβ antagonism is cell autonomous.

FGF21 is a hepatokine that regulates whole body insulin sensitivity, and glucose and lipid metabolism. In normal physiology, FGF21 levels are elevated by long term fasting to coordinate the adaptive starvation response (i.e., glucose homeostasis). Using gene expression and ChIP studies we showed that the GC activated-GR directly regulates the starvation hepatokine FGF21 in mouse liver (*Chapter 4*). In conclusion, we have identified novel modulators that contribute to GC-mediated gluconeogenesis.
Acknowledgments

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there for me and helped me any possible way they could. I feel extremely lucky to be surrounded by such supportive and kind people. Words cannot adequately express what they have done for me.
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette Transporter</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-CoA Cholesterol Acyltransferase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ Adenosine monophosphate-Activated Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASC-2</td>
<td>Activating signal cointegrator-2</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
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<tr>
<td>BRG1</td>
<td>Brahma-related gene 1</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT Enhancer Binding Protein</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate response element-binding protein</td>
</tr>
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<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>CD36</td>
<td>Cluster of Differentiation 36</td>
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<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>CMX</td>
<td>Cytomegalovirus-Based</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
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<tr>
<td>COUP-TF</td>
<td>Chicken ovalbumin upstream promoter transcription factor II</td>
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<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase I</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<td>CREB</td>
<td>cAMP Response Element-Binding</td>
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<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
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<td>Threshold cycle</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>Fatty Acid Synthase</td>
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<td>Fetal bovine serum</td>
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<td>Fibroblast Growth Factor 21</td>
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<td>Fat-specific protein 27</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
</tr>
<tr>
<td>G6Pc</td>
<td>Glucose-6-Phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>gAF</td>
<td>Glucocorticoid Receptor Accessory Factor Binding Site</td>
</tr>
<tr>
<td>GBSs</td>
<td>GR Binding Sequences</td>
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<td>Glucocorticoid</td>
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<td>Growth Hormone</td>
</tr>
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<td>GC-Induced Leucine Zipper</td>
</tr>
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<td>GRE</td>
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<td>Glucocorticoid Response Unit</td>
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<td>Glucose Tolerance Test</td>
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<td>Half maximal inhibitory concentration</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>Insulin-like Growth Factor 1</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>ko</td>
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<td>LC/MS/MS</td>
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<td>Low Density Lipoprotein Receptor</td>
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<tr>
<td>MTP</td>
<td>Microsomal Triglyceride Transfer Protein</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor coressor</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-pick C1-like 1</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>p300</td>
<td>E1A binding protein p300</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>pCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-Dependent Protein Kinase-1</td>
</tr>
<tr>
<td>Pepck</td>
<td>Phosphoenolpyruvate Carboxykinase</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGC1α</td>
<td>PPARγ Coactivator 1α</td>
</tr>
<tr>
<td>PH-domain</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5 tri-phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Pltp</td>
<td>Phospholipid Transfer Protein</td>
</tr>
<tr>
<td>Pnl</td>
<td>Pancreatic Lipase</td>
</tr>
<tr>
<td>Pnlrp 2</td>
<td>Pancreatic Lipase-Related Protein</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome Proliferator-Activated Receptor α</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator-Activated Receptor γ</td>
</tr>
<tr>
<td>PTM</td>
<td>posttranslational modification</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein Tyrosine Phosphatase Type 1B</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor-interacting protein 140</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luciferase unit</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Scad</td>
<td>Short-chain acyl-CoA Dehydrogenases</td>
</tr>
<tr>
<td>Sed-1</td>
<td>Stearoyl-CoA Desaturase-1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimeric partner</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoid and thyroid receptors</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>Spt</td>
<td>Serine Palmitoyltransferase</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid Receptor Coactivator</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol Regulatory Element-Binding Protein 1c</td>
</tr>
<tr>
<td>S1AR</td>
<td>Steroidogenic Acute Regulatory Protein</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal Transducer and Activator of Transcription 5</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWI/Sucrose NonFermentable</td>
</tr>
<tr>
<td>TAT</td>
<td>Tyrosine Aminotransferase</td>
</tr>
<tr>
<td>TFIIB</td>
<td>Transcription Factor IIB</td>
</tr>
<tr>
<td>TFIIF</td>
<td>Transcription Factor IIF</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGH</td>
<td>Triacylglycerol Hydrolase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TORC2</td>
<td>CREB Regulated Transcription Coactivator 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TRB3</td>
<td>mammalian homolog of <em>Drosophila tribbles</em></td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activating Sequence</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>YY-1</td>
<td>Yin Yang 1</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction

Some sections of this introductory chapter were published in a recent review:

1.1 Statement of the research problem

Metabolic diseases, including diabetes and obesity, are reaching epidemic levels in developed countries. Type 2 diabetes is an enormous health concern as there are an estimated 347 million people affected by diabetes worldwide (Danaei et al. 2011, WHO 2013). The complex interplay between genetic and environmental factors leads to decreased biological actions of insulin (termed insulin resistance) and resulting in type 2 diabetes (Taylor 2008). Chronic activation of the glucocorticoid receptor (GR) signaling pathway by glucocorticoids (GCs) has been known to cause diabetes and metabolic dysfunction (Long and Lukens 1936, Plotz et al. 1952, Shimomura et al. 1987, Friedman et al. 1997, Andrews et al. 2003, Liu et al. 2005). Patients with Cushing’s syndrome (a rare condition characterized by elevated endogenous GCs) develop abnormal fat distribution, insulin resistance, hyperglycemia and hypertension in 80-90% of cases (Howlett et al. 1985). Both endogenous and synthetic GCs such as, dexamethasone (DEX), betamethasone and prednisone have potent anti-inflammatory and immune suppressive effects. GCs have therefore been widely prescribed over the past 60 years for the treatment of chronic inflammatory diseases, autoimmune diseases, certain types of leukemia, and the prevention of organ transplant rejection (Lewis and Roger A 1940, Schimmer and Parker 2001, 2006, Pasero and Marson 2010, Coutinho and Chapman 2011). However, the long-term use of high dose GC drugs is limited due to undesirable side effects that include osteoporosis, obesity and type 2 diabetes (Rosen and Miner 2005, Coutinho and Chapman 2011). The potent induction of hepatic glucose production by GCs is partially responsible for the development of type 2 diabetes (Rizza et al. 1982, Andrews et al. 2003, Jacobson et al. 2005). Understanding the molecular mediators that are responsible for the metabolic effects of glucocorticoids in the liver will provide a mechanism to generate novel therapeutics to prevent or treat diabetes.

Like GR, Liver X Receptors (LXRs) are ligand activated transcription factors (Mangelsdorf and Evans 1995). Our laboratory has shown that despite having elevated corticosterone in the circulation, Lxra/β/-/- mice have lower plasma glucose levels and
are lean compared to wild-type (WT) controls (Cummins et al. 2006). Studies conducted by our lab found that following synthetic GC (DEX) administration, WT and \textit{Lxra-/-} mice developed hyperglycemia and hepatosteatosis whereas, \textit{Lxrβ-/-} and \textit{Lxra/β-/-} mice were protected against these detrimental effects of GC administration. In contrast, a similar degree of spleen atrophy (a measure of immune suppression) was present in both WT and each of \textit{Lxr-null} mice, suggesting that \textit{Lxrβ-/-} and \textit{Lxra/β-/-} mice were selectively resistant to some of the effects of GC administration. To determine how LXRβ is responsible for enhancing the hepatic gluconeogenic program in response to activated GR signalling, I conducted gene expression as well as liver chromatin immunoprecipitation (ChIP) studies following synthetic glucocorticoid DEX treatment of WT, \textit{Lxra-/-}, \textit{Lxrβ-/-} and \textit{Lxra/β-/-} mice (Chapter 2). Subsequently, I assessed whether pharmacological antagonism of LXRβ could suppress the gluconeogenic side-effects of GCs without affecting their immunosuppressive function by performing studies in primary cells as well as in mice (Chapter 3).

Studies have shown that a number of metabolic hormones including glucocorticoids, insulin, and FGF21 are elevated in mice and humans with metabolic syndrome (Shimomura et al. 1987, Dubuc 1992, Reynolds et al. 2001, Zhang et al. 2008, Mraz et al. 2009, Durovcova et al. 2010, Fisher et al. 2010). Recently, the physiologic regulation of FGF21, a liver derived hormone, has gained the attention due to its potential as a pharmacologic agent for the treatment of diabetes (Kharitonenkov and Shanafelt 2009, Mraz et al. 2009, Gaich et al. 2013). Additionally, endogenous FGF21 has been shown to regulate gluconeogenesis during fasting in mice (Potthoff et al. 2009, Liang et al. 2014). While analyzing microarray data from our laboratory, we discovered that hepatic FGF21 expression was increased in WT mice treated with DEX for 5 days compared to vehicle treated mice. Therefore, I wanted to investigate whether FGF21 was directly regulated by GCs (Chapter 4).
1.2 Metabolic syndrome

Metabolic syndrome is a term used to describe a group of factors that increase the risk of developing diabetes, atherosclerosis and heart disease. According to the current guidelines, a person can be diagnosed with metabolic syndrome if he or she exhibits three of the following metabolic risk factors: (1) high fasting blood glucose, (2) large waistline, (3) high blood pressure, (4) high triglyceride levels, and (5) low HDL cholesterol levels (Center 2011). Metabolic syndrome is becoming more common and approximately 1/4 of the adult American population exhibit some level of metabolic disease (Eckel et al. 2005, Grundy et al. 2005).

Metabolic syndrome originates from a combination of increased energy intake and decreased energy utilization. In normal physiology, circulating levels and actions of anabolic hormones such as insulin and leptin, and actions of catabolic hormones such as glucocorticoids and glucagon act in concert to maintain energy homeostasis. Under conditions where either an endocrine hormone level or the receptor through which the hormone works is dysregulated, metabolism of nutrient macromolecules is disturbed which causes imbalance in energy homeostasis (Eckel et al. 2005). For example, patients with Cushing’s syndrome, a disease characterized by chronic elevation of endogenous glucocorticoids, have increased abdominal obesity, hepatosteatosis and hyperglycemia (Plotz et al. 1952, Rockall et al. 2003).
1.3 Metabolic hormones

1.3.1 Insulin and Type 2 diabetes

Type 2 diabetes is a metabolic disease caused by decreased insulin action and secretion. Increased circulating blood glucose is a hallmark of diabetes (Eckel et al. 2005, Stumvoll et al. 2007). Uncontrolled hyperglycemia in type 2 diabetes may lead to numerous other complications in the long term including: retinopathy, neuropathy, nephropathy, hypertension, atherosclerosis and cardiovascular disease (Stumvoll et al. 2007).

Insulin is synthesised and secreted from pancreatic β-cells in response to elevated blood glucose. In pancreatic β-cells, glucose enters from the bloodstream and is metabolized through the glycolytic pathway generating ATP. ATP blocks ATP-sensitive K+ channels, which depolarize the cell membrane, causing Ca2+ influx and insulin secretion (Guyton 2006). In normal physiology, increased insulin in the circulation stimulates nutrient uptake in skeletal muscle and adipose tissue, and suppresses gluconeogenesis in the liver. Furthermore, insulin also stimulates cell growth and promotes energy storage. In an insulin resistant state, decreased glucose utilization and increased hepatic glucose production cause increased blood glucose levels. Pancreatic β-cells then secrete more insulin as a compensatory response to the elevated blood glucose (Mathis et al. 2001, Guyton 2006, Stumvoll et al. 2007). As the disease progresses, chronically elevated levels of glucose and fatty acids cause β-cells to undergo necrosis (Efanova et al. 1998, Maedler et al. 2001).

Generation of muscle, brain, adipocyte, liver and pancreatic islet-specific insulin receptor (IR) knockout mice have shown that only liver and pancreatic β-cell specific IR knockout animals become diabetic (Bruning et al. 1998, Kulkarni et al. 1999, Michael et al. 2000, Bluher et al. 2003, Stumvoll et al. 2007). This indicates that hepatic insulin resistance is a very crucial factor in the development of diabetes.
1.3.1.1 Insulin signalling cascade

Insulin is an anabolic hormone that promotes glucose utilization in the muscles, liver, and adipocytes, while inhibiting glucose production in the liver, postprandially (Guyton 2006). Insulin signalling begins with the binding of insulin to its plasma membrane receptor (IR), which consists of two α and two β subunits. Activation of the receptor stimulates its tyrosine kinase activity, whereby the IR phosphorylates its β subunit which acts as a docking site for adaptor proteins (Kido et al. 2001). Two principal pathways initiated by insulin are the PI3K-Akt pathway and the RAS-MAPK pathway. Since, metabolic effects of insulin are primarily mediated via the PI3K-Akt pathway, it will be discussed here (Taniguchi et al. 2006).

Insulin receptor substrate (IRS) proteins are the starting point of the PI3K-Akt pathway. IRS1 and IRS2 are important in regulating insulin-stimulated glucose and lipid metabolism. IRS proteins bind to phosphotyrosine-containing regions of the insulin receptor and are themselves phosphorylated by the IR (Wolf et al. 1995, Xu et al. 1995, He et al. 1996). Phosphorylated IRS proteins recruit the SH2-containing phosphoinositide 3-kinase (PI3K) (Myers et al. 1992). PI3K is a heterodimer comprised of regulatory (p85) and catalytic (p110) subunits that generates phosphatidylinositol 3,4,5 tri-phosphate (PIP3) at the plasma membrane (Geering et al. 2007). PIP3, in turn, serves as a membrane bound second messenger molecule that can activate proteins that contain a pleckstrin homology (PH) domain (Shepherd et al. 1998, Kido et al. 2001, Guyton 2006).

A major effector of insulin signalling downstream of PIP3 is Akt (also known as protein kinase B, PKB). Akt is involved in the regulation of many cellular processes, such as growth, apoptosis, angiogenesis and metabolism (Scheid et al. 2002, Manning and Cantley 2007). Akt activation depends on phosphorylation at threonine 308 by phosphoinositide-dependent protein kinase-1 (PDK1) and at serine 473 by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Scheid et al. 2002, Sarbassov et al.
2005). Recent studies indicate that another layer of regulation of Akt activation exists. Notably, Akt is acetylated by p300 at Lys 14 and Lys 20 in its PH domain, which leads to impaired binding of PIP3 and hence reduced Akt activity (Sundaresan et al. 2011). There are three isoforms of Akt (Akt1, Akt2, Akt3) and the ability of insulin to activate each isoform is tissue and species dependent (Kim et al. 2000). In mouse liver, 84% of Akt is Akt2 with the remainder being Akt1. Many of insulin’s downstream effects are mediated by phosphorylation of Akt. In agreement, acute hepatic knockdown of Akt 1 and 2 causes severe glucose intolerance, insulin resistance and augmented hepatic glucose production in mice (Lu et al. 2012).

In the liver, during times of energy deprivation, the transcription factors forkhead box-containing protein of O subfamily 1 (FOXO1), CREB regulated transcription coactivator 2 (CRTC2, also known as TORC2) and peroxisome proliferator-activated receptor γ coactivator protein 1α (PGC1α) upregulate gluconeogenic gene transcription (Herzig et al. 2001, Puigserver et al. 2003, Vegiopoulos and Herzig 2007). Insulin-activated Akt supresses hepatic gluconeogenesis by directly or indirectly phosphorylating these critical transcription factors, which promotes their degradation and dissociation from gluconeogenic gene promoters (Pepck, Phosphoenolpyruvate carboxykinase and G6Pc, Glucose-6-phosphatase) (Nakae et al. 1999, Puigserver et al. 2003, Dentin et al. 2007, Li et al. 2007). Following insulin administration, the degree of phosphorylated Akt in various tissues, including liver and muscle, is frequently measured to assess tissue specific insulin resistance.
1.3.2 Glucocorticoids and metabolic syndrome

1.3.2.1 Physiologic roles of glucocorticoids

Glucocorticoids (cortisol in humans and corticosterone in rodents) are endogenous stress hormones that affect almost every organ in the body, regulating diverse physiological processes including energy homeostasis (metabolism), the immune response, skeletal growth, reproduction, behaviour, cell proliferation and survival (Oakley and Cidlowski 2011). In response to stressful stimuli, the central nervous system increases the release of corticotropin releasing hormone (CRH) from the hypothalamus, leading to the elevated release of adrenocorticotrophic hormone (ACTH) from the pituitary gland which then induces GC production from the adrenal cortex. In normal physiology, stress-triggered GC synthesis and secretion from the adrenal cortex is tightly regulated by feedback inhibition of the hypothalamic-pituitary-adrenal (HPA) axis (Vegiopoulos and Herzig 2007). The essential role of GCs is to supply enough glucose into the circulation to fuel the brain and ensure survival of the organism under conditions of acute stress or reduced food intake and is intended to be for a short term. The mechanisms by which GCs orchestrate this effect include: (i) increased hepatic glucose production (Lewis and Roger A 1940, Vegiopoulos and Herzig 2007), (ii) decreased peripheral glucose uptake into muscle and adipose tissue (Weinstein et al. 1998, Sakoda et al. 2000), (iii) increased breakdown of fat and muscle to provide additional substrates for glucose production (Munck 1971, Odedra et al. 1983, Divertie et al. 1991, Guillaume-Gentil et al. 1993, Hasselgren 1999) and (iv) inhibition of insulin release from β-cells (Delaunay et al. 1997, Lambillotte et al. 1997, Gesina et al. 2004) (Figure 1.1).
Figure 1.1 Metabolic effects of glucocorticoids.

In response to elevated GC levels, liver, pancreas, muscle and adipose act in concert to increase circulating glucose levels to provide the requisite energy to maintain brain function. GCs increase the transcription of genes involved in de novo gluconeogenesis (liver); inhibit insulin secretion from pancreatic β-cells while also increasing protein catabolism (muscle) and lipolysis (adipose) to provide substrates for glucose production by the liver.

GCs are elevated basally in obese diabetic mouse models (ob/ob and db/db mice), and in some patients with insulin-resistance, and correlate with the occurrence of fatty liver and hyperglycemia (Ohshima et al. 1984, Reynolds et al. 2001, Andrew et al. 2002, Livingstone et al. 2009, Rose and Herzig 2013). Adrenalectomy of db/db mice mitigates the diabetic and obese phenotype of this genetic model of disease (Shimomura et al. 1987). Signaling through the GR is central to these phenotypes since administration of the GR antagonist RU-486 attenuates hyperglycemia in db/db mice (Friedman et al. 1997, Liu et al. 2005).
1.3.2.2 Glucocorticoids as therapeutic agents

Pharmacologic concentrations of GCs, such as cortisone acetate, DEX, and prednisone, exert their systemic therapeutic effects on the immune system by inducing anti-inflammatory cytokines, inhibiting pro-inflammatory cytokines, and inducing apoptosis of T-lymphocytes. Thus, these drugs have been widely prescribed over the past 60 years for the treatment of chronic inflammatory conditions, autoimmune diseases, certain types of leukemia and prevention of organ transplant rejection (Oakley and Cidlowski 2011, Oppong et al. 2013). It is estimated that 1.2% of the US population is currently being treated with a prescription GC drug (Overman et al. 2013). Chronic use of high doses of GCs, however, disturbs normal homeostasis and causes deleterious side-effects in humans and rodents including dysregulation of glucose and fat metabolism, hepatosteatosis, insulin resistance, diabetes, osteoporosis, muscle wasting, growth retardation, infertility, cognitive dysfunction, glaucoma, cataracts and topical skin thinning (Lewis and Roger A 1940, Wise et al. 1973, Rizza et al. 1982, Weinstein et al. 1998, Oakley and Cidlowski 2011, Brennan-Speranza et al. 2012, Scerif et al. 2013). In GC-treated rheumatoid arthritis patients (Hoes et al. 2011), primary renal disease patients (Uzu et al. 2007), and lymphoblastic leukemia patients (Gonzalez-Gonzalez et al. 2013) diabetes prevalence ranging between 20-40% is observed.

1.3.2.3 Glucocorticoid-induced insulin resistance

Systemic GC administration causes insulin resistance in skeletal muscle by reducing the expression of IRS-1 and simultaneously inducing the expression of two enzymes that inhibit insulin signalling, protein tyrosine phosphatase type 1B (PTP1B) and p38 MAPK (Almon et al. 2005). Moreover, an increased expression of p38 MAPK is also observed in rat liver after 7-day treatment with the synthetic GC, methylprednisolone (Almon et al. 2007). Notably, an earlier study by Saad et al demonstrated that insulin-mediated hepatic IR and IRS-1 phosphorylation is decreased following 5-day DEX-administration to the rats (Saad et al. 1993). DEX administration also decreases Akt and insulin-activated threonine 308 phosphorylation of Akt in the adipose and muscle of
rats (Buren et al. 2008). Additionally, factors such as adiponectin and osteocalcin, secreted by adipose and bone, respectively, can also promote insulin sensitivity of peripheral tissues. Adiponectin and osteocalcin expression is suppressed by GC administration, which also contributes to the insulin resistance seen with GC-therapy (Mazziotti et al. 2011, Brennan-Speranza et al. 2012, Sukumaran et al. 2012). Moreover, long term GC administration promotes proteolysis, lipolysis and hepatic-lipid accumulation, which all contribute to systemic insulin resistance (Mazziotti et al. 2011).

1.4 FGF21 and metabolic syndrome

Fibroblast growth factor 21 (FGF21) is an atypical member of the fibroblast growth factor family that acts in an endocrine fashion. FGF21 is predominantly secreted by the liver (Nishimura et al. 2000, Kharitonenkov et al. 2005, Badman et al. 2007). Unlike many other family members, FGF21 does not bind to heparin, allowing its circulation in the bloodstream. It exerts a physiologic response via the FGF receptors (FGFR1-4); however, FGF21 requires β-klotho as a co-receptor to achieve activation of the receptor. The limited tissue expression pattern of β-klotho dictates tissue specific FGF21 responsiveness (Goetz et al. 2007, Kharitonenkov et al. 2008, Fisher et al. 2011, Ding et al. 2012). Unlike the classical members of the FGF family, FGF21 does not exhibit mitogenic activity, but acts as an endocrine factor involved in the regulation of energy homeostasis, insulin sensitivity, and glucose and lipid metabolism (Kharitonenkov et al. 2005, Domouzoglou and Maratos-Flier 2011).

Physiologically, FGF21 plays a key role in coordinating the adaptive starvation response. Accordingly, chronically elevated FGF21 levels block somatic growth and sensitize mice to a hibernation-like state of torpor (Inagaki et al. 2007, Kliewer and Mangelsdorf 2010). In lean rodents, FGF21 expression is strongly induced in liver by prolonged fasting or in response to a ketogenic diet through a mechanism that involves the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) (Badman et
al. 2007, Inagaki et al. 2007). FGF21, in turn, induces the transcriptional coactivator protein PGC1α expression to induce hepatic gluconeogenesis, fatty acid oxidation, and ketogenesis (Estall et al. 2009, Potthoff et al. 2009). Recently, Liang et al demonstrated that fasting induced hepatic FGF21 enters into the brain to activate the HPA axis and increase corticosterone secretion, which in turn increases hepatic gluconeogenesis (Liang et al. 2014).

Similar to many other metabolic hormones, FGF21 levels are regulated by circadian rhythm and nutritional status in both rodents and humans (Tong et al. 2010, Wang et al. 2010, Andersen et al. 2011, Yu et al. 2011). A number of studies have demonstrated that PPARα is necessary for the basal expression of FGF21 and pharmacological activation of PPARα further induces FGF21 expression in the liver (Badman et al. 2007, Inagaki et al. 2007, Muise et al. 2008, Oishi et al. 2008). Accordingly, the adaptive starvation response is absent in Ppara-/− mice, and they exhibit severe hypoglycemia and hypoketonemia in response to a 24 hr fast (Kersten et al. 1999). Several other signaling molecules including insulin, glucagon, thyroid hormone, growth hormone and bile acids have also been shown to regulate FGF21 expression (Adams et al. 2010, Berglund et al. 2010, Tong et al. 2010, Chen et al. 2011, Cyphert et al. 2012).

Recent epidemiological studies have shown that circulating FGF21 concentrations were increased in subjects who were overweight, had type 2 diabetes or were glucose intolerant (Zhang et al. 2008, Eto et al. 2010, Yilmaz et al. 2010, Li et al. 2011, Mashili et al. 2011). Similarly, circulating endogenous FGF21 levels are elevated in mouse models of obesity and type 2 diabetes (db/db and ob/ob) (Lundasen et al. 2007, Zhang et al. 2008, Badman et al. 2009, Livingstone et al. 2009, Hale et al. 2012). Paradoxically, in obese diabetic rodents increasing FGF21 in the circulation, by either systemic infusion of the recombinant protein or transgenic expression, counteracts obesity-related metabolic disorders, including hyperglycemia, dyslipidemia, insulin resistance, and fatty liver disease (Kharitonenkov et al. 2005, Domouzoglou and Maratos-Flier 2011). Hence, a clear separation between the effects of endogenous vs pharmacological administration of FGF21, with respect to hepatic metabolism, exists.
1.5 Nuclear hormone receptors

Nuclear receptors (NRs) are a superfamily of ligand activated transcription factors. In humans and mice there are 48 and 49 members of this family, respectively (Mangelsdorf et al. 1995, Sonoda et al. 2008). NRs regulate diverse physiologic processes including energy homeostasis, metabolism, development, reproduction, cell growth, immunity, and inflammation by coordinating the transcription of genes involved in these pathways (Sonoda et al. 2008). The members of this superfamily are defined by the presence of two key domains, the central, highly conserved DNA-binding domain (DBD) of ~70 amino acids that targets the receptors to specific DNA sequences found in the promoters of regulated genes; and, the structurally conserved ligand binding domain (LDB) of approximately 250 amino acids (Evans 1988) (Figure 1.2 A). Small lipophilic compounds such as steroid hormones, dietary lipid derivatives, lipophilic xenobiotics, and cholesterol metabolites act as ligands for NRs (Mangelsdorf et al. 1995). Most NRs bind to specific DNA sequences called, hormone response elements (HRE). The canonical HREs are bipartite elements that are composed of two hexameric nucleotides ‘AGGTCA’ or its variants separated by several nucleotides, arranged in direct, inverted or everted repeats abbreviated DR$_x$, IR$_x$ or ER$_x$, respectively (Mangelsdorf et al. 1995, Sonoda et al. 2008) (Figure 1.2 B). The glucocorticoid receptor (GR) and liver X receptors (LXR$\alpha$ and LXR$\beta$) belong to this superfamily of proteins (Mangelsdorf et al. 1995, Chawla et al. 2001, Sonoda et al. 2008) and their role in metabolism will be discusses in section 1.5.2-1.5.3.

1.5.1.1 Structure

Although NRs differ significantly in their ability to respond to a wide variety of ligands, they share a conventional structure consisting of five or six functional modules called A, B, C, D, E and F (Figure 1.2 A) (Mangelsdorf et al. 1995).
At the amino-terminus, the A/B module consists of the ligand-independent transcriptional activation domain, called AF-1 (activation function-1). The C module represents the DBD, which contains two zinc fingers that are involved in DNA recognition and protein-protein interactions (Evans 1988). The D module acts as a hinge region between the DBD and LBD and imparts structural flexibility to the NR (Chandra et al. 2008). The E/F module is the LBD composed of 10 to 12 alpha-helices (Greschik and Moras 2003).

**Figure 1.2**  **Nuclear hormone receptors share common functional domains**

(A) Schematic representation of a typical nuclear hormone receptor. (B) NR response elements are composed of direct repeats (DRs), inverted repeats (IRs) or everted repeats (ERs) of hexameric nucleotides AGGTCA.

A typical LBD consists of a ligand-binding pocket and an activation function -2 (AF-2) regions at the carboxyl-terminus of the NR, which is responsible for ligand-mediated
changes in the transcriptional output by the NR. Ligand-binding to the NR causes a conformational change in the AF-2 region that creates a hydrophobic groove able to accommodate binding of a LXXLL peptide helix or an extended LXX IXX I/L peptide helix, found on co-activators and co-repressors, respectively (Hart 2002, Mahajan and Samuels 2005, Moore et al. 2006). This primary event leads to the build-up of multi-protein transcriptional machinery. The multi-protein complex can include various co-activators and co-repressors such as, SRC1-3 (NcoA1-3), PGC1α, ASC-2, RIP140, SMRT, NcoR, SHP; co-integrators such as, CBP/ P300 and pCAF; chromatin remodeling complex proteins such as, BRG1 and SWI/SNF that modify nucleosome positioning; and histone modifiers such as, histone acetyl transferases (HATs), histone deacetylases (HDACs), histone demethylases (HDMs) that add or remove acetyl and methyl groups on core histones (Eggert et al. 1995, Onate et al. 1995, Chakravarti et al. 1996, Chen et al. 1996, Kamei et al. 1996, Leers et al. 1998, Windahl et al. 1999, Glass and Rosenfeld 2000, Nissen and Yamamoto 2000, Rogatsky et al. 2002, De Martino et al. 2004, Burkhart et al. 2005, Lin et al. 2005, Liu et al. 2006, Kim et al. 2009). This dynamic multi-protein complex causes condensation or de-condensation of the local chromatin structure and propagates activating or repressive signals from the NR binding site to the basal transcriptional machinery (RNA polymerase II, TFIIB, TFIIF) at the promoter of NR-regulated genes (Glass and Rosenfeld 2000, Quack et al. 2002, Schwabish and Struhl 2007, Jakobsson et al. 2009) (Figure 1.3). In addition, posttranslational modifications (PTM) of NRs likely work in concert with co-regulators that recognize these specific PTMs, to help direct transcriptional output (Galliher-Beckley and Cidlowski 2009, Lee et al. 2009).
Figure 1.3  Transcriptional regulation by nuclear hormone receptors involves multi-protein complexes

(A) Ligand bound nuclear receptor recruits multiple co-activators and histone modifiers that de-condense local chromatin, which signal basal transcription machinery to induce target gene transcription. (B) Ligand bound nuclear receptor recruits multiple co-repressors and histone modifiers that condense local chromatin, which signal basal transcription machinery to repress target gene transcription.
1.5.1.2 Clinical relevance

The NRs regulate, in a ligand-dependent manner, the expression of important genes in many physiological processes such as glucose and lipid homeostasis, development, reproduction and immune function. Therefore, therapeutically targeting NRs to treat or manage many pathophysiological conditions is highly feasible, and indeed, NR targeted drugs represent a large proportion of marketed therapeutics. Currently, small molecule drugs targeting NRs have an estimated market of $50 billion, which makes the NRs the second largest category of druggable proteins (Gronemeyer et al. 2004).

Historically, the majority of the small molecule drugs that modulate NR activity were identified on an empirical basis. For example, cortisone was approved for the treatment of rheumatoid arthritis in 1950, long before the molecular basis of its immune suppressive actions were elucidated (Sprague et al. 1950). It is now widely recognized that cortisone is an endogenous ligand for GR and activation of GR regulates many genes involved in suppressing the inflammatory response (Uhlenhaut et al. 2013, Vandevyver et al. 2013). Currently, synthetic GCs targeting the GR are the most-prescribed anti-inflammatory drugs for treating immune-related conditions (Moore et al. 2006). Other very successful drugs that treat metabolic conditions include fibrates and thiazolidinediones. Fibrates activate PPARα and are widely prescribed as hypolipidemic drugs. Thiazolidinediones activate peroxisome proliferator-activated receptor gamma (PPARγ) and, until recently, were extensively used to manage diabetes (Moore et al. 2006).
1.5.2 Glucocorticoid Receptor (GR)

1.5.2.1 Discovery and biology

In the early 1980s, the glucocorticoid receptor (GR, NR3C1) became the first nuclear hormone receptor cloned, shortly after the availability of its radioactive ligand (Miesfeld et al. 1984, Hollenberg et al. 1985). GR regulates many physiologically important pathways including development, energy homeostasis and immune suppression. Proper functional activity of GR is critical for survival; as whole-body GR knock mice are not viable (Cole et al. 1995), and tissue-specific GR knock out mice exhibit compromised energy homeostasis (Tronche et al. 2004, Rose and Herzig 2013).

Systemically, GCs exert both therapeutic and adverse effects through the activation of GR. In most cell types, GR is primarily located in the cytoplasm in the absence of a ligand (Echeverria et al. 2009). GR exists as an oligomer associated with HSP90, HSP70, p23, and TPR (tetratricopeptide repeat)-containing immunophilins. This heteromeric complex is essential for ligand binding since the chaperones maintain GR in a competent folded state (Pratt et al. 2004). Ligand binding disassociates GR from this large multiprotein chaperone complex. This binding event unmasks the nuclear localization sequence and GR translocates into the nucleus. The detailed mechanism of this translocation is currently unknown but it has been shown to involve dynein motor and importin-β mediated translocation via nuclear pores (Grad and Picard 2007, Echeverria et al. 2009). In the nucleus, GC-GR complex activates or represses transcription of distinct sets of genes either by directly binding (cis-regulation) to specific DNA sequences (GRE, glucocorticoid response elements) as a homodimer; or via protein-tethering (trans-regulation) with other transcription factors bound to DNA (Figure 1.4)(Phuc Le et al. 2005, Kassel and Herrlich 2007, Uhlenhaut et al. 2013).
In the direct DNA binding pathway, ligand-activated GR homodimerizes and binds to GREs located within the regulatory regions of its target genes. In the indirect DNA binding pathway, ligand-activated GR interacts with DNA bound transcription factors, such as STAT 3/5 or NFκB to modulate gene expression.

1.5.2.2 Tissue-specific genome-wide binding of GR

A number of recent genome-wide studies using chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) have elucidated that a large number of GR binding sequences (GBSs) are not present in the typical promoter regions nearby the transcription start site (TSS) of any known genes. The majority of GBSs are actually located within intragenic regions and at distances greater than 25 kb from the TSS (Kuo et al. 2012). ChIP-seq studies performed in mouse liver found that genes activated and repressed by GR are actively remodelled upon GC treatment (i.e., have altered DNase hypersensitivity sites) (Grontved et al. 2013). Additionally, CCAAT/enhancer-binding
protein beta (C/EBPβ) appears to direct GR binding to the liver genome through a highly cooperative mechanism whereby 62% of GR-binding sites are pre-occupied by C/EBPβ prior to GR binding (Grontved et al. 2013).

Importantly, by performing an extensive comparison of genome-wide GR binding sites from mouse liver to previously published GR ChIP-seq data derived from cell lines (i.e., mouse mammary adenocarcinoma (3134) (John et al. 2011), pituitary gland tumour (AtT20) (Chakravarty et al. 2005), preadipocyte embryonic fibroblast (3T3L1) (Steger et al. 2010) and myoblasts (C2C12) (Kuo et al. 2012)), the authors found that only 0.5% of the 11,000 binding sites in liver are shared between the four other cell types and 83% of sites are unique to liver tissue (Grontved et al. 2013). Despite the unique locations of the GR binding sites in different tissues, the consensus GRE that was enriched in each of the above mentioned studies was very similar and consisted of an inverted repeat of 5’A/GGnACA3’ with 3 nucleotide spacer (IR3). Hence, the above mentioned studies reinforce the idea that cell/tissue specific expression of proteins dictates GR binding sites and transcriptional output.

1.5.2.3 Development of safer GR ligands

Glucocorticoid drugs are the most potent anti-inflammatory agents on the market but their long-term use is limited by undesirable metabolic effects including osteoporosis, obesity and type 2 diabetes. Earlier studies have suggested that glucocorticoids mediate their immunosuppressive effects through the differential tethering of GR to individual transcription factors such as NF-kB and c-Jun (Miner and Yamamoto 1992, Nissen and Yamamoto 2000). Based on these earlier gene specific studies, a general well accepted model was that “the trans-repressive effects of glucocorticoids were primarily responsible for the beneficial therapeutic effects on the immune system” (for example, repression of IL-6 (Ray et al. 1991) and IL-8 (Luecke and Yamamoto 2005)) and cis-activation of GR was responsible for the negative side effects of therapeutic glucocorticoids including hyperglycemia (Pepck, G6Pc), muscle catabolism (myostatin,
glutamine synthetase) and osteoporosis (receptor activator of NF-κB ligand) (De Bosscher and Haegeman 2009).

As such, the pharmaceutical industry has been interested in developing “dissociated” glucocorticoid receptor agonists that can separate the trans-repressive activity from the cis-activation activity (Schacke et al. 2002, Schacke et al. 2004). This strategy has provided numerous successful novel steroidal candidates \textit{in vitro} but the effects have not faithfully translated \textit{in vivo}. Complicating the application of the dissociated ligand strategy are genes that are trans-activated by GR but critical for its anti-inflammatory effects such as MAPK phosphatase 1 (MKP-1) and GC-induced leucine zipper (GILZ) (Abraham et al. 2006, De Bosscher and Haegeman 2009, Shipp et al. 2010). Notably, few of the non-steroidal GR modulators : AL-438, CpdA, LGD 5552, BBI 115, Quinol-4-ones, ZK 245186 and ZK 216348 have been shown to render beneficial anti-inflammatory actions \textit{in vivo} (animal) studies (De Bosscher et al. 2010). The selective GR agonist, mapracorat (also known as ZK 245186 and BOL-303242-X), has been shown to attenuate inflammatory conditions, dermatitis and allergic conjunctivitis while rendering lower adverse effect profile \textit{in vivo} compared to classical GCs (Schacke et al. 2004, Schacke et al. 2009, Cavet et al. 2013, Baiula et al. 2014). Notably, mapracorat also induces MAPK phosphatase 1 (MKP-1) via cis-regulation in \textit{in vivo} studies, despite being selectively chosen for its GR trans-repressive actions from \textit{in vitro} assays (Kassel et al. 2001, Vollmer et al. 2012). As such, this drug was evaluated in two clinical trials (NCT01228513, NCT01230125) for the treatment of atopic dermatitis and ocular inflammation. The results of these completed trials are not published (ClinicalTrials.gov).

Additionally, a recent genome-wide study examining the GR cistrome of LPS- induced macrophages following DEX treatment found that GR inhibits the transcription of inflammatory genes via both cis-repression and trans-repression; and simultaneously, GR activates multiple anti-inflammatory genes via both cis-activation and trans-activation (Uhlenhaut et al. 2013). This genome-wide study has shed some light on why
the dissociated ligand strategy was not universally successful at eliciting a potent immunosuppressive response \textit{in vivo}.

1.5.3 Liver X receptors (LXR\(_{\alpha}\) and LXR\(_{\beta}\))

1.5.3.1 Discovery and biology

LXRs were cloned prior to the discovery of their endogenous ligands and were classified as nuclear hormone receptors based on sequence similarity within their DBD compared to other family members. Human and rat LXR\(_{\beta}\) (NR1H2, also known as NER, RIP15, UR and OR-1) were first isolated and cloned by screening cDNA libraries and yeast two-hybrid technology (Shinar et al. 1994, Song et al. 1994, Seol et al. 1995, Teboul et al. 1995). Around the same time LXR\(_{\alpha}\) (NR1H3) was isolated and cloned from a human and rat liver cDNA library via low-stringency screens (Apfel et al. 1994, Willy et al. 1995). Crystal structure analysis of the LBD of the LXRs revealed a canonical \(\alpha\)-helical NR fold (Farnegardh et al. 2003, Svensson et al. 2003). LXR\(_{\alpha}\) is highly expressed in the liver, kidney and intestine, whereas LXR\(_{\beta}\) is ubiquitously expressed (Teboul et al. 1995, Repa and Mangelsdorf 1999, Repa and Mangelsdorf 2000). When compared using real-time quantitative PCR, the expression of mouse LXR\(_{\beta}\) in the liver is approximately 2-fold lower than that of LXR\(_{\alpha}\) (Bookout et al. 2006). Human LXR\(_{\alpha}\) (447 amino acids) and LXR\(_{\beta}\) (460 amino acids) proteins share 77\% sequence homology within their DBD and LBD (Repa and Mangelsdorf 2000). A single conservative amino acid change [Ile 277(LXR\(_{\beta}\))/Val 263 (LXR\(_{\alpha}\))] differentiates the ligand binding pocket for the two LXR subtypes (Hu et al. 2008, Viennois et al. 2011).

According to the conventional model, LXRs are mainly localized in the nucleus and as obligatory heterodimers with the retinoid X receptor (RXR), bind to a specific regulatory sequence, called the LXR response element (LXRE). A typical LXRE consists of direct repeats of the consensus hexameric sequence 5’-AGGTCA-3’ in which the half-sites are spaced by four nucleotides (DR\(_4\) motif) (Song et al. 1994, Willy
generally accepted that, like several other RXR heterodimer receptors, basally
LXR/RXR heterodimers are recruited to the regulatory regions in a multi-protein
complex with co-repressors such as silencing mediator of retinoic acid and thyroid
hormone receptor (SMRT) and nuclear receptor co-repressor (NCoR), and binding of a
ligand to either LXR or RXR causes a conformational change that facilitates the
exchange of the co-repressor complex for a co-activator complex to initiate
transcription of the target genes (Chen and Evans 1995, Horlein et al. 1995) (Figure
1.5). Many LXR target genes are involved in cholesterol metabolism, lipogenesis,
inflammation and gluconeogenesis (Mangelsdorf and Evans 1995, Joseph et al. 2003,
Commerford et al. 2007).

Figure 1.5  Canonical liver X receptor signalling

Unliganded LXR-RXR heterodimers along with co-repressors are bound to the regulatory
sequence (LXRE) within the target genes. Binding of either the LXR and/or RXR ligand to the
receptor causes a conformational change and facilitates exchange of the co-repressor complex
for a co-activator complex and induces the transcription of the target genes.
1.5.3.2 Genome-wide binding of the LXRs

In contrast to the canonical view of basal LXR signaling, recent ChIP-seq studies conducted from mouse livers and macrophages have shown that only a small percentage of LXR-RXR binding sites contains a well-defined cognate DR4 element, and instead these heterodimers bind to rather degenerate DR motif sequences; and the majority of the binding sites are not within a promoter region near any gene (Heinz et al. 2010, Boergesen et al. 2012). Additionally, the liver ChIP-seq study found that the majority of genome-wide LXR binding required ligand activation, since a large number of binding sites (15,782) were identified following LXR agonist treatment compared to the vehicle treatment (3,771). These genome-wide cistrome analyses in liver and macrophages suggest that active chromatin modifications are a prerequisite for LXR-mediated response (Heinz et al. 2010, Boergesen et al. 2012).

1.5.3.3 LXR ligands

After the discovery of LXRs using cDNA cloning techniques, cell-based reporter assays were used to screen organic tissue extracts to look for potential ligands of these orphan receptors. Janoswki et al discovered that oxysterols could activate the RXR/LXR heterodimer (Janowski et al. 1996). Subsequently, oxidative metabolites of cholesterol such as, 22(R)-hydroxycholesterol in the adrenal; 24(S), 25-epoxycholesterol in the liver; 24(S)-hydroxycholesterol in the brain and plasma; 25-hydroxycholesterol and 27-hydroxycholesterol in macrophages and plasma, were identified as endogenous LXR ligands (Repa and Mangelsdorf 2000).

In 2003, the crystal structures of ligand bound LXR sub-types were published and revealed a very flexible ligand binding pocket. (Farnegardh et al. 2003, Svensson et al. 2003, Williams et al. 2003). Farnegardh et al demonstrated that the ligand binding pocket of LXRβ almost doubles from 560–680 A° for T0901317 to 980–1090 A° for GW3965. This study also showed that the LXRβ can orient fundamentally different
ligands in unique directions and still result in activation (Farnegardh et al. 2003). Most of the studies investigating consequences of pharmacological activation of LXR in various disease models have used either T0901317 or GW3965, and these compounds potently activate both isoforms of LXR. A major liability associated with activation of LXR in the liver is the induction of hepatic lipogenesis.

The pharmaceutical industry had been keenly interested in the development of safer LXR ligands, due to the well-established atheroprotective (described below is section 1.5.1) and newly discovered anti-cancer effects of LXR activation (Jakobsson et al. 2012). Recently, partial agonists and tissue and isoform-selective LXR modulators have been characterized to try to overcome the limitations associated with full dual agonists. These compounds include: (1) the LXRβ specific agonist WYE-672 that promoted cholesterol efflux from macrophages without inducing hepatic lipogenesis in Ldlr -/- mice (Hu et al. 2010); (2) the intestine specific agonist GW6340 that promoted reverse cholesterol transport without inducing lipogenesis in mice (Yasuda et al. 2010); (3) LXR-623, which lowered LDL cholesterol in Syrian hamsters and monkeys, and decreased atherosclerotic lesions in Ldlr -/- mice without inducing hepatic lipogenesis (Quinet et al. 2009); and (4) GSK9772, which showed separation of anti-inflammatory and lipogenic activities in human macrophage and liver cell lines (Chao et al. 2008). Notably, a promising atheroprotective LXR modulator, LXR-623, underwent a phase I clinical trial, but it was terminated due to neurological side effects that were observed in healthy humans (Loren et al. 2013).

As mentioned above, developing novel LXR agonists has been an active area of research, but the discovery of potent LXR antagonists has not been characterized until very recently. In an effort to characterize novel partial LXR agonists, Zuercher et al inadvertently discovered GSK2033, the first potent synthetic LXR antagonist in cell based assays (Zuercher et al. 2010). An ester-modified derivative of GSK2033 termed SR9238 was recently shown to potently suppress hepatic lipogenesis, inflammation, and hepatic lipid accumulation, in a mouse model of non-alcoholic hepatosteatosis (Griffett
et al. 2013). I used GSK2033 for the pharmacological LXR antagonist and DEX co-treatment studies shown in Chapter 3 of this thesis.

1.6 Metabolic pathways regulated by GR and LXRs

1.6.1 Role of LXRs in cholesterol homeostasis

The LXRαβ–/– mice by removing the DBD of the receptors from these gene loci (Peet et al. 1998, Repa et al. 2000, Alberti et al. 2001). LXR-null mice are viable and do not exhibit detrimental abnormalities when fed normal chow diet, and are resistant to obesity when challenged with high fat high cholesterol diet (Peet et al. 1998, Repa et al. 2000, Alberti et al. 2001, Kalaany et al. 2005).

Bile acid synthesis is a major pathway for cholesterol catabolism and excretion in mammals. The rate-limiting enzyme in the classical pathway of bile acid synthesis is cholesterol 7α-hydroxylase (CYP7A1). Dietary cholesterol modulates CYP7A1 expression via activation of LXRα in mice. When LXR-null mice are challenged with a high cholesterol diet the Lxra−/− and Lxra/β−/−-mice accumulated hepatic cholesterol esters which lead to significant liver dysfunction, whereas Lxrb−/− mice were protected from the gross liver anomalies caused by a high cholesterol diet (Peet et al. 1998, Alberti et al. 2001). These studies suggested that LXRα is an essential regulator of cholesterol homeostasis and no other physiological mechanism exists to compensate for its loss.

Cholesterol can be transported by high density lipoproteins (HDL) and low density lipoproteins (LDL). LDL is responsible for the delivery of cholesterol to the peripheral
organs and tissues, whereas, HDL removes excess cholesterol from the peripheral organs and macrophages, and delivers it to the liver where it can be eliminated through metabolism and secretion into the bile. A number of retrospective human studies have shown that elevated LDL levels and low HDL levels are independent risk factors for coronary heart disease (Gordon et al. 1977, Martin et al. 1986, Brunner et al. 1987, Grundy 1995). LXR activation via synthetic ligands increases HDL-cholesterol in the circulation. In contrast, LXR-null mice have a lower level of HDL compared to the WT mice following a high fat high cholesterol diet. (Schultz et al. 2000, Cao et al. 2002, Miao et al. 2004, Kalaany et al. 2005). Furthermore, utilizing mice injected with macrophages loaded with tritiated cholesterol tracer, Naik et al showed that LXR agonist GW3965 treatment for 10 days significantly increases fecal excretion of the cholesterol tracer and enhanced the production of tritiated bile acids. This in-vivo study clearly demonstrated that pharmacological activation of LXRs induces reverse cholesterol transport to the liver for subsequent excretion and metabolism in vivo (Naik et al. 2006). Activated LXR-RXR heterodimers directly bind to the regulatory regions of ATP binding cassette (ABC) transporter genes (Abca1, Abcg1, Abcg5, and Abcg8), phospholipid transfer protein (Pltp), and several apolipoproteins (the structural components of circulating lipoproteins) including ApoE, ApoCI/II/IV and ApoD to induce their expression, which promotes the removal of excess cholesterol from the body (Costet et al. 2000, Repa et al. 2000, Venkateswaran et al. 2000, Laffitte et al. 2001, Mak et al. 2002, Mak et al. 2002, Repa et al. 2002, Hummasti et al. 2004, Sabol et al. 2005). Additionally, LXR agonist treatment down-regulates the expression of an important intestinal cholesterol absorption transporter, NPC1L1 (Niemann-pick C1-like 1) in both mouse intestine and human enterocytes (Duval et al. 2006). These LXR-mediated effects prevent the formation of lipid laden macrophages in blood vessels and thus, are athero-protective.

In agreement, LXR agonist (GW3965) treatment for 12 weeks reduced atherosclerotic lesion formation by approximately 50% in Ldlr/- and ApoE/- mice (murine models of atherosclerosis) (Joseph et al. 2002). LXR dual agonist-treatment studies in Lxra/- mice demonstrated that the activation of LXRβ can induce reverse cholesterol transport
and increase HDL-cholesterol in mice without changing plasma triglyceride levels (Lund et al. 2006, Quinet et al. 2006). In complementary studies, administration of GW3965 for 11 weeks to Lxra-/- apoE-/- double-knockout mice significantly reduced atherosclerosis (Bradley et al. 2007). These studies provide proof-of-concept validation that LXRβ-specific ligands may be beneficial for the treatment of atherosclerosis.

The above mentioned studies indicate that LXRs control cholesterol homeostasis in the body by inhibiting cholesterol uptake in the intestine, promoting reverse cholesterol transport back to the liver and enhancing bile acid synthesis.

### 1.6.2 Role of LXRβs in lipogenesis

It should be noted that activation of LXR either by dietary cholesterol or synthetic compound, T0901317 and GW3965, induces lipogenesis in the liver, adipose tissue and pancreas (Repa et al. 2000, Schultz et al. 2000, Laffitte et al. 2003, Efanov et al. 2004). SREBP1-c (sterol regulatory element-binding protein-1c) is a master regulator of fatty acid synthesis and its activation leads to increased triglyceride synthesis in tissues. Activated LXR-RXR heterodimers bind to the regulatory regions within lipogenic genes, Srebp-1c, acetyl-CoA carboxylase (Acc), fatty acid synthase (Fas) and stearoyl-CoA desaturase-1 (Scd-1) to induce hepatic expression of these genes in mice (Repa et al. 2000, Schultz et al. 2000, Joseph et al. 2002, Wang et al. 2004, Talukdar and Hillgartner 2006). Notably, activation of LXRβ in Lxra-/- mice fails to increase the expression of hepatic lipogenic genes or plasma triglyceride levels in mice (Quinet et al. 2006). In addition, the basal expression of Srebp-1c, Acc, Fas and Scd-1 genes are decreased in Lxra-/- and Lxra/β-/- mice compared to WT mice, whereas, Lxrβ-/- mice retain normal expression of these lipogenic genes. These data suggest that LXRα is the dominant regulator of hepatic lipogenesis in vivo (Peet et al. 1998, Repa et al. 2000, Schultz et al. 2000, Alberti et al. 2001, Kalaany et al. 2005, Quinet et al. 2006, Korach-Andre et al. 2010).
Carbohydrate response element-binding protein (ChREBP) is a glucose sensitive transcription factor that induces conversion of excess carbohydrates to lipids in the liver. Glucose-mediated activation of ChREBP induces the expression of liver-type pyruvate kinase (L-PK) (Kawaguchi et al. 2001). This enzyme provides carbon sources necessary for de novo lipogenesis in the liver (Towle 2001). Cha et al have shown that ligand activated LXR-RXR heterodimers directly upregulate ChREBP mRNA expression. Conversely, the mRNA expression of ChREBP and its target gene L-PK is significantly attenuated in the Lxra/β-/- mice livers following a 10 days T0901317 treatment (Cha and Repa 2007). However, Denechaud et al demonstrated using Lxra/β/-/ - mice that the nuclear accumulation of ChREBP protein and activation of its target gene L-PK in response to a high carbohydrate diet was not dependent on LXR expression (Denechaud et al. 2008).

A number of studies have demonstrated that short-term treatment with an LXR agonist increases plasma triglyceride levels in mice (Schultz et al. 2000, Grefhorst et al. 2002, Beyer et al. 2004, Quinet et al. 2006). Grefhorst et al demonstrated this is a result of increased secretion of triglyceride-rich, large VLDL particles from the liver. The authors found that following LXR agonist treatment, the increase in mean particle size was identical to the increase in VLDL-TG production; suggesting the number of VLDL particles produced does not change but their diameter increases due to the augmented amount of triglycerides per particle (Grefhorst et al. 2002). The hyper-triglyceridemic actions of LXR agonists are likely the result of increased lipogenesis, but may also be partially mediated by Pltp, which promotes VLDL secretion from the liver (Okazaki et al. 2010). Activation of LXRs has been shown to directly up-regulate the expression and activity of Pltp in WT mice (Zhang et al. 2001, Cao et al. 2002, Laffitte et al. 2003).

Lipogenesis is a detrimental side effect that goes against the beneficial effects of LXR activation in reverse cholesterol transport. However, lipogenesis may actually be an important component of a complex defence mechanism to protect cells from elevated intracellular cholesterol, since it promotes the conversion of extremely toxic free
cholesterol to less toxic cholesterol esters within a cell. Indeed, monounsaturated fatty acids produced by SCD-1 enzyme are the preferred substrates for the cholesterol esterifying enzyme acyl-CoA: cholesterol acyltransferase (ACAT) (Miyazaki et al. 2000).

### 1.6.3 Role of GR in hepatic lipogenesis

Elevated GC levels modulate genes involved in lipolysis and triglyceride (TG) synthesis in a tissue specific manner. GCs acts in the liver to increase fatty acid synthesis, decrease fatty acid oxidation and increase VLDL secretion (Cole et al. 1982). The effects of GCs in the liver are thought to be partially responsible for hypertriglyceridemia and hepatic steatosis seen in Cushing’s patients (Taskinen et al. 1983, Rockall et al. 2003, Shibli-Rahhal et al. 2006).

GC dosing studies performed in normal and streptozotocin-treated rats, perfused livers, and isolated primary hepatocytes, have shown that GCs require the presence of insulin to exert their full lipogenic potential (Altman et al. 1951, Wendel 1954, Diamant and Shafrir 1975, Kirk et al. 1976, Mangiapane and Brindley 1986, Cai et al. 2011). In vivo administration of GCs increases circulating insulin levels as well as, induces the hepatic expression of Acc1, Acc2 and Fas; key enzymes that control de novo lipogenesis (Berdanier 1989, Cai et al. 2009). A ChIP-seq study performed in 3T3-L1 cells found GR binding to the mouse Acc1, Acc2 and Fas genes; however, the functionality of these binding sites has not yet been validated (Yu et al. 2010).

Dolinsky et al observed that treatment of mice with DEX caused accumulation of TGs in the liver by reducing TG lipolysis and increasing TG synthesis (Dolinsky et al. 2004). By blocking in vivo VLDL metabolism, the authors showed that DEX treatment for 4 days had no effect on hepatic VLDL secretion rates in vivo or in isolated primary hepatocytes. In contrast, hepatocyte turnover was decreased by 50%, in hepatocytes isolated from DEX-treated mice compared to vehicle treatment. They also observed that
the proportion of secreted TG derived from *de novo* sources is increased with DEX, while utilization of stored TG for secretion was reduced (Dolinsky et al. 2004). Triacylglycerol hydrolase (TGH, also known as Ces3) is a lipase that hydrolysates intracellular TG within hepatocytes prior to incorporation into VLDL. Although GR does not regulate *Tgh* transcriptionally, *Tgh* mRNA stability is decreased by DEX treatment (Dolinsky et al. 2004).

A study by Lemke et al showed that hepatic overexpression of Hes1 (hairy inducer of split-1) completely protects mice from liver steatosis caused by GR activation. GC-treatment represses the expression of *Hes1*, a transcriptional repressor, in the liver. Adenoviral-mediated overexpression of Hes1 in the liver induces the expression of pancreatic lipase (Pnl) and pancreatic lipase-related protein (Pnlrp 2) in mice treated with DEX for 3 weeks (Lemke et al. 2008). Hepatic Pnl and Pnlrp2 promote TG hydrolysis, fatty acid oxidation and ketogenesis (Inagaki et al. 2007). Moreover, liver specific knockdown of GR in *db/db* mice induces Hes1 expression and improves hepatic steatosis. These data suggest that GC-mediated downregulation of *Hes1* causes decreased expression of Pnl and Pnlrp2, which leads to hepatosteatosis. Furthermore, the authors showed that *Hes1* is a direct target of GR using ChIP and promoter reporter studies (Lemke et al. 2008). This study suggests that *Hes1* is a GR primary target gene that regulates hepatic TG accumulation by inducing genes involved in TG hydrolysis. A recent study using DEX-treated *Hes1* liver specific knockout mice found that Hes1 plays a more global role as a master repressor that must first be downregulated for GR to have its effects on carbohydrate metabolism (Revollo et al. 2013).

MED1, a component of the mediator cofactor complex, co-activates a number of NRs including GR via its interaction with their LBD (Chen and Roeder 2007, Chen and Roeder 2011). In *Med1/-* mouse embryonic fibroblasts, DEX-induced IRF8 expression is attenuated, whereas DEX-induced GILZ expression is maintained compared to WT cells, suggesting that MED1 is selectively required for transcriptional activation of GR (Chen and Roeder 2007). Moreover, liver-specific *Med1/-* mice are protected from DEX-induced hepatosteatosis compared to WT mice. Hepatic expression of fatty acid β-
oxidation enzymes and medium-and short-chain acyl-CoA dehydrogenases (Mcad and Scad) are sharply decreased following 3 days of DEX administration in WT mice. In contrast, hepatic expression of these enzymes in Med1-liver specific knockout mice is not affected by DEX treatment (Jia et al. 2009). Currently, it is not clear whether the expression of Mcad and Scad is directly regulated by GR, as functional GR binding sites in the promoters of the Mcad or Scad genes have not yet been identified.

### 1.6.4 Role of GR in gluconeogenesis

Hepatic glucose production by gluconeogenesis is the primary source of glucose for survival during stress and prolonged fasting. Augmented hepatic glucose production is a significant contributor to hyperglycemia in diabetic individuals. Hepatic and whole-body glucose metabolism is tightly controlled by a number of endocrine factors including insulin, epinephrine, glucagon, and glucocorticoids. The generation of liver-specific GR knockout mice (Liv.GR ko) revealed that GR is a major player contributing to hepatic gluconeogenesis, since 50% of Liv.GR ko die within 2 days after birth due to hypoglycemia, and adult Liv.GR ko exhibit fasting hypoglycemia. Moreover, hyperglycemia is diminished in Liv.GR ko following streptozotocin-induced diabetes (Opherk et al. 2004).

Several metabolic genes regulated by GR in the liver include Pepck, G6Pc, insulin like growth factor binding protein, and tyrosine aminotransferase (TAT). The Pepck and G6Pc enzymes catalyze, respectively, the first committed step and final step in hepatic glucose production (Hers and Hue 1983). Pepck is highly regulated at a transcriptional level by complex hormonal and dietary stimuli. Under normal conditions, after a meal, the pancreas senses the increase in circulating glucose, and enhances insulin secretion from the β-cells. At the level of the liver, increased circulating insulin inhibits Pepck transcription through Akt-mediated phosphorylation of FOXO1, inactivating this transcription factor which results in the suppression of hepatic glucose production.
Conversely, Pepck expression is induced in response to fasting through the actions of increased circulating glucagon and GC levels (Chakravarty et al. 2005).

### 1.6.5 Molecular regulation of the *Pepck* promoter

Functional GR binding sites have been characterized in the promoter regions of Pepck and G6Pc (Short et al. 1986, Imai et al. 1990, Vander Kooi et al. 2005). Pepck and its regulation have been extensively studied due to its importance in hepatic glucose output during fasting and in diabetes. A recent ChIP-seq study on human, mouse, dog, short-tailed opossum, and chicken livers demonstrated that the *Pepck* promoter regulatory regions are highly conserved among these vertebrates (Puigserver and Spiegelman 2003, Rhee et al. 2003, Schmidt et al. 2010). Multiple interactions of regulatory factors and co-activators have been characterized within the first 1.5 kb upstream of the transcription start site on the rat *Pepck* promoter (Chakravarty et al. 2005) (Figure 1.6). The increase in Pepck gene expression by GCs is mediated through the complex glucocorticoid response unit (GRU). The GRU is composed of two low affinity, non-consensus GR binding sites (GRE1 and GRE2), a cAMP response element (CRE) binding site, three adjacent glucocorticoid receptor accessory factor binding sites (gAF1, gAF2, gAF3) and two distal accessory binding sites (dAF1, dAF2) (Imai et al. 1990, Imai et al. 1993, Hall et al. 1995, Scott et al. 1996, Scott et al. 1998, Sugiyama et al. 1998, Cassuto et al. 2005). The low affinity GR binding sites GRE1 and GRE2 are at -387 to -374 and at -367 to -353, respectively, in the rat *Pepck* promoter where the ligand activated GR homodimers bind (Imai et al. 1990, Imai et al. 1993).

A consensus CRE is located at -100 bp relative to the TSS of the rat *Pepck* promoter. CREB (CRE binding protein) regulates cAMP-dependent as well as basal *Pepck* promoter reporter activity (Short et al. 1986, Roesler et al. 1989, Park et al. 1993). Importantly, this CRE site has been described as an essential accessory site for maximal GR-mediated induction of the rat-*Pepck* promoter following DEX treatment, as deletion of this site from a WT rat-*Pepck* promoter reporter construct decreases its activation by
The accessory factors HNF4α, COUP-TF, PPARγ2 and RXRα/RAR heterodimers bind to gAF1 at -451 to -439 in the rat Pepck promoter (Hall et al. 1995, Scott et al. 1996, Scott et al. 1998). The accessory factors HNF-3β, PPARα and FOXO1 bind to gAF2 at -416 to -407 in the rat Pepck promoter (Sugiyama et al. 1998, Cassuto et al. 2005). The accessory factors COUP-TF and RXRα/RAR heterodimers bind to gAF3 at -337 to -321 in the rat Pepck promoter (Sugiyama et al. 1998). Additionally, Cassuto et al. (2005) have found that HNF4α binds to dAF1 at -993 and FOXO1 and PPARα bind to dAF2 at -1365 in the rat Pepck promoter (Cassuto et al. 2005).

**Figure 1.6 Depiction of the glucocorticoid response unit (GRU) in the Pepck gene promoter**

The location of regulatory elements in the rat Pepck gene promoter (−1500 to +73 bp) and the transcription factors known to bind to these sites are shown. The glucocorticoid regulatory unit (GRU) consists of the AF1, AF2, dAF1, and dAF2 accessory factor binding sites. CRE, cAMP regulatory element; GRE, GR response element.

Mutation in any one of the gAF1, gAF2 or gAF3 regions of the Pepck promoter impairs its accessory factor binding and reduces GC-induced Pepck transcription by 50-60% in rat hepatoma H4IIE cells (Imai et al. 1993, Scott et al. 1998). Moreover, any
combination of two mutations in the gAF1, gAF2 or gAF3 abolishes the GC response of the promoter reporter construct (Scott et al. 1998, Wang et al. 2000). Additionally, using labelled oligonucleotides and real-time fluorescence anisotropy measurements of protein-DNA interactions in nuclear extracts, Stafford et al demonstrated that the GR homodimer binds to the Pepck non-consensus GREs (GRE1 and GRE2) poorly compared to the consensus GRE and, inclusion of the gAF1 and gAF2 sites within the labelled oligonucleotides slowed the dissociation rate of GR from the Pepck GREs (Stafford et al. 2001).

Moreover, to assess the position dependent functions of gAF1 and gAF2 within the GRU, as well as determine the important regulatory regions of its corresponding accessory factors, HNF-4α and HNF-3β, Wang et al carried out co-transfection promoter reporter assays. WT or mutated Pepck promoter plasmids that either swapped or replaced the gAF1 or gAF2 elements along with various deletion constructs of the HNF-4α and HNF-3β receptors were co-transfected. These experiments showed that gAF1 and gAF2 were not interchangeable, because the exchange of these elements within the Pepck GRU caused a 30-50% reduction of the GC response. They also showed that the conserved carboxyl-terminus of HNF-4α (amino acids 128-374) and HNF-3β (amino acids 361-458) are necessary of the activation of gAF1 and gAF2, respectively (Wang et al. 1999).

The above mentioned studies demonstrate that activation of the Pepck promoter by GCs requires complex interactions between GR and accessory factors at various elements within the GRU to ensure maximal activation.

Several co-activators are also involved in modulating Pepck transactivation in response to GC administration including, SRC1 (steroid receptor co-activator p160 family member), p300, CBP, p300/CBP/cointegrator-associated protein (p/CIP) and PGC1α (Friedman et al. 1997, Yoon et al. 2001, Puigserver and Spiegelman 2003, Herzog et al. 2004, Wang et al. 2004, Hall et al. 2007).
1.6.5.1 SRC-1

The co-activator SRC-1 has intrinsic histone acetyltransferase activity. It directly interacts with a number of nuclear hormone receptors to stimulate hormone-dependent transcriptional activity. GR interacts with several nuclear hormone receptors within the GRU, causing a synergistic activation of the Pepck promoter following GC administration. In fact, accessory factors HNF-4α, COUP-TF and HNF-3β interact independently with SRC-1, which may contribute to the synergistic activation of the Pepck gene. However, following GC treatment, SRC-1-mediated activation of the Pepck promoter reporter construct requires GR, which could be explained by direct protein-protein interactions between SRC-1 and GR (Stafford et al. 2001, Kucera et al. 2002). In agreement with these in vitro data, SRC1-null mice have reduced hepatic glucose output following overnight fasting (Louet et al. 2010).

1.6.5.2 p300/CBP

The proteins p300 and CBP are two closely related family members of coactivators with intrinsic HAT activity. Chromatin modifying enzymes are responsible for maintaining the Pepck promoter in an open conformation thereby allowing transcription factors to access chromatin. Insulin strongly represses the induction of Pepck, in part, through the loss of p300/CBP, resulting in chromatin condensation (Hall et al. 2007). In agreement, RNAi–mediated knock down of either CBP or p300 in a rat hepatic cell line strongly diminished Pepck promoter activity, demonstrating the essential role of HATs for Pepck gene regulation (Wang et al. 2004).
1.6.5.3 PGC1α

PGC1α is an important co-activator regulating the expression of gluconeogenic genes in the liver. In agreement, obese diabetic mouse models (ob/ob and db/db mice) have constitutively elevated levels of hepatic PGC1α (Yoon et al. 2001). Conversely, RNAi-mediated hepatic knockdown of PGC1α improves fasting glycemia and glucose tolerance in db/db mice (Koo et al. 2004). In normal physiology, the expression of hepatic PGC1α and Pepck is potently induced during fasting and following GC treatment. Importantly, hepatic Pepck expression is attenuated in fasted liver-specific Pgc1α knockout mice and in WT mice that have RNAi-mediated knockdown of PGC1α in their livers (Koo et al. 2004, Handschin et al. 2005).

PGC1α has been shown to directly interact and co-activate multiple gluconeogenic transcription factors including GR, HNF4α, CREB and FOXO1 in the liver (Yoon et al. 2001, Puigserver et al. 2003). PGC-1α does not exhibit HAT activity itself, however, following docking to transcription factors, it provides a platform for the recruitment of additional co-activators possessing HAT activity, such as SRC-1, CBP, and p300. The final outcome is acetylation of histone proteins to increases the accessibility of DNA for the transcription initiation complex (Knutti and Kralli 2001, Puigserver and Spiegelman 2003). GCs also directly induce PGC1α expression in hepatocytes via a GRE on its promoter (Lemberger et al. 1994).

Taken together, the above mentioned studies indicate that the dynamic interactions of various co-activators within the GRU are essential for synergistic control of Pepck transcription in response to nutritional and hormonal stimuli.
1.6.6 Additional regulators of glucocorticoid-induced gluconeogenesis

In a unique mechanism, Nadar et al recently found that phosphorylation of GR at serine 232 by p38 MAPK can discharge p300 and the SWF/SNF chromatin remodelling complex component SNF2 from gluconeogenic GR bound promoters in rat liver (Nader et al. 2010). Since AMP-activated kinase (AMPK) activates p38 MAPK, this provides a mechanism by which the organism ‘senses’ its low energy status and turns-off ATP-consuming metabolic programs including gluconeogenesis (Kahn et al. 2005). Indeed, when rats were treated with the AMPK activator AICAR, DEX-induced hepatic steatosis and gluconeogenesis were attenuated (Nader et al. 2010).

Several transcription factors have been shown to regulate GR levels directly. Farnesoid X receptor (FXR, NR1H4) is a ligand activated transcription factor that responds to endogenous bile acids. Recently, Renga et al found that FXR activation up-regulates GR, Pepck and G6Pc expression, only when the mice were in the fasted state. In the fed state, FXR activation results in a decrease of GR, Pepck and G6Pc expression, likely through the mechanism of induction of the small heterodimer partner (SHP), a nuclear receptor repressor regulated by FXR (Lu et al. 2000, Renga et al. 2012). The authors further describe a distal FXR binding site (ER8) on the human GR promoter and show that activation of FXR is necessary to up-regulate GR transcription in response to bile acids. They have also shown, following fasting, Fxr-/- mice exhibit hypoglycemia and have decreased expression of GR, Pepck and G6Pc compared to WT mice. Moreover, decreased expression of GR in Fxr-/- mice protected them from hyperglycemia caused by 4 days of DEX treatment (Renga et al. 2012). Similarly, Lu et al found that Yin Yang 1 (YY-1), a TF involved in cell proliferation and differentiation, also regulates hepatic gluconeogenesis. YY-1 expression is induced in mice during fasting by cAMP/PKA/CREB signalling and in the state of insulin resistance (in db/db mice). Under these conditions, YY-1 along with the SRC-1 co-activator complex is recruited to the promoter of GR to induce its expression resulting in increased GR signalling and increased hepatic gluconeogenesis (Lu et al. 2013).
Altering the nuclear translocation of GR is another mechanism of changing the GC/GR liver response. Inhibition of histone deacetylase 6 (HDAC6) activity results in hyperacetylation of HSP90 and impaired chaperone-dependent activation of GR (Kovacs et al. 2005, Murphy et al. 2005). Recently, Winkler et al found that Hdac6-/-mice are protected from DEX-induced glucose intolerance, insulin resistance and hyperglycemia due to decreased nuclear translocation of GR. They further showed that HDAC6 inhibition by tubacin had no effect on DEX-mediated suppression of inflammatory gene expression in LPS-induced human THP-1 monocytes, suggesting that this may be a new mechanism to dissociate the positive and negative actions of GR (Winkler et al. 2012).

It is well recognized that catabolic hormones, such as GCs, oppose the actions of insulin in the liver. It is therefore not surprising that GCs modulate the expression of proteins and lipids that inhibit the insulin signaling pathway and contribute to GC-induced hepatic glucose production. For example, GCs have been shown to increase the expression of Trb3, a mammalian homolog of Drosophila tribbles, in hepatocytes (Du et al. 2003, Stayrook et al. 2005). Du et al found that TRB3 functions as a negative modulator of insulin signalling where it binds directly to AKT, preventing its phosphorylation and activation in diabetic db/db mouse livers, in turn promoting hyperglycemia and glucose intolerance (Du et al. 2003). In a second example, Holland et al showed that DEX treatment increases ceramide synthesis and accumulation in the liver by inducing the expression of ceramide synthetic genes including serine palmitoyltransferase (Spt), the rate-limiting enzyme in ceramide synthesis. Reducing ceramide levels by myriocin (SPT- inhibitor) pre-treatment protects mice from developing DEX-induced insulin resistance and glucose intolerance. In addition, mice that are heterozygous for dihydroceramide desaturase-1 (Des1), an enzyme that converts inactive dihydroceramide into active ceramide, are protected from DEX induced insulin resistance (Holland et al. 2007). Together these studies highlight new mechanisms regulated by GCs that impact hepatic gluconeogenesis and insulin resistance.
1.6.7 Role of LXRαs in glucose homeostasis

Short-term treatment of obese diabetic rodent models (db/db mice, ob/ob mice, fa/fa rats, and high-fat diet-induced obese mice and rats) with either T0901317 or GW3965 have shown improved glucose tolerance, decreased plasma glucose levels, and reduced hepatic glucose output compared to vehicle-treated mice. These beneficial effects of pan-LXR agonist treatment were attributed to the down-regulation of the gluconeogenic genes, Pepck, G6Pc, Pgc1α, Hsd11β1(11β hydroxysteroiddehydrogenase type1) and GR in the liver and the induction of the glucose uptake transporter Glut-4 in adipocytes (Cao et al. 2003, Laffitte et al. 2003, Grefhorst et al. 2005, Liu et al. 2006, Commerford et al. 2007). Utilizing Lxrα-/- and Lxrβ-/- mice, Commerford et al demonstrated that LXRα was responsible for mediating the anti-diabetic effect of LXR agonist-treatment by suppressing hepatic gluconeogenesis (Commerford et al. 2007). Mechanistically, using ChIP and gel shift techniques, Herzog et al found that treatment of cells with T0901317 results in the recruitment of LXRα and the co-repressor RIP140 to the endogenous Pepck promoter at the gAF3 region to repress Pepck transcription (Herzog et al. 2007).

It was recently shown that treatment of human hepatoma (HEPG2) cells with LXR agonist also suppressed GC-mediated Pepck and G6pc expression (Nader et al. 2012). In agreement, dosing rats with the LXR ligand GW3965 for three days attenuated the increase in plasma glucose that is observed after a single dose of DEX (Nader et al. 2012). Using gel shift and ChIP experiments, the authors demonstrated that these effects were mediated by LXRα, as LXRα/RXRα heterodimers competed with GR to bind to the GRE of the rat G6Pc gene (Nader et al. 2012).

The role of LXRβ in hepatic glucose metabolism was not apparent prior to the studies presented in this thesis. In the context of glucose metabolism, glucose tolerance tests on WT and Lxrβ-/- mice and glucose stimulated insulin secretion studies on pancreatic islets isolated from WT and Lxrβ-/- mice found that LXRβ was necessary for glucose-
stimulated insulin secretion (Gerin et al. 2005, Zitzer et al. 2006, Gabbi et al. 2008). A recent genetic study has shown that an LXRβ SNP (rs17373080) is associated with type 2 diabetes and obesity in humans; however, the functional consequence of this SNP has not yet been clarified (Solaas et al. 2010).
1.7 Rationale and Hypotheses

There are many genetic and environmental factors (i.e., food intake, lifestyle and prescription drugs) that lead to insulin resistance and type 2 diabetes (Taylor 2008). Endogenous GCs (cortisol) and exogenous GC drugs are among these factors. Patients with chronic stress or Cushing’s syndrome have increased risk of developing type 2 diabetes and central obesity. Likewise, while GC drugs are widely prescribed for their potent anti-inflammatory and immunosuppressive actions, the long-term use of GC drugs is limited by undesirable side effects including osteoporosis, obesity, and type 2 diabetes. The potent induction of hepatic glucose production by GCs is known to contribute to the development of type 2 diabetes. Understanding the molecular mediators contributing to the metabolic effects of GCs in the liver will provide a basis from which to generate novel therapeutics to treat this disease.

Like GR, LXRα and LXRβ are ligand activated transcription factors. As mentioned in the introduction GR and LXRs regulate overlapping pathways to maintain energy homeostasis. It has previously been shown by our laboratory that Lxrα/β-/- mice, despite having elevated corticosterone in the circulation (Cummins et al. 2006), have lower plasma glucose levels and are lean compared to WT controls. Studies conducted in our lab found that following synthetic GC administration (DEX treatment), WT and Lxrα-/- mice developed hyperglycemia and hepatosteatosis whereas, Lxrβ-/- and Lxrα/β-/- mice were protected against these detrimental effects of GC administration. In contrast, a similar degree of spleen atrophy (a measure of immune suppression) was present in both WT and each of Lxr-null mice, suggesting that Lxrβ-/- and Lxrα/β-/- mice were selectively resistant to some of the effects of GC administration. At the time that this was discovered, little was known of the role of LXRβ in the liver or in glucose homeostasis. In our DEX-induced insulin resistance model it was not clear how Lxrβ-/- mice were protected, as GCs are not LXR ligands and do not activate LXRs. Moreover, neither GR nor LXRβ regulated each other’s expression basally or after DEX administration. Thus, the molecular mechanism governing pathway selective inhibition of GR activity in Lxrβ-/- mice was unclear. I hypothesized that LXRβ was an essential
positive regulator of GC-induced gluconeogenesis (acting either directly or indirectly) through the regulation of a key gluconeogenic gene (Pepck), and conversely, that pharmacological antagonism of LXRβ would result in selective GC resistance as seen in the Lxrβ-/- mice.

Studies have shown that a number of metabolic hormones including GCs, insulin, and FGF21 are elevated in mice and humans with metabolic syndrome (Shimomura et al. 1987, Dubuc 1992, Reynolds et al. 2001, Zhang et al. 2008, Mraz et al. 2009, Durovcova et al. 2010, Fisher et al. 2010). Recently, physiologic regulation of FGF21 has gained attention from the scientific community due to its potential as a pharmacologic agent for the treatment of diabetes (Kharitonenkov and Shanafelt 2009, Mraz et al. 2009, Gaich et al. 2013). Early fasting hormones such as glucagon and growth hormone have been shown to regulate FGF21 expression (Tong et al. 2010, Chen et al. 2011, Yu et al. 2012). GCs and FGF21 are both endogenous catabolic hormones. The strikingly similar rise in serum cortisol and FGF21 during early morning in humans, and activation of hepatic gluconeogenic genes by both GC and FGF21 administration suggested that the FGF21 and GC signaling pathways were interconnected. I hypothesized that GCs were directly regulating FGF21 expression.
1.8 Objectives

To test the hypotheses listed above, I had the following objectives:

1. To identify the molecular mechanism controlling GC-dependent hepatic glucose production by the nuclear receptor LXRβ. (Chapter 2)
2. To assess if pharmacological antagonism of LXRβ could suppress the gluconeogenic side-effects of GCs without affecting their immunosuppressive function (mimic genomic knockout of LXRβ). (Chapter 3)
3. To investigate if FGF21, the liver synthesized starvation hormone, is transcriptionally regulated by GR. (Chapter 4)
Chapter 2  LXRβ is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice

This chapter was published in an original research article.

Author contributions:

Rucha Patel: Animal study in Fig 1 A. Generated data in Fig 4 C-D, Fig 6 A-C, Fig 7C, Supp Fig 1 and Supp Fig 3. Developed a novel method for ChIP and established primary hepatocytes and macrophage culture in the laboratory. Actively involved, in data analysis and interpretations, significantly contributed to the discussion and response to reviews.

Monika Patel: Contributed to Fig 4A, Fig 5D. Participated in animal studies and tissue collection.

Ricky Tsai: Performed western blots shown in Fig 4A and Fig 5D. Contributed to glucose tolerance studies presented in Supp Fig 1.

Vicky Lin: Assisted with WT and Lxrα/β-/- OGTT and ITT studies shown in Fig 3 C-E.

Angie L. Bookout, Yuan Zhang and Tingting Li: Assisted with tissue collection and sample preparation.

Lilia Magomedova: Performed LXRα, LXRβ and GR co-immunoprecipitation studies in transiently transfected HEK cells (Supp Fig 2.d).

Jessica F. Chan: validated QPCR primers for gene expression analysis, and performed differential white blood cell count shown in Fig 7B

Conrad Budd: Developed mass spectrometry assay to measure plasma corticosterone levels shown in Fig 1A.

David J. Mangelsdorf: Provided access to LXR-null mice and edited the manuscript.

Carolyn L. Cummins: Conceived all of the studies presented in this manuscript and performed most of the in-vivo treatment studies.
2.1 Introduction

Glucocorticoids (GCs) and their synthetic analogs are among the most widely prescribed drugs in the world (2006). GC drugs have profound anti-inflammatory and immunosuppressive properties that are critical for the treatment of rheumatoid arthritis, cerebral edema, allergic reactions, asthma, and certain types of cancer. They are also employed as potent immunosuppressants to prevent organ transplant rejection and graft-versus-host disease (Schimmer and Parker 2001). Unfortunately, the development of major metabolic side-effects remains the key limitation for the long-term therapeutic use of GCs. Common side effects requiring dosage adjustment or cessation of treatment include diabetes, hypertension, osteoporosis and muscle wasting (Rosen and Miner 2005).

Glucocorticoids were first recognized as important determinants in diabetes when it was found that adrenalectomy of diabetic animals decreased hyperglycemia (Long and Lukens 1936). Since then, there have been numerous reports linking elevated GCs with the metabolic syndrome, obesity, and insulin resistance (Levitt et al. 2000, Duclos et al. 2001, Rask et al. 2001, Andrew et al. 2002, Vegiopoulos and Herzig 2007). Patients with Cushing’s syndrome (a rare condition characterized by elevated endogenous GCs) develop an abnormal fat distribution, insulin resistance, hyperglycemia and hypertension in 80-90% of cases (Howlett et al. 1985). Fatty liver (hepatic steatosis) has also been characterized in Cushing’s patients (Shibli-Rahhal et al. 2006) and several studies have found that hepatic steatosis is an independent risk factor for the development of insulin resistance (Marchesini et al. 1999, Seppala-Lindroos et al. 2002, Nguyen-Duy et al. 2003, Kotronen et al. 2008).

The role of endogenous glucocorticoids is to supply the body with enough glucose to survive under conditions of acute stress or reduced glucose intake. The physiologic response to stress is mediated by the release of cortisol (in humans) or corticosterone (in rodents) into the bloodstream. The increase in glucocorticoid hormone then acts on
multiple metabolic tissues via its receptor to increase circulating glucose levels. The mechanisms by which GCs achieve this effect are multifactorial and involve: i) increased hepatic glucose production (gluconeogenesis) (McMahon et al. 1988) ii) decreased peripheral glucose uptake into muscle and adipose (Weinstein et al. 1998, Sakoda et al. 2000) iii) breakdown of muscle and fat to provide additional substrates for glucose production (Divertie et al. 1991, Hasselgren 1999) and iv) inhibition of insulin release from pancreatic β-cells (Delaunay et al. 1997, Vigiopoulos and Herzig 2007). The stress response is intended to be of short duration to reset the balance of plasma glucose. If prolonged GC exposure is present (as with therapeutic use of GCs or in Cushing’s syndrome), insulin secretion will increase to compensate for the excess glucose and ultimately result in severe insulin resistance and metabolic dysfunction.

The glucocorticoid receptor (GR) and liver X receptors LXRα (NR1H3) and LXRβ (NR1H2) are members of the nuclear receptor superfamily of transcription factors that regulate distinct but overlapping transcriptional programs (Kalaany and Mangelsdorf 2006, Gross and Cidlowski 2008). GR and LXRβ are expressed at relatively high levels throughout the body, whereas LXRα expression is highest in liver, kidney, intestine, adipose, and adrenal gland (Bookout et al. 2006). GCs act by binding to GR in the cytoplasm causing translocation of the ligand-bound receptor to the nucleus. There, GR homodimerizes and activates the carbohydrate metabolic pathway through the direct binding and activation of GR response elements in key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6Pc) (Hanson and Reshef 1997, van Schaftingen and Gerin 2002). In addition, activation of GR represses the expression of several genes involved in inflammation (e.g. IL-1, TNF-α, IL-6, COX-2), a function which accounts for the widespread therapeutic use of synthetic GCs. The endogenous ligands of LXRs are oxidative metabolites of cholesterol, also called oxysterols. As such, the LXRs are known for their important role in modulating whole-body cholesterol homeostasis by acting as sensors of the intracellular cholesterol load (Janowski et al. 1996, Janowski et al. 1999). Upon activation, LXR increases the expression of an array of genes involved in cholesterol efflux (Costet et al. 2000, Venkateswaran et al. 2000, Repa et al. 2002), cholesterol
metabolism (Lehmann et al. 1997, Peet et al. 1998) and fatty acid synthesis (Repa et al. 2000). Interestingly, like GR, LXR s have potent anti-inflammatory actions that are due to repression of a number of overlapping target genes (Ogawa et al. 2005). However, in contrast to GR, activation of LXRα has been shown to have beneficial effects in diabetic rodent models by improving glucose control (Cao et al. 2003, Laffitte et al. 2003, Zelcer and Tontonoz 2006). This improvement has been attributed to inhibition of hepatic Pepck (Cao et al. 2003, Laffitte et al. 2003), G6Pc (Laffitte et al. 2003, Grefhorst et al. 2005), and 11β-hydroxysteroid dehydrogenase (Stulnig et al. 2002); and increased skeletal GLUT4 expression (Dalen et al. 2003, Laffitte et al. 2003) collectively resulting in decreased circulating glucose.

Taken together, the studies above highlight striking parallels between the genes regulated by GR and LXR. Recently, we showed that circulating GCs (corticosterone) are elevated in Lxrα/β-/--mice (Cummins et al. 2006). This hypercorticism correlated with a basal derepression of the LXRα-target gene StAR (steroidogenic acute regulatory protein), which is rate-limiting for glucocorticoid synthesis from cholesterol. Intriguingly, despite having higher circulating GCs, the Lxrα/β-/--mice did not exhibit hallmarks of GC excess, such as obesity and insulin resistance. In this study, we investigated whether there was a functional requirement for LXR in eliciting the metabolic effects of exogenous GC administration. Here, we demonstrate that LXRβ is required for the development of GC-mediated hyperglycemia and hepatic steatosis, but not GC-mediated anti-inflammatory effects.
2.2 Materials and Methods

2.2.1 Materials

Dexamethasone and dexamethasone-21-acetate were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade from Caledon Laboratories (Georgetown, ON).

2.2.2 Animal experiments

WT, *Lxrα−/−, Lxrβ−/−* and *Lxrα/β−/−* mice (Peet et al. 1998, Repa et al. 2000) were maintained on a mixed strain background (C57Bl/6:129SvEv) and housed in a temperature and light-controlled environment. All mice used in the studies were male aged-matched between 3 and 8 mo as indicated. The wildtype mice used in these studies were littermates derived from the original *Lxrα/β−/−* crosses. For all the experiments described herein the mice were bred homozygously changing breeder pairs every 4 mo to avoid fertility problems in the *Lxrα/β−/−* mice. Mice were fed *ad libitum* the 2016S or 2018S diet from Harlan Teklad (Madison, WI). To minimize the production of endogenous glucocorticoids from environmental stress, mice were sacrificed in the fed state (unless otherwise indicated) within 1 min of initial handling by decapitation and within 2 h after the start of the light cycle. Animals were treated with dexamethasone (DEX) or dexamethasone-21-acetate at 2.5 mg/kg in sesame oil (vehicle) twice daily for 5-7 days (s.c.) or 14 days (i.p.). From the different dosing regimens it was determined that s.c. injections of DEX yielded higher intra-liver dexamethasone levels compared to i.p. injections. There were only minor or insignificant changes in body weight after DEX treatment (Supplementary Table 2.1). All animals were maintained according to the recommendations of the Faculty Advisory Committee on Animals at the University of Toronto (Toronto, ON) and the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center (Dallas, TX).
2.2.3 Plasma analyses

Trunk blood was collected in EDTA-tubes on ice and plasma was stored at -80°C. Insulin and corticosterone levels were measured by RIA (Millipore, Billerica, MA). Blood glucose was measured using a handheld glucometer (FreeStyle, Abbott) or after separation of plasma using the enzymatic kit from Wako (Richmond, VA). Free fatty acids were measured from plasma using an enzymatic assay (Wako).

2.2.4 Corticosterone and dexamethasone LC/MS/MS analysis

Plasma corticosterone (Figure 2.1A only) and liver dexamethasone levels were measured by LC/MS/MS. Plasma samples (100 µL) were spiked with internal standard and precipitated with four volumes of acetonitrile. After centrifugation, the supernatant was washed with 100 µL of saturated KCl and 100 µL of water. The glucocorticoids were extracted in four volumes of 6:4 MTBE:CH₂Cl₂ and evaporated to dryness. Samples were resuspended in 250 µL of methanol for analysis. To the ~200 mg of liver (same piece that was used for triglyceride analysis) 84 ng of the internal standard triamcinolone acetonide was added prior to homogenization. After washing and measurement of cholesterol and triglyceride levels, as described above, the remaining sample was dried under N₂ and resuspended in 500 µL of methanol for analysis. Ten µL was then injected onto the LC/MS/MS triple quadrupole instrument (Agilent Technologies, Mississauga, ON) running in ESI positive ion mode. Samples were loaded onto a Zorbax C18 column (4.6 × 50 mm, 5 µm, Agilent) and run using a mobile phase of methanol (A) and water (B), both containing 5 mM ammonium acetate increasing from 20% A to 60% A over 12 min then flushing with 100% A for 2 min before returning to initial conditions. Positive ions [M+H]⁺ for corticosterone (m/z 347→329, RT 10.4 min), dexamethasone (m/z 393→373, RT 10.1 min) and triamcinolone acetonide (m/z 435→415, RT 10.9 min) were monitored in multiple reaction monitoring mode.
2.2.5 Glucose and insulin tolerance tests

For the oral glucose tolerance test, mice were fasted 4 h before receiving an oral gavage of 20% D-glucose (Sigma) (2 g/kg body weight). At 0, 15, 30, 60, and 120 min after injection, 20 μl of blood was collected from the tail into an EDTA coated microvette tube. After centrifugation at 1500g for 20 min the plasma was stored at -80°C until glucose and insulin analysis. For the insulin tolerance test, mice were fasted for 4 h and given an i.p. injection of human insulin (0.75 U/kg, Sigma). At 0, 15, 30, 60 and 90 min blood glucose was sampled from a tail nick as described above.

2.2.6 RNA isolation, cDNA synthesis and real-time QPCR analysis

Total RNA was extracted from tissues using RNA STAT-60 (Tel-Test, Inc.), treated with DNase I (RNase-free, Roche), and reverse transcribed into cDNA with random hexamers using the High Capacity Reverse transcription system (Applied Biosystems, ABI). Primers used are shown in Table 2.1 and were validated as previously described (Bookout et al. 2006). Real-time QPCR reactions were performed on an ABI 7900 in 384-well plates containing 12.5 ng cDNA, 150 nM of each primer, and 5 μl 2X SYBR Green PCR Master Mix (ABI) in a 10 μl total volume. Relative mRNA levels were calculated using the comparative Ct method normalized to cyclophilin mRNA.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbrev.</th>
<th>Accession#</th>
<th>Forward and Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36 (fatty acid translocase)</td>
<td>CD36</td>
<td>NM_007643.3</td>
<td>5’ggaactgtgggtgctatgc3’ 5’catgagaatgtctcacaac3’</td>
</tr>
<tr>
<td>Carnitine Palmitoyltransferase I</td>
<td>CPT1</td>
<td>NM_013495.1</td>
<td>5’tgattggctcctctgg3’ 5’cagcagtagctcatactca3’</td>
</tr>
<tr>
<td>Cyclophilin B</td>
<td>cyclophilin</td>
<td>NM_011149.2</td>
<td>5’ggatggccagaggaa3’ 5’gccgtagtgctccgtct3’</td>
</tr>
<tr>
<td>Fat specific gene 27 (CIDEC)</td>
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### 2.2.7 Primary hepatocyte isolation

Mouse primary hepatocytes were isolated by collagenase perfusion and purified by centrifugation as described (Horton et al. 1999). Freshly prepared hepatocytes were seeded at a final density of 0.5x10^6 cells per well onto type I collagen coated 6-well plates in attachment medium (William’s E medium, 10% charcoal stripped FBS, 1X Penicillin/Streptomycin and 10 nM insulin). Medium was exchanged 3 h after plating and all the experiments were performed on the second day. Ligands were added to the cells in M199 medium without FBS and cells were harvested 16 h after ligand treatment for RNA extraction.

### 2.2.8 Western blot analysis

Protein extracts were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4°C with primary polyclonal antibodies against Akt (Cell Signaling Technologies, 1:1000), P-Akt (Ser 473) (Cell Signaling
Technologies; 1:1000), GR (M-20, Santa Cruz Biotechnology, Inc. 1:200), or lamin B1 (Abcam, 1:1000), followed by a 1 h incubation with a peroxidase-conjugated anti-rabbit IgG (1:2000). Peroxidase activity was measured using ECL Plus (GE Healthcare) and visualized using the Storm phosphoimager (GE Healthcare). Quantitation was performed using ImageQuant from GE Healthcare.

2.2.9 Chromatin immunoprecipitation

ChIP was carried out using the EZ ChIP kit (Millipore). Livers were perfused in situ for 30 min via the portal vein with either vehicle or 10 nM DEX. Whole liver was minced and cross-linked in 1% formaldehyde containing PBS, 1 mM DTT and 1 mM PMSF for 10 min at RT. Cross-linking was stopped by addition of glycine to final concentration of 125 mM for 5 min at RT, followed by centrifugation and washing the pellet twice in ice-cold PBS containing 1mM DTT and protease inhibitors (Complete, Roche). Liver nuclei were recovered by dounce homogenization in a hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2% Nonidet P-40, 0.2 mM sodium orthovanadate, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose, 1 mM DTT and protease inhibitors) and layered onto a cushion buffer (10 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, 1 mM DTT and protease inhibitor) followed by centrifugation. The nuclei pellet was washed with cold PBS and resuspended in 2 mL sonication buffer (0.75% SDS, 2 mM EDTA and 50 mM Tris-HCl, pH 8.0). The chromatin was sheared to 200-1000 bp by sonication. The sonicated chromatin was diluted 7.5 fold in dilution buffer (Millipore), 800 µL of diluted sample per immunoprecipitation was used. After 1 h pre-clearing with protein G-agarose beads (100 µL/IP), 10 µg of GR (M-20), SRC-1 (C-19) or C/EBPβ (M-341) antibody (Santa Cruz Biotechnology, Inc) was added for overnight incubation. Protein G agarose (60 µL) was used to recover the immune complexes (2 h at 4°C). Washes and elutions were performed in accordance with the ChIP kit. DNA was reverse-crosslinked overnight at 65°C, RNase treated for 30 min at 37°C, proteinase K treated for 2 h at 45°C and purified using a spin column to a final volume of 50 µL. The eluate was diluted 5-fold with water and QPCR was performed.
using 5 μL of template DNA with the following primers: PEPCKpr GRE (0kb) F, tgcagccacacaatagaa, R, tgatgcaactcagctct, -3kb PEPCKpr F, tggagacacacactttatcca, R, gtcctcttatagaacctcagaca and TAT GRE (-2.5kb) F, cgcaaacaagagaagctaa, R, catgacacccaaagtcttc. Quantitation was performed by QPCR (standard curve method) using serial dilutions of the input as standards.

2.2.10 Histology

Oil red O staining of liver sections was performed from formalin fixed and sucrose protected tissues. Cryosectioning and staining with oil red O was performed by the University of Texas Southwestern Medical Center Histology Core (Dallas, TX).

2.2.11 Liver triglycerides

Lipids were extracted from liver (0.2 g) in chloroform:methanol (2:1, v/v) using the Folch method (Folch et al. 1957). Extracts were washed once in 50 mM NaCl and twice in 0.36 M CaCl$_2$/methanol. The organic phase was separated and brought up to 5 mL final volume with chloroform. Dried aliquots of standards and samples were re-dissolved in 10 μL of 1:1 chloroform:TritonX-100 and evaporated overnight. Samples were assayed for triglycerides using a commercial colorimetric assay (Thermo).

2.2.12 White blood cell differential

Blood (20 μL) was thinly smeared on a glass slide, air-dried and dipped in Hemastain (Hema 3 stain set, Fisher). A differential count was then performed under brightfield microscopy on 100 white blood cells.
2.2.13 Thioglycolate-elicited peritoneal macrophages

Macrophages were collected by peritoneal lavage using 10 mL of PBS 4 days after i.p. injection of mice with 2 mL of 3% thioglycolate. Isolated cells were washed in PBS and pelleted at 1700xg. Cells were plated to a density of 2 x 10^6 cells/well in 6-well plate in DMEM + 10% FBS. After allowing the macrophages to adhere overnight, pre-treatment with vehicle or 100 nM DEX was initiated in DMEM containing 10% charcoal stripped-FBS. After 6 h, wells were spiked with vehicle or LPS to a final concentration of 10 ng/mL and left an additional 18 h before harvesting for RNA and QPCR.

2.2.14 Statistical analyses

For comparison between two groups the unpaired Student’s t-test was performed. One-way ANOVA followed by the Student-Newman-Keuls test was used to compare more than two groups. P<0.05 was considered significant. All tests were performed using the software program Primer of Biostatistics (McGraw Hill, New York, NY).
2.3 Results

2.3.1 \textit{Lxrα/β-/-} mice exhibit hypercorticosteronemia without Cushing’s Disease symptoms.

We previously reported that \textit{Lxrα/β-/-} mice develop adrenomegaly and have two-fold higher basal morning corticosterone levels in plasma compared to WT mice (Cummins et al. 2006). These basal differences persist throughout the circadian cycle because corticosterone levels remained 2-fold higher in \textit{Lxrα/β-/-} mice compared to WT mice in the evening (Figure 2.1 A). In agreement with our previous work (Kalaany et al. 2005), \textit{Lxrα/β-/-} mice were lean (Figure 2.1 B and 2.1 C) and had a similar percentage of fat relative to WT mice (Figure 2.1 D). In addition, \textit{Lxrα/β-/-} mice had lower fasting glucose levels compared to WT mice (Figure 2.1 E).
Figure 2.1  Lxrα/β−/− mice are hypercorticosteronemic but do not exhibit symptoms of Cushing’s syndrome.

(A) Circulating corticosterone from WT and Lxrα/β−/− mice (n=4) measured at the peak of the circadian rhythm (10 PM, 2 h after lights off). Plasma was analyzed for corticosterone level by LC/MS/MS. (B) Size comparison of 15 mo old male WT and Lxrα/β−/− mice. The size differential between the genotypes is most pronounced after 1 year of age. (C) Body weights of WT (n=17) and Lxrα/β−/− (n=36) mice at 6 mo of age. Body fat (D) and plasma glucose (E) levels from WT and LXR-null mice measured by NMR minispec and tail nick, respectively (Avg±SEM, n=10). Mice used in C-E were male mice (6 mo) fed a chow diet. * P<0.05 vs. WT (Student’s t-test).
2.3.2 *Lxra/β*-/- mice are resistant to glucocorticoid-induced hyperglycemia.

To determine whether *Lxra/β*-/- mice are resistant to the metabolic effects of elevated GCs, we treated WT and *Lxra/β*-/- mice with dexamethasone (DEX) or vehicle for 5 days and sacrificed the animals on day 6. DEX was chosen for these studies because it is a potent glucocorticoid receptor agonist with insignificant mineralocorticoid receptor activity and it has been well studied in humans and animals. As expected, the DEX-treated WT mice developed hyperglycemia (3-fold increase in circulating glucose compared to vehicle), whereas DEX-treated *Lxra/β*-/- were refractory to this effect (Figure 2.2 A). Notably, *Lxra/β*-/- mice were still sensitive to feedback repression of corticosterone by DEX (Figure 2.2 B). Spleen weight was measured as a secondary marker of immune function since lymphoid organ atrophy is commonly observed after chronic GC treatment. Both WT and *Lxra/β*-/- mice exhibited significant atrophy of the spleen with chronic DEX treatment (Figure 2.2 C). Thus, the *Lxra/β*-/- mice represent a unique animal model that exhibits dissociated glucocorticoid actions (i.e., resistance to the glycemic response of DEX but sensitive to feedback repression of the HPA-axis and lymphoid organ atrophy).
WT and Lxrα/β−/− mice (6 mo, male) were treated with 2.5 mg/kg dexamethasone (DEX) or vehicle (sesame oil) twice a day for 5 days (A, B) or 14 days (C). Mice were sacrificed by decapitation at lights on in the fed state. Trunk blood was collected and organs were harvested. Plasma glucose (A) and plasma corticosterone (B) levels were measured using commercial kits. (C) The spleen weight was used as a marker of lymphoid organ atrophy. Data shown represent Avg±SEM, n=4-6. *P<0.05 by ANOVA and Student-Newman-Keuls.
2.3.3 \( Lxra/\beta/-\) mice are refractory to DEX-induced insulin intolerance.

To further investigate the role of LXRs in the DEX-mediated effects on carbohydrate metabolism, circulating insulin levels and insulin tolerance were assessed. As expected, insulin levels increased dramatically in response to DEX-induced hyperglycemia in WT and \( Lxra/\beta--/\) mice (Figure 2.3A). However, insulin levels remained lower in \( Lxra/\beta-/\)-mice treated with DEX relative to WT mice. As previously reported (Kalaany et al. 2005), \( Lxra/\beta-/\)-mice also had lower basal levels of insulin compared to WT mice (Figure 2.3 A). There were no significant differences in plasma free fatty acids between WT and \( Lxra/\beta-/\)-mice treated with DEX (Figure 2.3 B), although there was a trend toward increased levels with DEX, consistent with previous studies (Guillaume-Gentil et al. 1993). Glucose and insulin tolerance tests were performed on WT and \( Lxra/\beta-/\)-mice after a 7 day treatment with DEX. A trend towards decreased glucose tolerance was seen in both WT and \( Lxra/\beta-/\)-mice treated with DEX compared to mice that received vehicle (Figure 2.3 C), consistent with the known effects of DEX on decreasing peripheral insulin sensitivity (Vegiopoulos and Herzig 2007). Although there was no significant difference in plasma glucose between \( Lxra/\beta-/\)-and WT mice during the glucose tolerance test, plasma insulin levels were markedly higher in WT animals treated with DEX (Figure 2.3 D), demonstrating that LXR-null mice required less absolute levels of insulin to achieve a similar level of glucose excursion.

Furthermore, under both basal and chronic DEX treatment, there was significant preservation of insulin tolerance in the \( Lxra/\beta-/\)-mice compared to WT mice, especially at the early time points (15 and 30 min) but also over the entire time course as assessed by AUC (Figure 2.3 E). This suggested that the enhanced insulin tolerance in the \( Lxra/\beta-/\)- mice favorably contributes to the prevention of hyperglycemia under chronic DEX treatment. In support of this conclusion, we noted that the level of hepatic phosphorylated Akt (normalized to total Akt) was enhanced 2.5-fold in \( Lxra/\beta-/\)- mice relative to WT mice after 5 min exposure to i.p. insulin (Figure 2.3 F). Taken together, these data demonstrate that \( Lxra/\beta-/\)- mice remain more insulin tolerant compared to WT mice when challenged with DEX, despite having similar plasma FFA levels.
Figure 2.3  Insulin tolerance is maintained in DEX treated Lxra/β-/- mice.

A and B. WT and Lxra/β-/- mice were treated with DEX or vehicle for 5 days. Plasma insulin (A) and free fatty acids (B) were measured in the fed state using commercially available kits. (C-E) Mice were treated with DEX or vehicle for 7 days and fasted for 4 h prior to performing the OGTT and ITT tests. C and D. Mice were gavaged with 20% D-glucose (2g/kg) and blood sampled from a tail nick at different time points. Plasma glucose (C) was assayed using a colorimetric kit and plasma insulin (D) from the glucose tolerance test was assayed by RIA. E. Insulin was injected i.p. at 0.75 U/kg and plasma was assayed at regular intervals to measure glucose as described in C. The figure legend from C is used for D and E. Mice were injected with saline or insulin (1.5 U/kg, i.p.) 5 min prior to sacrifice. Liver extracts
(20 µg) were immunoblotted using antibodies against P-Akt and normalized against total Akt (F). Samples were run on the same gel but were noncontiguous. Data shown are Avg±SEM, (A and B, n=4), (C and D, n=7), (E, n=6-7). *,# P<0.05, ANOVA followed by Student-Newman-Keuls. C-E, * is significantly different from Lxra/β-/- mice with the same treatment regimen, # is significantly different from vehicle treated control of same genotype, n.s., not statistically significant.
2.3.4  *Lxra/β/-* mice differentially activate GR target genes in liver.

To determine whether the selective effects of DEX observed in the *Lxra/β/-* mice were due to differential expression or activation of GR, DEX and GR levels were measured in the liver. There was no significant difference in the hepatic exposure to DEX in WT and *Lxra/β/-* as measured by LC/MS/MS (Figure 2.4A). In addition, the translocation of GR from the cytoplasm to the nucleus in response to ligand was intact since GR nuclear protein levels remained similar in liver of WT and *Lxra/β/-* mice treated with DEX (Figure 2.4B). Furthermore, GR mRNA levels were unchanged between WT and *Lxra/β/-*, and DEX treatment decreased GR mRNA significantly in the liver of both genotypes (Figure 2.4C). Consistent with these findings, the classic GR target gene tyrosine aminotransferase (TAT) (Jantzen et al. 1987, Schmid et al. 1987) was induced to a similar extent in WT and *Lxra/β/-* mice (Figure 2.4C). Remarkably, expression of phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6Pc) was differentially induced in WT vs. *Lxra/β/-* animals. In agreement with known regulation by GR, DEX increased Pepck expression 3-fold and G6Pc expression 1.8-fold in WT mice but not in *Lxra/β/-* mice (Figure 2.4D). Analysis of PGC-1α and FOXO1, two other known transcriptional regulators of Pepck and G6Pc, showed DEX-dependent increases in their expression in WT mice (Figure 2.4D). In *Lxra/β/-* mice, DEX still induced expression of FOXO1 and PGC-1α but this induction was diminished significantly compared to WT mice. Together, these data point to a role for LXR in modulating gene-selective GR activation.
Figure 2.4  Selective regulation of GR target genes in Lxra/β-/- mice treated with DEX.

WT and Lxra/β-/- mice (6 mo, male) were treated with 2.5 mg/kg dexamethasone (DEX) or vehicle (sesame oil) b.i.d. for 5 days. (A) Organic extracts of liver (~100-200 mg) were analyzed by LC/MS/MS for intra-hepatic dexamethasone (DEX) levels after chronic treatment with DEX (Avg ± SEM, n=5, n.s., not statistically significant). (B) Expression of GR from liver nuclear extracts of mice treated with DEX. Protein (40 µg/lane) was normalized for loading using the nuclear marker lamin B. Values represent the average GR protein level normalized to lamin B. (C - D) Real-time quantitative PCR was performed on liver RNA. Gene expression changes were calculated using the comparative Ct (cycle time) method with cyclophilin as the reference gene and WT, vehicle as the calibrator. Samples exhibiting non-LXR selective (C, n=4-6) and LXR-selective (D, n=8-11) changes in gene expression (Avg±SEM, *P<0.05 by ANOVA and Student-Newman-Keuls).
2.3.5  *Lxrβ*-/- mice are protected from GC-induced hyperglycemia and hyperinsulinemia.

To explore the contribution of the two LXR proteins to the selective hepatic GC resistance, *Lxrα*-/- and *Lxrβ*-/- mice were subjected to chronic DEX administration and phenotypic characterization was performed. LXRα is regarded as the most active LXR isoform in the liver because of its high expression and critical role in the regulation of lipogenesis and cholesterol homeostasis (Peet et al. 1998, Repa and Mangelsdorf 2000, Alberti et al. 2001, Zhang et al. 2001, Tobin et al. 2002). Interestingly, after 14 days of DEX treatment, resistance to hyperglycemia was only observed in *Lxrβ*-/- and *Lxrα/β*-/- mice, and not in WT or *Lxrα*-/- mice (Figure 2.5 A). Consistent with these data, the WT and *Lxrα*-/- mice became severely hyperinsulinemic after DEX treatment (Figure 2.5 B). In oral glucose tolerance tests, *Lxrα*-/- mice were less glucose tolerant after DEX treatment relative to vehicle (measured by the AUC), whereas *Lxrβ*-/- mice actually had improved glucose tolerance after DEX (Supplementary Figure 2.a A). Furthermore, the corresponding insulin levels were dramatically increased in the *Lxrα*-/- mice treated with DEX (Supplementary Figure 1.a B), reminiscent of the response seen in WT mice (Figure 2.3 D). In contrast, this insulin response was severely dampened after a glucose bolus in the DEX-treated *Lxrβ*-/- mice (Supplementary Figure 2.a B). These data suggest that, similar to the *Lxrα/β*-/- mice, the *Lxrβ*-/- mice require less absolute levels of insulin to achieve a similar level of glucose excursion. In addition, the insulin data are consistent with a previous report that demonstrated that islets isolated from *Lxrβ*-/- mice exhibited decreased glucose-stimulated insulin secretion (Gerin et al. 2005).

Taken together, the glucose and insulin profiles after OGTT suggest that the *Lxrα*-/- mice are potentially more susceptible to developing GC-induced hyperglycemia than WT mice. Consistent with this notion, activation of LXRα by synthetic LXR agonists has been shown to reverse hyperglycemia in mouse models of insulin resistance (Cao et al. 2003, Laffitte et al. 2003). Therefore, loss of the protective effect of LXRα under these conditions would be expected to result in an exacerbation of the GC effect.
2.3.6  *Lxrβ*-/ mice are protected from GC-induced hepatic steatosis.

The development of insulin resistance is highly correlated with the presence of fatty liver. We explored whether the preserved glucose tolerance in the *Lxrβ*-/ and insulin tolerance in the *Lxra/β*-/ mice after DEX treatment correlated with decreased fat accumulation in the liver. Histologic examination of liver sections was performed with oil red O staining to detect neutral lipids. Significant lipid droplets were observed in livers of WT and *Lxra*-/ mice treated with DEX, whereas no change was detected in the liver histology of *Lxrβ*-/ and *Lxra/β*-/ mice between vehicle and DEX treatment (Figure 2.5 C). Liver triglyceride levels were significantly increased with DEX in all genotypes except *Lxrβ*–/–, although the same trend was observed in this genotype (Figure 2.5 D). These data correlate strongly with the insulin levels measured after DEX treatment (Figure 2.5 B) supporting the essential role of insulin in the development of GC-induced hepatic steatosis (Altman et al. 1951, Diamant and Shafrir 1975).
**Figure 2.5**  *Lxrβ/-* mice are resistant to developing GC-induced hyperglycemia, hyperinsulinemia and hepatic steatosis.

WT, *Lxrα/-*, *Lxrβ/-* and *Lxrα/β/-* were treated with DEX (2.5 mg/kg, b.i.d.) or vehicle (V) for 14 days (by i.p. injection).  

A. Blood glucose levels were measured by tail nick 6 h after lights on in fed mice using a handheld glucometer *P<0.05* t-test.  

B. Plasma insulin was measured using a sensitive rat insulin RIA kit.  

C. Liver sections were stained with oil red O and examined under brightfield microscopy (100x magnification, representative image from *n=3/treatment group). Pre-weighed liver sections were homogenized in chloroform:methanol (2:1) and lipids extracted by the Folch method. Dried aliquots were assayed for liver triglycerides (D) using a commercial kit.  

(A, B, D, Avg ± SEM, *n=4-6), (B, D) *P<0.05* ANOVA followed by Student-Newman-Keuls.
2.3.7 Recruitment of GR to its target genes is decreased in livers of \textit{\textit{Lxr{\beta}^{-/-}}} and \textit{\textit{Lxr{\alpha}/\beta^{-/-}}} mice

To determine whether the requirement for LXRs in the GC regulation of \textit{\textit{Pepck}} was autonomous to the liver, gene expression was investigated in primary mouse hepatocytes. \textit{\textit{Pepck}} was selectively induced by DEX in WT and \textit{\textit{Lxr{\alpha}^{-/-}}} but not in \textit{\textit{Lxr{\beta}^{-/-}}} and \textit{\textit{Lxr{\alpha}/\beta^{-/-}}}-primary hepatocytes (Figure 2.6 A). In contrast, the induction of TAT was similar in all genotypes. Two additional GCs were tested (cortisol and triamcinolone acetonide) in primary hepatocytes and showed the same result demonstrating that this effect is not DEX-specific but represents a general GC-mediated effect (data not shown). These data indicate a hepatocyte autonomous role for LXR in contributing to GC-induced hepatic gluconeogenesis.

To address the mechanism of these promoter-specific actions of GCs, we next investigated promoter occupancy by \textit{in vivo} chromatin immunoprecipitation of GR bound to the glucocorticoid response element (GRE) of either the \textit{\textit{Pepck}} or the TAT gene promoters. As expected, there was a robust recruitment of GR to both the \textit{\textit{Pepck}} and TAT GREs in response to DEX treatment in WT mice (Figure 2.6 B and 2.6 C). Remarkably, GR occupancy of the \textit{\textit{Pepck}} promoter, but not the TAT promoter, was dramatically decreased in the \textit{\textit{Lxr{\beta}^{-/-}}} and \textit{\textit{Lxr{\alpha}/\beta^{-/-}}}-mice in response to DEX. These data strongly support the notion that LXR{\beta} is required for the selective binding of GR to the \textit{\textit{Pepck}} promoter.
Figure 2.6  Recruitment of GR to the Pepck promoter is decreased in livers of Lxrβ−/− and Lxra/β−/− mice.

A. Primary hepatocytes were seeded on 6-well collagen-coated plates and treated with vehicle (DMSO) or DEX (10 nM) for 16 h. RNA was extracted, reverse transcribed and analyzed for gene expression by real-time QPCR. Data pooled from two independent experiments (Avg±SEM, n=5-6). B and C. Chromatin immunoprecipitation of GR protein from mice perfused with vehicle or 10 nM DEX through the portal vein for 30 min. The negative control region for Pepck was at -3 kb relative to the transcription start site. Chromatin was pooled from 2 mice per treatment and results are expressed relative to % input and normalized to GR Veh for each genotype. Error bars represent PCR amplification variability (Avg±SD, n=3). The values for the GR Ab Pepck and TAT GREs prior to normalization were as follows: WT (0.08, 0.05); Lxrα−/− (0.06, 0.08); Lxrβ−/− (0.06, 0.02); Lxra/β−/− (0.08, 0.02). *P<0.05 ANOVA and Student-Newman-Keuls, n.s., not statistically significant. Fold changes are indicated on the graphs.
2.3.8 The immunosuppressive effects of DEX are maintained in the *Lxr-null* mice.

GCs are widely used therapeutically for their immunosuppressive and anti-inflammatory effects. Since hepatic steatosis and hyperglycemia were differentially induced in the *Lxrβ-/-* mice in response to DEX, it was of interest to determine whether the anti-inflammatory response to GCs was preserved. Examination of the lymphoid tissues and white blood cells from LXR-null mice treated with DEX for 14 days confirmed that the loss of LXRβ does not influence the ability of GCs to suppress the immune response (as previously shown for *Lxra/β-/-* mice in Figure 2.1 C). Spleen atrophy was apparent in both *Lxra-/-* and *Lxrβ-/-* mice (Figure 2.7 A). A differential white cell count definitively demonstrated that lymphocyte extravasation and neutrophil demargination were present with DEX treatment regardless of the LXR genotype (Figure 2.7 B). To directly assess the molecular effects of DEX on the immune system, we measured the mRNA expression of cytokines from thioglycolate-elicited peritoneal macrophages. As expected, after stimulation with LPS, there was a significant increase in the expression of key pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α from the primary macrophages (Figure 2.7 C). The response to LPS in the *Lxra-/-*, *Lxrβ-/-* and *Lxra/β-/-* macrophages was attenuated compared to the WT mice. These data are consistent with both LXR isoforms playing a role in the immune response and support previous reports that *Lxra/β-/-* mice are more susceptible to infection than WT mice (Joseph et al. 2003, Joseph et al. 2004, Korf et al. 2009). In addition, pre-treatment with DEX dramatically decreased the cytokine expression in response to LPS for all LXR genotypes (Figure 2.7 C). Therefore, while there is an LXRβ-dependent GC response in the liver on glucose metabolism, the response to GCs in the immune system appears to be independent of LXR.
**Figure 2.7** *Lxrβ/-* mice remain sensitive to the immunosuppressive effects of DEX.

(A) WT, Lxrα−/−, Lxrβ−/− and Lxrα/β−/− were treated with DEX (2.5 mg/kg, b.i.d.) for 14 days (by i.p. injection). The spleen was weighed at the time of sacrifice using an analytical balance. (B) Blood smears were obtained 6 days after initiating DEX treatment by tail nick. After staining, a differential white blood cell (WBC) count was performed under 40x magnification. B, basophils; E, eosinophils; N, neutrophils; L, lymphocytes; M, monocytes. (C) Thioglycolate-elicited peritoneal macrophages were treated with veh or 100 nM DEX (6 h) followed by LPS stimulation (10 ng/mL) for an additional 18 h. RNA was extracted, reverse transcribed and analyzed for gene expression by real-time QPCR. A,B: Avg±SEM, n=4-6, C: Avg±SD n=3. *P<0.05 Student’s t-test for comparison of groups within one genotype.
2.4 Discussion

In this report, we detail the discovery of LXRβ as an important potentiating factor in glucocorticoid-mediated hyperglycemia and hepatic steatosis. As discussed below, these findings are of both physiologic and pharmacologic interest. On the physiologic side, this work reveals an unexpected crosstalk between LXR and glucocorticoid regulation of glucose and lipid metabolism. Glucocorticoids potently upregulate hepatic glucose production under conditions of stress to protect the body from excessive nutrient depletion. This outcome is achieved through the upregulation of numerous genes in the gluconeogenic pathway that include Pepck and G6Pc. Pepck is considered to be the rate-limiting enzyme in gluconeogenesis and as such the molecular factors controlling the expression of this gene have been extensively studied (Park et al. 1990, Imai et al. 1993, Hall et al. 1995, Hanson and Reshef 1997, Wang et al. 1999, Chakravarty et al. 2005, Chakravarty and Hanson 2007). Pepck transcription is strongly activated by GCs and inhibited by insulin (Hall et al. 2007). In addition to GR, numerous proteins are bound to accessory factor sites on the Pepck promoter either directly or indirectly, and are essential for maximal GC response including PPARα (Cassuto et al. 2005), FOXO1 (Puigserver et al. 2003), and PGC1α (Puigserver et al. 2003). We found the induction of liver Pepck, FOXO1 and PGC1a after DEX treatment was significantly attenuated in the Lxrα/β-/- mice compared to WT mice. In addition, Lxrα/β-/-mice remained more insulin tolerant than WT mice after 5 days DEX treatment determined from basal insulin values and an insulin tolerance test, and this effect was specifically due to loss of LXRβ. From experiments performed in primary hepatocytes, we found that the selective transcriptional effect of GCs was cell autonomous and required LXRβ despite the fact that the enhanced insulin tolerance of the Lxrα/β-/-mice would also contribute to protection against GC-induced hyperglycemia. Together, these data suggest that LXRβ in the liver contributes selectively to the regulation of key gluconeogenic enzymes including Pepck.
Non-alcoholic fatty liver results from an imbalance of fat metabolism in the liver. This can arise if there is excessive fatty acid uptake or synthesis, decreased fatty acid oxidation or decreased secretion of VLDL particles. Glucocorticoids are known to contribute to fatty liver production through a combination of increased fatty acid synthesis and decreased fatty acid $\beta$-oxidation (Letteron et al. 1997). The effect of GCs on promoting fatty acid synthesis has been shown to require the presence of insulin. In fact, GCs alone, in the absence of insulin, do not promote fatty acid synthesis (Diamant and Shafrir 1975). However, when combined with insulin, GCs synergistically increase de novo lipogenesis (Altman et al. 1951). While extensive studies have been performed to understand the molecular regulation of gluconeogenesis by GCs (Chakravarty et al. 2005); the detailed molecular mechanisms by which GCs induce fatty liver have not been well characterized (Vegiopoulos and Herzig 2007). It was recently reported that downregulation of the transcriptional repressor HES1 by GR is important in promoting fatty liver because reconstitution of HES1 promoted lipolysis through the ectopic expression of pancreatic lipases (Lemke et al. 2008). These same lipases were recently reported to be upregulated by PPAR$\alpha$ activation (Inagaki et al. 2007). Our data suggest that this mechanism is not involved in the resistance to fatty liver seen in the $Lxra/\beta$-/-mice since the expression of HES1 was not significantly altered by DEX and we were unable to detect the expression of these pancreatic lipases in our liver samples (Supplementary Figure 2.b and data not shown). PPAR$\alpha$, a key transcriptional regulator of fatty acid oxidation, has been found to be essential for DEX-mediated induction of hypertension and insulin resistance in $Ldlr^{-/-}/Ppara^{-/-}$-animals but its role in hepatic steatosis was not analyzed in that study (Bernal-Mizrachi et al. 2003). Here, we found that PPAR$\alpha$ expression was unchanged between WT and $Lxra/\beta$-/-mice and downstream target genes were not dramatically or differentially altered with DEX in WT and $Lxra/\beta$-/-mice (Supplementary Figure 2.b). PPAR$\gamma$ has been recently implicated in hepatic steatosis through the regulation of fat specific protein 27 (FSP27), a direct PPAR$\gamma$ target gene (Matsusue et al. 2008). In our study, PPAR$\gamma$ expression itself was not significantly changed by DEX in both WT and $Lxra/\beta$-/-mice whereas FSP27 was induced equally by DEX in WT and $Lxra/\beta$-/-mice suggesting this pathway was not involved in selective fatty liver production (Supplementary Figure 2.b).
transcription factor SREBP-1c has been previously shown to be critical for inducing an overall program of lipogenesis and promoting fatty liver (Shimano et al. 1997). As expected, due to the known direct regulatory effect of LXR\(\alpha\) on SREBP-1c expression (Repa and Mangelsdorf 2000, Schultz et al. 2000), basal expression of SREBP-1c was greatly decreased in \(Lxra/\beta^{-/-}\)-mice (Supplementary Figure 2.b). However, DEX actually decreased the expression of SREBP-1c in WT mice suggesting that this pathway cannot account for the increased fatty liver seen in WT mice treated with DEX. In summary, the mechanism by which the LXR\(\beta\) and LXR\(\alpha/\beta\) knockout mice are protected from fatty liver appears to be independent of the known pathways affected by GC regulation. This is the first LXR\(\beta\)-specific role that has been ascribed in the liver, an organ in which LXR\(\alpha\) has been recognized as the critical mediator of both cholesterol metabolism and lipogenesis. A plausible hypothesis that is the subject of future studies, is that the LXR\(\beta\) effects on hepatic steatosis may be related to the enhanced glucose tolerance in the \(Lxr\beta^{-/-}\)-mice after chronic DEX treatment (Supplementary Figure 2.a A).

Glucocorticoids mediate their anti-inflammatory effects through the differential tethering of GR to individual transcription factors such as NFkB and c-Jun (Miner and Yamamoto 1992, Nissen and Yamamoto 2000). The transrepressive effects of glucocorticoids are primarily responsible for the beneficial therapeutic effects on the immune system (for example, repression of IL-6 and TNF-\(\alpha\)). Transactivation of GR is believed to be responsible for the negative side effects of therapeutic glucocorticoids including hyperglycemia (Pepck, G6Pc), muscle catabolism (myostatin, glutamine synthetase) and osteoporosis (receptor activator of NF-\(\kappa\)B ligand) (De Bosscher and Haegeman 2009). As such, the pharmaceutical industry has been interested in developing “dissociated” glucocorticoid receptor agonists that can separate the transrepression from transactivation activities (Schacke et al. 2002, Schacke and Rehwinkel 2004). This strategy has provided successful novel steroidal candidates \textit{in vitro} but the effects have not faithfully translated \textit{in vivo}. More recent compounds with a non-steroidal structure have begun to be used \textit{in vivo} with an enhanced side effect profile compared to steroidal ligands (Schacke et al. 2007). Complicating the
application of the dissociated ligand strategy are genes that are transactivated by GR but critical for its anti-inflammatory effects such as MAPK phosphatase 1 (MKP-1) and GC-induced leucine zipper (GILZ) (De Bosscher and Haegeman 2009). The finding that LXRβ selectively promotes gene-specific transactivation opens a new avenue for the development of GC-selective ligands that does not depend on separating transactivation from transrepression.

To capitalize on the discovery that LXRβ is potentiating some of the negative metabolic effects of GCs, a detailed molecular mechanism for how selective transactivation occurs must be uncovered. While an improved insulin tolerance in LXRα/β-null mice would provide protection against DEX-induced hyperglycemia, the cell autonomous effect of Pepck regulation in the primary hepatocytes suggests a more direct mechanism is involved. We have shown that the GC levels in the livers of WT and Lxrα/β-/--mice treated with DEX are similar, yet there is differential regulation of GR target genes that points to a promoter specific mechanism. To that end, we have shown that GR is differentially recruited to the Pepck promoter in the absence of LXR. This effect is not due to a general decrease in recruitment of co-factors to the Pepck promoter in the Lxrα/β-/- mice since two other factors important for Pepck activation (C/EBPβ and SRC-1) were similarly recruited (Supplementary Figure 2.c). Co-immunoprecipitation studies in HEK293 cells overexpressing GR and Flag-tagged LXRα or LXRβ uncovered an interaction between GR and each isoform of LXR (Supplementary Figure 2.d). Therefore, this protein-protein interaction is unlikely to account for the LXRβ-specific mechanism demonstrated herein unless competition is occurring between the two receptors for binding to GR. Ongoing studies are currently being directed at understanding the basis for the selective recruitment of GR to the Pepck promoter. In addition, it will be of interest to explore whether other detrimental effects of long-term glucocorticoids, including osteoporosis and muscle wasting, show LXR-selectivity.

In summary, the discovery that the Lxrα/β-/--mice were hypercorticosteronemic without exhibiting Cushing’s like symptoms prompted our investigation into whether the mice
were resistant to the effects of glucocorticoids. Through the use of the potent synthetic ligand dexamethasone we discovered that Lxrα/β-/mice were selectively resistant to some of the effects of glucocorticoids—most notably the metabolic effects—but were still sensitive to the immunosuppressive effects (Figure 2.8). Furthermore, we uncovered the liver as a key organ influencing the effect of LXRβ on GC-mediated induction of Pepck. The data presented herein renew the optimism that a more selective GC agonist can be designed to provide exceptional anti-inflammatory action without the development of negative metabolic effects.
Figure 2.8  Selective activation of the hepatic GC-response pathway.

LXRβ is modulating the GC-dependent induction of hyperglycemia and hepatic steatosis by increasing hepatic glucose production and decreasing glucose tolerance contributing to insulin resistance and diabetes. Lxrβ-/- (and Lxra/β-/-) mice remain sensitive to the immunologic effects of glucocorticoids.
2.5 Acknowledgments

We thank D. Ferguson for critically reading the manuscript and K. Gauthier, M. Hawkes and J. Repa for their expert advice. This work was supported by the Howard Hughes Medical Institute (DJM), the National Institutes of Health (DJM, U19DK62434), the Robert A. Welch Foundation (DJM, I-1275), Natural Sciences and Engineering Research Council of Canada (CLC, 356873-08), the Canadian Institutes of Health Research (CLC, MOP-89361 and 97904), Canada Foundation for Innovation (CLC). MP and LM are supported by Ontario Graduate Scholarships. ALB is supported by a pre-doctoral fellowship (GM007062). VL is a Howard Hughes Medical Institute research associate and DJM is a Howard Hughes Medical Institute investigator.
2.6 Supplementary Figures

**Supplementary Figure 2.a** Glucose tolerance is preserved in DEX treated *Lxrβ/-* mice.

*Lxrα/-* and *Lxrβ/-* mice were treated with DEX (2.5 mg/kg b.i.d.) or vehicle for 7 days and fasted for 4 h prior to performing the OGTT test. Mice were gavaged with 20% D-glucose and blood sampled at regular intervals into EDTA coated microvette tubes. Plasma glucose (A) was assayed using a colorimetric kit. Plasma insulin (B) from the glucose tolerance test was assayed by RIA. B shares the same figure legend as A (Avg±SEM, *n*=6-7). *P<0.05, ANOVA followed by Student-Newman-Keuls. * is significantly different from *Lxrβ/-* mice of the same treatment regimen, # is significantly different from vehicle treated control of same genotype, n.s., not statistically significant.
Liver RNA was extracted from WT and Lxrα/β-/-mice treated with DEX (2.5 mg/kg b.i.d.) for five days, reverse-transcribed and real-time QPCR was performed using SYBR Green I chemistry. CD36, fatty acid translocase; CPT-1, carnitine palmitoyl-transferase I; FSP27, fat specific protein; HES1, hairy and enhancer of split 1. (Avg±SEM, n=4-6). *P<0.05 by ANOVA and Student-Newman-Keuls, n.s., not statistically significant.
Supplementary Figure 2.c Recruitment of C/EBP\(\beta\) and SRC-1 to the Pepck promoter is similar in WT and \(Lxr\beta^{-/-}\) mice.

Chromatin immunoprecipitation of GR protein from mice perfused with vehicle or 10 nM DEX through the portal vein for 30 min. Chromatin was pooled from 2 mice per treatment and results are expressed relative to % input. Error bars represent PCR amplification variability (Avg±SD, \(n=3\)). *\(P<0.05\) (Student’s t-test, relative to vehicle control of same genotype).
Supplementary Figure 2.d  LXRα and LXRβ co-immunoprecipitate with GR in transiently transfected HEK cells

Co-immunoprecipitations from HEK cells transiently over-expressing GRα and LXRα or LXRβ isoforms following vehicle or 500 nM DEX treatment overnight.
Table 2.2  Average change in body weight after DEX treatment as a function of dosing route and length of treatment

Data shown represent the Avg±SEM

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>s.c. injection</th>
<th>s.c. injection</th>
<th>i.p. injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DEX 5 day</td>
<td>DEX-21-Acetate</td>
<td>DEX 14 day</td>
</tr>
<tr>
<td>Change in BW</td>
<td>(g) (% of initial)</td>
<td>(g) (% of initial)</td>
<td>(g) (% of initial)</td>
</tr>
<tr>
<td>WT Veh</td>
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<td>-0.3 ± 0.6</td>
</tr>
<tr>
<td>DEX</td>
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<td>-2.4 ± 0.9</td>
</tr>
<tr>
<td>Lxrα/β−/− Veh</td>
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<td>-0.2 ± 0.4</td>
<td>-0.3 ± 1.0</td>
</tr>
<tr>
<td>DEX</td>
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<td>-1.5 ± 0.5</td>
<td>-0.6 ± 0.5</td>
</tr>
<tr>
<td>Lxrα−/− Veh</td>
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<td>-0.5 ± 0.6</td>
<td>-3.0 ± 1.4</td>
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<tr>
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<tr>
<td>Lxrβ−/− Veh</td>
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<td>-2.9 ± 0.6</td>
</tr>
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</table>

*Significantly different from Veh of same genotype P<0.05 (Student’s t-test)

^P=0.05; ^P=0.07
Chapter 3  LXRβ antagonism mitigates glucocorticoid-induced gluconeogenesis without altering immune suppression.

Portions of the data in this chapter appear in a patent application and are currently being formatted for future publication.


Author contributions

Rucha Patel: Planned, conducted and analysed all of the studies.

Lilia Magomedova: Conducted the GR and LXRβ unbiased proteomic studies using the T-Rex Bir-A system (data not shown).

Ricky Tsai: Injected the mice with thioglycollate for primary macrophage studies and helped with primary hepatocyte isolation.

Melanie Robitaille: Took confocal images for the co-localization study.

Stephane Angers: Provided experimental guidance and expertise.

Arturo Orellana: Developed synthesises of GSK2033, Amgen54 and DMHCl and provided compounds for in vitro and in vivo analysis.

Carolyn L. Cummins: Principal Investigator on the project and contributed to experimental design and analysis.
3.1 Introduction

Glucocorticoids (GCs), such as cortisol, dexamethasone (DEX), and prednisone, are potent anti-inflammatory and immune suppressive drugs that are widely used in clinics for the treatment of chronic conditions such as rheumatoid arthritis, lupus erythematosus, inflammatory bowel disease, asthma and certain types of leukemia. However, long term use of GCs results in deleterious side effects such as hyperglycemia, hepatosteatosis, and insulin resistance. In glucocorticoid-treated patients with rheumatoid arthritis (Hoes et al. 2011), primary renal disease (Uzu et al. 2007), and lymphoblastic leukemia (Gonzalez-Gonzalez et al. 2013), diabetes prevalence ranges between 20-40%. Moreover, Cushing’s syndrome and chronic stress also cause elevated endogenous GCs levels that lead to metabolic syndrome (Vegiopoulos and Herzig 2007).

GCs exert both their therapeutic and adverse effects through the glucocorticoid receptor (GR) in the body. GR belongs to the nuclear hormone receptor family of transcription factors. In most cells, ligand activation dissociates GR from the HSP90/ HSP70 complex and then translocates into the nucleus. In the nucleus, GR is recruited to the regulatory elements of GC-responsive genes via either directly binding to a GRE or interacting with other transcription factors bound to activate or repress unique sets of genes (Vegiopoulos and Herzig 2007, Uhlenhaut et al. 2013).

A key desirable effect of GR activation is a potent repression of several genes (e.g. IL-1β and IL-6) involved in inflammation (Coutinho and Chapman 2011). However, GCs also promote hepatic glucose production by increasing transcription of phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6Pc), key enzymes involved in gluconeogenesis.

Two isoforms of LXRs, LXRα and LXRβ, are known as sterol sensors, also belong to the nuclear hormone receptor superfamily. LXRα is primarily expressed in the liver,
 Similar to GR, activation of LXR also suppresses genes involved in the immune response. Moreover, LXRα activation via synthetic ligands has been shown to improve glycemia in diabetic rodent models by reducing expression of gluconeogenic genes, Pepck and G6Pc in the liver and inducing Glut4 expression in the white adipose tissue (Cao et al. 2003, Laffitte et al. 2003, Commerford et al. 2007, Herzog et al. 2007, Nader et al. 2012). However, a major deleterious effect of LXRα activation is a potent increase in hepatic lipogenesis in rodent models (Repa et al. 2000, Quinet et al. 2006). In agreement with LXRα having a dominant role in mediating lipogenesis, basal expression of lipogenic genes SREBP-1c, ACC, FAS and SCD-1 are reduced in Lxrα-/mice (Peet et al. 1998, Repa et al. 2000).

Recently, we have shown that the whole body Lxrβ-/ mice are protected against DEX-induced hyperglycemia and hepatosteatosis, without affecting DEX-mediated inflammatory suppression. We demonstrated that LXRβ is essential for full recruitment of GR to the GRE of Pepck and hepatic Pepck induction following GC-administration (Patel et al. 2011).

As highlighted above, GR, LXRα, and LXRβ regulate overlapping target genes to control important physiological pathways, making them attractive druggable candidates. Efforts to create newer generation ‘target gene selective small molecule ligands’ of GR,
LXRα and LXRβ is an active field of research since full pan-agonist activation of these NRs has been spoiled by additional deleterious metabolic effects of receptor activation. Recently, GSK2033, AMGEN 54 and 22-S-hydroxycholesterol were published as synthetic LXR antagonists (Kase et al. 2006, Zuercher et al. 2010, Jiao et al. 2012); with GSK2033 as the most potent antagonist in cell-based assays (Zuercher et al. 2010). In this proof of principle study, we assessed whether co-treatment of an LXRβ antagonist in combination with GC administration dissociates the gluconeogenic effects from the anti-inflammatory effects of GCs. Here we show that LXRβ antagonism along with GC administration inhibits the gluconeogenic program without affecting the immune suppressive actions of GR.

3.2 Material and Methods

3.2.1 Reagents

Dexamethasone, high glucose DMEM, low glucose DMEM, zero glucose DMEM, sodium lactate, glucose HK assay kit, penicillin/streptomycin, 0.25% trypsin and thioglycollate were purchased from Sigma. Fetal bovine serum (FBS), Charcoal-treated FBS (ss FBS), liver digestion medium, liver perfusion medium, medium M199, William’s E medium, insulin, sodium pyruvate, non-essential amino acids and Lipofectamine 2000 were purchased from Invitrogen. Tissue culture treated plates and rat collagen type I coated plates and cover slips were purchased from BD Bioscience. Complete protease inhibitor cocktail tablets were purchased from Roche. Dual-luciferase reporter assay kit was purchased from Promega. RNA-stat was purchased from Tel-Test Inc. Poly acrylamide 4-20% gradient gels were purchased from Bio-Rad. LXR agonist T0901317 was purchased from Cayman chemicals. GSK2033 was synthesised by Dr. Arturo Orllena (York University, Toronto, ON).

The antibodies for GR (M-20), RNA polymerase II (C-21), LXRβ (N-20), SRC-1 (M-341), P300 (N-15), RXR (ΔN 197), C/EBP-β (C-19), and rabbit IgG were purchased
from Santa Cruz Biotechnology, Inc. The antibodies against H3K9Ac (Ab4441), H3K27Ac (Ab4729), H4 (Ab7311), H3 (Ab1791) and Lamin B (Ab16048) were purchased from Abcam. A pan-AcH4 antibody was purchased from Millipore. The antibodies for HA (C29FA) and Flag (F1804) were purchased from Cell Signaling and SIGMA, respectively. Monoclonal LXRβ (PP-K8917-10, PPMX) antibody was a kind gift from Dr. Jason Matthews (University of Toronto, ON). Breeder pairs of LXR-null mice; stock vials of HEK293 and H4IIE cells; and the following plasmids GAL4-hLXRα, GAL4-hLXRβ, GAL4-hGR, CMX-mLXRα, CMX-mLXRβ, CMX-mGR, UAS_luciferase promoter and CMX control were kindly provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical Centre, Dallas, TX). The rat Pepck promoter (-484 to +63, with respect to TSS) was cloned into the PGL3 basic plasmid using rat tail genomic DNA as a template with the forward primer 5’gctcgagagagatctcagagcgtctcg3’ and reverse primer 5’cggggtacctgaatcctctctcatgacc3’. The plasmids necessary to make third generation lenti-virus constructs (PSL3, PSL4, PSL5, PSL9 and PLKO1) were a kind gift from Dr. Stephane Angers (University of Toronto). The H4IIE overexpressing HA-tagged-mLXRβ cell line was generated by transducing H4IIE cells with a third generation lenti-virus encoding HA-mouse LXRβ coding sequence followed by puromycin selection.

3.2.2 Cell culture conditions

HEK293 cells were grown under aseptic conditions in 10-cm plates using high glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. During active use, HEK293 cells were passaged at >80% confluency every 2-3 days. H4IIE cells were maintained similarly. H4IIE medium was made of low glucose DMEM, with 10% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin, and 1% HEPES buffer.
3.2.3 Mouse primary hepatocytes

Primary hepatocytes were isolated from 7 to 10 week old WT and Lxr-null mice. After the mouse was anesthetized with isoflurane, the portal vein was cannulated using a 24G angiocatheter (BD science) and the liver was perfused with perfusion and collagenase digestion buffer via peristaltic pump at a flow rate of 4 mL/min for 10 mins. Thereafter, the liver surface capsule was peeled off with forceps and the cells were forced to separate in a low glucose DMEM wash medium. The hepatocytes were separated via low gravity (50xg) centrifugation for 3 minutes. The cells were washed three times, counted, checked for viability, and then plated on collagen I–coated plates in William’s E medium supplemented with 10% ssFBS, 10 nM insulin and 1% penicillin/streptomycin. After 3 hours, cells were washed with PBS twice and the medium was changed to M199 supplemented with 5% ssFBS, 1nM insulin and 1% penicillin/streptomycin. The cells were allowed to grow overnight in a 37°C incubator and all of the experimental treatments were started the next day. For luciferase reporter assays, cells were plated in 48-well plates at a density of 8 x 10⁴ cells/well. For gene expression and glucose production analysis cells were plated in 6-well plates at a density of 4 x 10⁵ cells/well and 1 x 10⁶ cells/well, respectively. Hepatocytes were treated with vehicle, 10 µM GSK2033, 100 nM DEX or DEX + GSK2033 in M199 without FBS for 16-18 hours for gene expression studies. For co-localization experiments, cells were plated on glass coverslips, placed in 6-well plates at a density of 4 x 10⁵ cells/well. For protein expression and ChIP analysis 4 x 10⁶ cells/treatment were plated in 10 cm plates.

3.2.4 Transient co-transfection reporter assays

Primary hepatocytes grown in 48-well plates were transfected with Lipofectmine 2000 in OPTI-MEM medium using the manufacturer’s protocol. For IC₅₀ experiments with GSK2033, the total amount of plasmid DNA (250 ng/well) included 150 ng UAS-reporter, 50 ng Renilla, 25 ng of either pCMX-GAL4hLXRα, pCMX-GAL4hLXRβ or
pCMX-GAL4hGR and 25 ng of CMX filler plasmid. For *Pepck* promoter activation assays, the total amount of plasmid DNA (250 ng/well) included 125 ng rat *Pepck* (484 bp) promoter reporter, 50 ng *Renilla*, and following combination of nuclear receptors:

1. 25 ng of pCMX-mGR, 50 ng of CMX,
2. 25 ng of pCMX-mGR, 25 ng pCMXm-LXRα and 25 ng pCMX-mRXRα
3. 25 ng of pCMX-mGR, 25 ng pCMXm-LXRβ and 25 ng pCMX-mRXRα.

Six hours after transfection, medium was changed to M199 medium supplemented with 5% ssFBS, 1 nM insulin and 1% penicillin/streptomycin. The next evening, ligands were added in M199 without FBS. Cells were harvested 20 hours later in passive lysis buffer (Promega) and the luciferase and *Renilla* activities were measured on Victor^3^ 1420 Multilabel counter (PerkinElmer) using the Promega dual luciferase kit. Luciferase values were normalized to *Renilla* to control for transfection efficiency and expressed as Relative Luciferase Units (RLU).

### 3.2.5 Co-localization studies

Primary hepatocytes grown on cover slips were co-transfected with 1.25 µg of Cherry-LXRβ and 1.25 µg Venus-GR using the Lipofectmine 2000 protocol in OPTI-MEM medium. Six hours after transfection, medium was changed to M199 medium supplemented with 5% ssFBS, 1 nM insulin and 1% penicillin/streptomycin. After 48 hours, the cells were treated with indicated ligands for 4 hours and fixed using freshly made 4% PFA (pH 7.4). Vectashield (Vector labs) mounting medium with DAPI was used to mount the coverslips on glass slides. Sub-cellular localization of GR and LXRβ proteins was visualized using a LSM 700 Zeiss confocal laser scanning microscope.

### 3.2.6 Glucose production

Glucose production experiments were carried out as described by Chutkow et al (Chutkow et al. 2008). Briefly, the hepatocytes were stimulated overnight with indicated ligands in M199 without FBS. Zero glucose DMEM supplemented with L-glutamine 0.584(g/L), NaHCO3 (3.7 g/L) and HEPES (3.57 g/L) pH7.3 (glucose-free
medium) was prepared. The following day, the cells were washed twice with glucose-free medium and then incubated in glucose-free medium with ligands for 30 min to deplete intracellular glycogen. The medium was then replaced with fresh ligand and zero glucose medium supplemented with 20 mM lactate and 2 mM pyruvate. After 6 hours, the culture medium was removed, lyophilized and reconstituted to a final volume of 100 µL. The glucose content of the concentrated medium was measured using the glucose hexokinase (HK) assay kit (Sigma).

3.2.7 In-vivo study

Lxra-/- mice (male, 4-5 months old) were treated with 5 mg/kg DEX or 5% EtOH in sesame oil twice daily (s.c.) and 40 mg/kg GSK2033 once daily (i.p.) for 5 days alone or in combination. To minimize the production of endogenous GCs from environmental stress, mice were sacrificed within 1 minute of handling in the fed state by decapitation within 2 hours after the start of the light cycle. Trunk blood was collected in K+EDTA containing tubes, and plasma was separated by centrifugation. Livers were immediately flash-frozen in liquid nitrogen for gene expression analysis. Spleen weights were measured. Plasma and tissues were stored at -80°C until assayed. Plasma glucose was measured using a glucose oxidase kit (Wako).

3.2.8 RNA isolation, cDNA synthesis, and real-time QPCR analysis

Total RNA was extracted from cells and tissues using RNA STAT-60. Following quantification at 260 nm by UV spectroscopy, 2µg of RNA was treated with DNase I (RNase-free; Roche), and reverse transcribed into cDNA with random hexamers using the High Capacity Reverse Transcription System (ABI; Applied Biosystems). The primers used are shown in Table 3.1. Real-time quantitative PCR (QPCR) reactions were performed on an ABI 7900 in 384-well plates containing 12.5 ng cDNA, 150 nM of each primer, and 5 µl 2X SYBR Green PCR Master Mix (ABI) in a 10 µl total
volume. Relative mRNA levels were calculated using the comparative Ct method (Bookout et al. 2006).

Table 3.1 QPCR primer sequences

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<th>Abbrev.</th>
<th>Accession#</th>
<th>Forward and Reverse Primers</th>
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<td>cyclophilin</td>
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<td>GR</td>
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<td>Liver X receptor beta</td>
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<td>Gilz</td>
<td>NM_001077364.1</td>
<td>5'tagcggagaccccaactact3' 5'ctcgggacgccgctt3'</td>
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<td>NM_031168.1</td>
<td>5'tctgggaatgagaaaaagttt3' 5'agtcatactctgtctttcataca3'</td>
</tr>
</tbody>
</table>

3.2.9 Protein extraction and Western blotting

Hepatocytes were treated for 4 hours with indicated ligands, and cells were harvested for protein extraction. Nuclear and cytoplasmic fractions were prepared. Briefly, cells were washed twice with cold PBS, scraped, centrifuged and lysed in 5 x pellet volume of Buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na-orthovanadate, 0.5 mM PMSF and 1X protease inhibitor) on ice for 15 minutes. NP-40 was added to the cell suspension at a final
concentration of 0.5% and vortexed hard for 15 sec. The lysates were centrifuged at 6500xg for 30 seconds, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in 2 x pellet volume buffer B (20mM HEPES (pH 7.9), 1.5 mM MgCl2, 420 mM NaCl, 2 mM EDTA, 25% glycerol, 10 mM NaF, 1 mM Na-ortovanadate, 0.5 mM PMSF and 1X protease inhibitor). Sonication was performed on ice and stored at -80°C until analysis. Protein concentration was measured using the BCA assay (Pierce). Nuclear extracts (50µg) or whole cell lysates (50µg) were electrophoresed on 4-20% gradient gels (Bio-Rad) and transferred to nitrocellulose using standard techniques. The blot was blocked for 1 hour in 5% non-fat milk and incubated overnight at 4°C with primary monoclonal GR S111 antibody (1:500; Cell Applications), lamin B1 (1:1000; Abcam), Pepck (1:1000; Abcam), actin (1:5000; Abcam) in 1% non-fat milk. The membrane was washed three times with T-PBS (phosphate buffered saline containing 0.05% Tween-20) and incubated with secondary HRP-conjugated anti-rabbit IgG (1:5000) or anti-goat IgG (1:5000) for 1-hour. The membrane was washed three times with T-PBS and the signal was observed using ECL prime (GE health care). The blots were quantified using Image J software. Three individual sets of experiments and Western blots were performed.

3.2.10 Chromatin immunoprecipitation (ChIP)

*lxra/-* livers were perfused in-situ for 30 minutes via the portal vein with vehicle, 10µM GSK2033, 100 nM DEX or DEX + GSK2033 ligands dissolved in perfusion buffer. Whole liver was minced and cross-linked in 1% formaldehyde PBS for 10 minutes at room temperature. Cross-linking was stopped by addition of glycine to a final concentration of 125 mM for 5 minutes at room temperature, followed by centrifugation and the pellet was washed twice in ice-cold PBS. Liver nuclei were recovered by dounce homogenization in a hypotonic buffer (10 mM Hepes [pH 7.9], 1.5 mM MgCl2, 10 mM KCl, 0.2% Nonidet P-40, 0.2 mM sodium orthovanadate, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose, 1 mM DTT and protease inhibitor) and layered onto a cushion buffer (10 mM Tris-HCl [pH 7.5], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, 1 mM
DTT, and protease inhibitor) followed by centrifugation. The nuclear fraction was washed with cold PBS and resuspended at 2 x pellet volume with sonication buffer (0.2% SDS, 1% Triton-X, 0.1% Na-deoxycholate, 1 mM EDTA and 50 mM Tris-HCl, 8.0, 150 mM NaCl and protease inhibitor). The chromatin was sheared to 200–1000 bp by sonication using Misonix microtip (419) 15 x 30 sec pulses, 1sec on 1 sec off/ pulse, at 24-27 W. The soluble chromatin was collected by centrifugation and diluted 2-fold in dilution buffer (1% Triton X-100, 0.1% Na-deoxycholate, 50mM Tris-HCL (pH 8.0), 150mM NaCl, EDTA 1mM and protease inhibitor). The lysates were incubated by rotation with 80 μl of protein A agarose for 2 h at 4°C and an aliquot of the chromatin was put aside that represented the input fraction. The diluted lysate (250 μl per immunoprecipitation) was transferred to a new micro centrifuge tube to which one of the following antibodies was added (10 μg of GR, LXRβ, RNA Pol II, RXR, SRC-1, C/EBP β, P300 or 2 μg of H3, H4, H3K27Ac, H3K9Ac) and incubated overnight at 4°C. The next morning, 50 μl of Protein A agarose beads was added and incubated for 3 hrs at 4C with rotation. The beads were successively washed for 10 min in 1 ml of TSE 1 buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), 1 ml of TSE 2 buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), 1 ml of LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mMEDTA, 1% NP-40, 1% Na-deoxycholate), and 2 × 1 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Protein-DNA complexes were eluted using 2 x 50 μl of elution buffer (100 mM NaHCO3, 1% SDS) for 30 min, and the cross-links were reversed by addition of 5 μL 5M NaCl and overnight incubation at 65°C. The next day, samples were RNase treated for 30 minutes at 37°C, proteinase K treated for 2 hrs at 45°C, and purified using a PCR purification kit (QIAGEN) and eluted in 50 μl. The eluate was diluted 5-fold with water, and QPCR was performed using 1.2 μl of template DNA with the following primers:

Pepck GRE (0kb) F, 5’tgcagccagcaacatatgaa3’, R, 5’tgatgcaaaactgcaggctct3’,
Pepck neg. (-3kb) F, 5’tgggagacacacatcttacctca3’, R, 5’gtccctctatatgactcgcagcaca3’,
TAT GRE (-2.5kb) F, 5’cgcaaaacacaggaagctaa3’, R, 5’catgacacccaaagcctcetctc3’and
SREBP-1c LXRE (-1kb) F, 5’gcaaccatccccgaaag3’, R, 5’acagagttcgggatcaa3’.
For ChIP experiments from mouse primary hepatocytes transfected with the rat *Pepck* promoter and H4IIE cells over expressing HA-tagged LXRβ dilution buffer was used to lyse the cells prior to sonication and the assay was conducted as described above. The following rat specific primers were used for QPCR:

Pepck GRE F, 5’gtttcacgtctcagctga3’, R, 5’acctgtgactgttgctgatgc3’,
Pepck neg. F, 5’ttctctctcctccatcattgg’3 R, 5’tgcacctcgagaaggttaag’3 and
SREBP-1c LXRE F, 5’aagtgcctgggttcggaat3’, R, 5’acagagctctcgggaga3’.

Quantitation was performed by QPCR (standard curve method) using serial dilutions of a 10% input as standards. The results were first normalized to 3% total chromatin input, and then GR recruitment to the Pepck GRE with DEX treatment was set as 100. The results are expressed in arbitrary units, relative to 3% input and normalized to GR recruitment to the Pepck GRE for each protein and genomic location tested in the experiment. The liver ChIP results are average enrichments from three independent sets of experiments expressed as arbitrary units.

### 3.2.11 Mouse primary macrophage isolation

WT and Lxr-null mice were i.p. injected with 1 mL of 3% thioglycollate 4 days prior to macrophage isolation. Macrophages were collected by peritoneal lavage using 10 ml of cold PBS. Isolated cells were pelleted at 1700 x g and washed in PBS supplemented with 3% FBS three times. Cells were resuspended in high glucose DMEM medium supplemented with 10% FBS insulin and 100U/mL penicillin/streptomycin, counted, checked for viability and plated to a density of 2 million cells/well in 6-well plates. After allowing the macrophages to adhere overnight, pre-treatment with 10ng/mL LPS was initiated. Four hrs later, cells were co-treated with 10 µM GSK 2033, 100 nM DEX, DEX + GSK 2033, 250 nM T0901317, T0901317 + GSK2033 and T0901317 + DEX for 16-18 hrs. The next morning the cells were harvested for RNA.
3.2.12 Statistical analyses

Data are presented as mean ± SEM unless otherwise indicated. GraphPad Prism was used for ANOVA followed by multiple comparisons testing as appropriate; or by two-tailed Student’s t-test. \( P<0.05 \) was considered significant.
3.3 Results

3.3.1 GSK2033 is an LXR antagonist with increased selectivity for LXRβ compared to LXRα.

In 2010, Zuercher et al reported the characterization of GSK2033, a potent LXR antagonist in cell based assays. However, the utility of the compound for in vivo studies was questioned because of human liver microsome studies that suggested GSK2033 was rapidly metabolized by the liver cytochrome P450 enzymes. Therefore, in order to determine an optimal concentration for dosing in my studies, I assessed GSK2033’s potency and selectivity in mouse primary hepatocytes. In transient co-transfection reporter studies, GSK2033 co-treatment antagonized T0901317 (LXR agonist)-induced activity of both isoforms of LXRs without affecting DEX induced activity of the GR (Figure 3.1A,B). GSK2033 showed slightly more selectivity for LXRβ with an IC$_{50}$ of 853 nM for LXRα and 191 nM for LXRβ. However, this 4.5-fold selectivity is not anticipated to be sufficient to differentiate between the two receptors within cells.

3.3.2 LXRβ antagonism represses GC-induced Pepck expression and glucose production in mouse primary hepatocytes.

A major undesirable side effect of GC treatment is increased glucose production by the liver. To investigate whether antagonism of LXRβ along with DEX treatment alters GR-mediated Pepck expression and glucose production in vitro, we conducted a number of gene expression and glucose production studies on mouse primary hepatocytes isolated from WT, Lxra-/- and Lxrβ-/- mice.
Figure 3.1  GSK2033 is a potent LXR antagonist. Selectivity and potency of LXR antagonist GSK2033 was assessed for LXRα and LXRβ in mouse primary hepatocytes.

Mouse primary hepatocytes were co-transfected with GAL4-hLXRα, GAL4-hLXRβ or GAL4-hGR with UAS-luciferase reporter plasmid. A dual LXR ligand T0901317 was dosed at the EC₈₀ for each receptor (1 µM for GAL4-hLXRα and 250 nM for GAL4-hLXRβ). (A) The IC₅₀ of GSK2033 against LXRα and LXRβ in a mouse primary hepatocyte transfection assay. GSK2033 did not antagonize the activity of GAL4-hGR (B) when activated with 100 nM DEX in primary hepatocytes. (A-B) Data show a representative experiment mean ± SEM (N=3). Similar results are obtained in at least three independent experiments, performed on hepatocytes isolated on different days. IC₅₀ values were determined using a sigmoidal curve fit (Prism, GraphPad).
3.3.2.1 Gene expression studies

As expected, DEX treatment significantly increased the expression of Pepck, a key gluconeogenic gene, in WT and Lxra/- primary hepatocytes. DEX induction of Pepck was significantly inhibited by co-treatment with the LXR antagonist GSK2033 in both WT (from 8.5-fold to 1.9-fold) and Lxra/- (from 15.7-fold to 3.2-fold) hepatocytes. As expected, DEX treatment did not increase Pepck expression in Lxrb/- primary hepatocytes, and this remained unchanged with co-treatment of GSK2033 (Figure 3.2A). In contrast to Pepck, the expression of another GR target gene, TAT, was induced significantly in each of the WT (20.8-fold), Lxra/- (12.7-fold) and Lxrb/- (9.7-fold) hepatocytes, and unchanged with GSK2033 co-treatment (Figure 3.2B). As expected, the LXR target gene Srebp-1c was induced by T0901317 (T09) in hepatocytes from WT (37.7-fold), Lxra/- (3.7-fold) and Lxrb/- (42-fold) mice, and this induction was significantly down-regulated by GSK2033 co-treatment, independent of genotype (Figure 3.2C). To determine whether GSK2033 may indirectly contribute to suppression of DEX-mediated Pepck expression through the regulation of another protein, we performed similar experiments in the presence of the protein translation inhibitor cycloheximide. Under these conditions, GSK2033 maintained its ability to diminish DEX-induced Pepck expression in Lxra/- primary hepatocytes, suggesting that de novo protein synthesis is not required to exert this inhibitory effect (Supplementary Figure 3A).
Figure 3.2  Co-treatment of GSK2033 with DEX results in repression of Pepck gene expression in mouse primary hepatocytes.

Expression of (A) Pepck, (B) TAT and (C) Srebp-1c genes in WT, Lxrα−/− and Lxrβ−/− mouse primary hepatocytes following 18 hr treatment with vehicle (V), 10 μM GSK2033 (G), 250 nM T09 (LXR agonist), 100 nM DEX (D) alone or in combination. Data show a representative experiment Avg ± SD, N= 3, repeated at least 4-5 times (A-C). *P<0.05 by One way ANOVA, Student-Newman-Keuls.
In addition, gluconeogenic gene inhibition by LXR antagonist GSK 2033 was not limited to DEX; GSK2033 co-treatment with other glucocorticoids such as cortisol, prednisone and triamcinolone acetonide, repressed expression of both Pepck and G6Pc in Lxra/- primary hepatocytes (Supplementary Figure 3.b A-B). Moreover, I showed that Amgen54 (a recently published LXR antagonist) and DMHCl (an LXR antagonist made by Dr. Arturo Orellana’s lab) were also able to decrease DEX induced Pepck and G6Pc expression in Lxra/- primary hepatocytes (Supplementary Figure 3.b C-D). In summary, these results indicate that antagonism of LXRβ is able to attenuate GC-induction of the gluconeogenic programme independent of the drug (not only DEX and GSK2033) used in mouse primary hepatocytes.

3.3.2.2 Glucose production studies

To determine whether changes we observed in gluconeogenic enzymes were impacting gluconeogenesis, glucose production studies were carried out in WT, Lxra/- and Lxrβ/- primary hepatocytes. As expected, only WT and Lxra/- primary hepatocytes were able to secrete significant glucose in the medium with DEX treatment. Furthermore, GSK2033 co-treatment significantly attenuated glucose production in WT and Lxra/- primary hepatocytes (Figure 3.3).
Figure 3.3  Co-treatment of GSK2033 with DEX results in repression of glucose production in mouse primary hepatocytes

Glucose output over 6 hrs from WT, Lxra−/− and Lxrb−/− hepatocytes following DEX 500 nM ± 10 μM GSK2033 ligand treatments. Data represent the average of 3 to 5 independent experiments performed in triplicates Avg ± SEM, N= 3-5. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033. One way ANOVA followed by Student- Newman-Keuls.
3.3.3 LXRβ antagonism represses the DEX-induced gluconeogenic programme in vivo

GSK2033 is a dual LXR antagonist. Therefore, to assess the impact of antagonizing LXRβ alone, Lxra-/- mice were used for GSK2033 studies in vivo. Lxra-/- mice were subjected to 5 mg/kg DEX (b.i.d., subcutaneous injection) and 40 mg/kg GSK2033 once daily (intraperitoneal injection) for five days, and sacrificed at lights on. The dose of GSK2033 was determined based on an in-vivo pilot study in Lxra-/- mice, in which the dose dependent repression of liver LXR target genes, ABCG5 and ABCG8, was observed when LXR agonist (T09, 5 mg/kg) and GSK2033 (0, 20 or 40 mg/kg) were co-administered (Supplementary Figure 3.c).

Liver gene expression analyses showed that the DEX-induced expression of several gluconeogenic genes (Pepck, G6Pc, PGC1α and FOXO1) was significantly dampened with GSK2033 co-treatment compared to DEX treatment alone (Figure 3.4 A). FGF21, a starvation hormone that is also a target gene of GR (described in Chapter 4) showed a similar trend. In contrast, DEX-mediated induction of the anti-inflammatory gene Gilz was unaffected by GSK2033 co-treatment. The expression of the nuclear receptors GR and LXRβ remained unaffected by the treatments, indicating that GR and LXRβ do not regulate each other’s mRNA expression (Figure 3.4 A). In this experiment, however, we failed to see any significant changes in the blood glucose levels with DEX treatment compared to vehicle treated mice (Figure 3.4 B). In contrast, treatment with DEX was effective as judged by significant spleen atrophy; a marker of immune suppression, in both DEX and DEX + GSK2033 treated mice relative to the vehicle treated group (Figure 3.4 C). Despite the lack of hyperglycemia, this in vivo study successfully demonstrated that co-treatment of the LXR antagonist GSK2033 with DEX modulates gene selective GR activation in mouse liver.
Figure 3.4  Co-treatment of GSK2033 with DEX results in repression of gluconeogenic gene expression in Lxra−/− mice.

Lxra−/− mice were treated with vehicle (Veh), DEX (5 mg/kg) b.i.d. or GSK2033 40 mg/kg (once daily) along with DEX for 5 days. (A) Liver Pepck, PGC1α, FOXO1, G6Pc,FGF21, Gilz, LXRβ and GR and gene expression measured by RT-QPCR. (B) Plasma glucose levels were measured by a colorimetric kit (Wako). (C) Normalized spleen weight at the sacrifice. Data represent the Avg ± SEM, N=5-7. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033. One way ANOVA followed by Student- Newman-Keuls.
3.3.4 LXRβ antagonism represses DEX-induced \textit{Pepck} promoter activity

To assess whether the interplay between LXRβ and GR was occurring at the level of the promoter, I conducted rat \textit{Pepck} (484bp) -promoter-reporter co-transfection studies in WT primary hepatocytes. As expected with DEX treatment, the \textit{Pepck} promoter was activated in cells transfected with GR alone or in combination with LXR isoforms and RXRα. More importantly, this DEX-mediated activation was significantly dampened by co-treatment with GSK2033 only in the hepatocytes co-transfected with GR, LXRβ, and RXRα receptors, but not with GR, LXRα, and RXRα or GR alone (Figure 3.5). Taken together, these data suggest that LXRβ antagonism disturbances a transcriptional complex required for GR-mediated Pepck activation. In agreement with the literature, these results also imply isoform specific roles for LXRα vs. LXRβ in the regulation of the \textit{Pepck} promoter.
Figure 3.5  Co-treatment of GSK2033 with DEX attenuates Pepck promoter activity in mouse primary hepatocytes when LXRβ is present.

Mouse primary hepatocytes were co-transfected with Pepck promoter luciferase along with the indicated combinations of receptors and Renilla control plasmid. Following 18 hr 100 nM DEX ±10 µM GSK2033 ligand treatment, Pepck promoter activity was measured using the dual luciferase reporter kit. Data show a representative experiment Avg ± SD, N= 3, repeated in 4 to 5 independent experiments. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033. One way ANOVA followed by Student-Newman-Keuls.
3.3.5 How does LXRβ antagonism with GC- treatment supress Pepck transcription?

There are numerous mechanisms by which LXRβ antagonism may decrease GC-mediated upregulation of Pepck. For example, LXRβ could potentially: (1) alter the subcellular localization of GR; (2) inhibit the post-translational modification/activation of GR; (3) alter the recruitment of GR; (4) modulate LXRβ recruitment to the reported LXRα binding site within the Pepck promoter glucocorticoid response unit; (5) alter the recruitment of coregulators; (6) change the chromatin conformation near the Pepck gene; or indirectly, (7) alter the expression or function of a GR co-regulator important for Pepck expression. Below I detail the systematic investigation of these various possibilities.

3.3.5.1 Sub-cellular localization of GR and LXRβ within hepatocytes

Under basal conditions GR is complexed to heat shock proteins in the cytoplasm, and upon DEX treatment, GR undergoes nuclear translocation and accumulation (Stavreva et al. 2009). Therefore, altering the nuclear translocation of GR is one mechanism by which the GC/GR liver response can be modulated. Inhibition of histone deacetylase 6 (HDAC6) activity results in hyperacetylation of Hsp90 and impaired chaperone-dependent activation of GR (Kovacs et al. 2005, Murphy et al. 2005). Recently, Winkler et al found that Hdac6/- mice are protected from DEX-induced gluconeogenesis, insulin resistance and hyperglycemia due to decreased nuclear translocation of GR (Winkler et al. 2012).

To assess whether co-treatment with GSK2033 and DEX disturbed nuclear accumulation of GR, I performed localization studies in primary hepatocytes co-transfected with mouse Venus-GR and mouse Cherry-LXRβ followed by treatment with vehicle, GSK2033, DEX, or DEX + GSK2033. Under basal conditions, LXRβ was primarily localized in the nucleus and GR was both cytoplasmic and nuclear. Upon
DEX stimulation, GR accumulated in the nucleus whereas no change in LXRβ localization was observed (Figure 3.6). Moreover, GSK2033 co-treatment with DEX did not cause export of GR from the nucleus to the cytoplasm or alter LXRβ localization (Figure 3.6). Similar results were also obtained in HEK293 cells co-transfected with mouse Venus-GR and mouse Cherry-LXRβ following treatment with vehicle, GSK2033, DEX, and DEX + GSK2033 (data not shown). Thus, down-regulation of gluconeogenesis by LXRβ antagonism is unlikely to be caused by decreased nuclear translocation of GR.

### 3.3.5.2 Direct interactions with GR

To assess whether GR and LXR were present as part of a protein complex, I performed immunoprecipitation assays followed by LC/MS/MS in collaboration with Dr. Stephane Angers’ laboratory. I generated a stable cell line overexpressing TAP-tagged LXRβ in a rat hepatoma cell system that was responsive to GCs (H4IIE-LXRβ). The GR was not found to be interacting with LXRβ in TAP-purified cell lysates in this high throughput assay (data not shown).

To circumvent the possibility that any protein-protein interactions between GR and LXRβ may be transient and difficult to detect by standard affinity purified IP-LC/MS/MS, Lilia Magomedova (another graduate student in the Cummins lab) performed proteomic profiling of Bir-A tagged GR or LXRβ stably overexpressed in HEK293 cells. Utilizing either LXRβ or GR as bait, neither GR nor LXRβ was found to be interacting with one another following vehicle, GSK2033, DEX, or DEX + GSK2033 treatment in this cell system (data not shown). Taken together, these data support the idea that GR and LXRβ are not directly interacting with one other in this extra-hepatic cell model.
Figure 3.6  Co-treatment of GSK2033 with DEX does not change nuclear accumulation of GR in mouse primary hepatocytes.

Mouse primary hepatocytes were co-transfected with Cherry-LXRβ and Venus-GR. Following 4 hr 100 nM DEX ±10 µM GSK2033 ligand treatments, the cells were fixed and coverslips were mounted on glass slides. The images were taken using ZEISS LSM 700 confocal laser scanning microscope.
3.3.5.3 Phosphorylation status of GR

It was recently shown by Nader et al (2010) that activation of AMPK by AICAR in rats reversed DEX (single dose)-induced gluconeogenesis and hyperglycemia in rats. They have demonstrated that activation of AMPK activates p38MAPK, which hyper-phosphorylates GR at serine 211 to attenuate GC-induced upregulation of Pepck promoter activation in HepG2 human liver carcinoma cells. They also showed a marked increase in GR phosphorylation at serine 232 (the conserved rat GR orthologous site) and activation of p38MAPK in the livers of rats co-treated with AICAR and DEX compared to treatment with DEX alone (Nader et al. 2010). Similarly, since my studies found that GSK2033 co-treatment with DEX inhibited gluconeogenesis in mouse primary hepatocytes, I also assessed nuclear GR phosphorylation (at serine 220, the mouse GR orthologous site) in Lxrα/- primary hepatocytes following DEX± GSK2033 treatment. The results showed nuclear GRS220 phosphorylation did not change with GSK2033 co-treatment compared to DEX alone; whereas, Pepck protein expression was reduced under the same conditions (Figure 3.7 A-B). Thus, the down-regulation of Pepck by LXRβ antagonism is not mediated via hyper-phosphorylation of the mouse GR at S220.
Figure 3.7  Co-treatment of GSK2033 with DEX does not change phosphorylation of GR at S220 in Lxra-/- mouse primary hepatocytes.

(A) Phosphorylation of GR S220 in Lxra-/- mouse primary hepatocytes treated with vehicle (V), 10 μM GSK2033 (G) 100 nM DEX (D) alone or in combination for 4 hrs. (B) Ppck protein expression in Lxra-/- mouse primary hepatocytes treated similarly as (A) for 24 hrs. The blots were quantified using Image J software and normalized to lamin B (A) or β-actin (B). Three independent sets of experiments were performed.
3.3.5.4 *Lxrα*-/- mouse liver perfusion and chromatin immunoprecipitation

To define whether antagonism of LXRβ was selectively modulating the chromatin near the *Pepck* promoter I conducted ChIP studies in *Lxrα*-/- livers perfused in situ via the portal vein with vehicle or DEX ± GSK2033 for 30 minutes. These experiments were performed to test the hypotheses that LXRβ antagonism 1) altered GR recruitment to the *Pepck* promoter in response to DEX; 2) recruited LXRβ to the reported LXRα binding site within the *Pepck* promoter’s glucocorticoid response unit; 3) altered the recruitment of coregulators or 4) closed the chromatin conformation around the *Pepck* promoter.

3.3.5.5 Recruitment of GR to the Pepck GRE

As expected, there was potent recruitment of GR to both the Pepck and TAT GREs in response to DEX (Figure 3.8 A-B). Strikingly, GR occupancy of the Pepck GRE, but not the TAT GRE, was significantly decreased in response to co-treatment of GSK2033 with DEX (Figure 3.8 A-B). RNA polymerase II recruitment to the *Pepck* promoter was also attenuated in response to co-treatment of GSK2033 with DEX (Figure 3.8 C). Taken together, the ChIP and *Pepck* promoter-reporter studies support the notion that LXRβ antagonism destabilizes the GR transcriptional complex at the *Pepck* promoter to dampen GC-induced Pepck expression.
Figure 3.8 Co-perfusion of GSK2033 with DEX decreases GR recruitment to the *Pepck* promoter in *Lxra-/-* mouse liver.

GR recruitment to the (A) Pepck GRE, *Pepck* promoter negative control region and (B) TAT GRE, and (C) RNA polymerase II (RNA Pol II) recruitment to the Pepck GRE assessed by ChIP analysis of *Lxra-/-* livers following 30 min 100 nM DEX ±10 μM GSK2033 ligand perfusion via portal vein. The results are expressed relative to 3% input and normalized to GR recruitment to the Pepck GRE for each protein and genomic location. Data show the average of three independent sets of liver ChIPs (three livers/treatment) performed on different days, error bars represent SEM of N = 3. *P*<0.05 Veh vs. DEX and #*P*<0.05 DEX vs. DEX + GSK2033, one way ANOVA followed by Student-Newman-Keuls.
3.3.5.6 Is LXRβ recruited to the *Pepck* promoter?

To assess whether LXRβ was binding to the promoter we performed LXRβ ChIPs using the DEX ± GSK2033 *Lxra*-/ livers. We were unable to show any significant recruitment of LXRβ beyond background levels at the Pepck proximal promoter; whereas, a significant recruitment of LXRβ to the LXRE of SREBP-1c was observed (Figure 3.9 A-B). These data suggest that LXRβ is not significantly recruited to the *Pepck* promoter in response to DEX or GSK2033.

To circumvent the possibility that the LXRβ antibody was not able to pick up low levels of endogenous *Pepck* promoter-LXRβ interactions, I performed ChIPs from *Lxra*-/ hepatocytes overexpressing the ratPepck (484bp) promoter-reporter construct along with mGR, mLXRβ and mRXRα. Using both a monoclonal (PPMX) and a polyclonal (Santa Cruz) antibody against LXRβ for the ChIP experiments, I still found no significant recruitment of LXRβ to the transfected rat *Pepck* promoter (Supplementary Figure 3.d). However, GR recruitment to the transfected promoter was decreased by GSK2033 co-treatment with DEX compared to DEX alone (Supplementary Figure 3.d).
Figure 3.9  LXRβ is not recruited to the Pepck promoter in Lxrα-/ mouse livers.

LXRβ recruitment to the (A) Pepck GRE, Pepck promoter negative control region and (B) SREBP-1c LXRE assessed by ChIP analysis of LXRα-/ livers following 30 min perfusion of 100 nM DEX ±10 µM GSK2033 via the portal vein. The results are expressed relative to 3% input and normalized to GR recruitment to the Pepck GRE for each genomic location. Data show the average of three independent sets of liver ChIPs (three livers/treatment) performed on different days, error bars represent SEM of N = 3. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033, one way ANOVA followed by Student-Newman-Keuls.
Furthermore, to rule out non-specificities related to the endogenous polyclonal LXRβ antibody, ChIPs were conducted from H4IIE cells stably overexpressing HA-tagged-mLXRβ, using an anti-HA antibody. A robust recruitment of LXRβ was observed at the SREBP-1c LXRE; however, we still saw no recruitment of LXRβ to the Pepck proximal promoter compared to a negative control region upstream (Supplementary Figure 3.e A-B). More importantly, similar to the ChIP results from Lxra/-livers and transfected hepatocytes, GR recruitment to the Pepck GRE was attenuated following GSK2033 co-treatment compared to DEX alone in H4IIE cells (Supplementary Figure 3.e A). Taken together, these results indicate that the nuclear receptor LXRβ is not bound to the proximal Pepck promoter; and LXRβ antagonism may be inhibiting recruitment of a co-regulator that is required for GC-induced Pepck activation.

3.3.5.7 Recruitment of other co-regulators to the Pepck promoter

The Pepck promoter has two low affinity GR binding sites (-387 to -374 and -367 to -353) (Petersen et al. 1988) and therefore, the presence and proper assembly of several co-regulators (including HNF 4α, CREB, C/EBP β, PPARα, RAR, RXR, Torc2, FOXO1, PGC1α, P300 and SRC-1) is a requisite for potent induction of Pepck following GC-treatment (Imai et al. 1993, Chakravarty and Hanson 2007) (Figure 3.10 A). In response to co-perfusion of GSK2033 with DEX, no significant changes in the recruitment of P300, SRC-1, C/EBP β, Torc 2, or RXR were observed at the Pepck promoter (Figure 3.10 B). These data suggest that LXRβ is not influencing the recruitment of these co-regulators to the Pepck promoter.

3.3.5.8 Histone acetylation near the Pepck gene

The promoter of the Pepck gene is normally in an open chromatin conformation that allows GR and co-regulatory proteins to fully engage in gene transcription. Hall et al have shown that basally, the histones associated with the Pepck gene are highly acetylated and a further increase in the acetylation level is not observed after DEX
treatment despite the recruitment of multiple histone acetyl transferases (HATs) to the Pepck gene promoter (Hall et al. 2007). More importantly, the authors showed that the degree of acetylation of histones (H4Ac and H3k9Ac) rapidly decreases after the addition of insulin to DEX-treated H4IIE cells (Hall et al. 2007). Insulin is a well-known dominant suppressor of gluconeogenesis therefore this result is consistent with insulin’s effects. Since the treatment of DEX with GSK2033 also supresses GC-induced gluconeogenesis and Pepck expression; I examined the histone acetylation status in the Lxrα-/ livers treated with DEX ± GSK2033. Three open chromatin conformation marks were examined normalized to total histone content in the same region of DNA: H4Ac/H4, H3k9Ac/H3 and H3K27Ac/H3. The results showed that the acetylation-status of the chromatin near the Pepck promoter remained unchanged in response to DEX and DEX + GSK2033 treatment (Figure 3.10 C). Therefore, in contrast to the effect of insulin, these data suggest that the down regulation of Pepck in response to GSK2033 co-treatment with DEX is not caused by decreasing histone acetylation near the Pepck gene.
Figure 3.10  Co-perfusion of GSK2033 with DEX does not change the acetylation status of the Pepck proximal promoter.

(A) Depiction of the Pepck gene proximal promoter. (B) SRC-1, P300, Torc2, and RXR recruitment to the Pepck +0kb promoter and (C) open chromatin marks: H3K9Ac/H3, H3K27Ac/H3 and AcH4/H4 at the Pepck promoter in livers of Lxra/- mice following 30 min 100 nM DEX ±10 µM GSK2033 ligand perfusion via the portal vein. The results are expressed relative to 3% input and normalized to GR recruitment to the Pepck GRE for each protein. Data show the average of three independent sets of liver ChIPs (three livers/treatment) performed on different days, error bars represent SEM of N = 3. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033, one way ANOVA followed by Student-Newman-Keuls.
3.3.6 GC-mediated immune suppression is not affected by GSK2033 co-treatment in mouse primary macrophages.

Immune suppression is a desired therapeutic effect of GC drugs on the market. Activation of GR by GC represses the expression of genes important for inducing inflammation, and at the same time, increasing the expression of genes involved in the anti-inflammatory response. To assess the ability of GSK2033 to affect GR-mediated inflammatory suppression, I conducted a number of gene expression studies on primary macrophages (thioglycollate-elicited peritoneal macrophages) isolated from WT, \( Lxr\alpha^{-/-} \) and \( Lxr\beta^{-/-} \) mice. As expected, DEX treatment potently decreased LPS induced expression of the inflammatory genes IL-1\(\beta\) and IL-6. This DEX-mediated IL-1\(\beta\) and IL-6 repression was unaffected by GSK2033 co-treatment with DEX (Figure 3.11). Moreover, GR-mediated induction of the anti-inflammatory gene glucocorticoid-induced leucine zipper (GILZ) was unaffected by GSK2033 co-treatment with DEX (Figure 3.11). Thus, DEX-mediated immune suppression is unaffected by LXR antagonism in mouse primary macrophages. As expected, LXR agonist (T09) mediated suppression of IL-1\(\beta\) and IL-6 was observed, and this suppression was antagonized by co-treatment of GSK2033 (Figure 3.11). These data strongly support the idea that although GSK2033 is targeting LXR\(\beta\) in the macrophages it did not antagonize the anti-inflammatory actions of GCs in this system.
Expression of genes important for immune suppression (IL-1β, IL-6 and GILZ) were measured following an overnight treatment with 100 nM DEX ± 250 nM T0901317 (T09) ± 10 μM GSK2033 in LPS activated WT, Lxra/- and Lxrb/- mouse primary macrophages by QPCR. Data show a representative experiment Avg ± SD, N= 3, repeated 3 times. *P<0.05 relative to vehicle, One way ANOVA, Student-Newman-Keuls.
3.4 Discussion

The findings of the present study show that LXRβ antagonism prevents DEX-induction of gluconeogenesis without affecting the immunosuppressive actions of GR.

Previous studies by others have shown that agonism of LXRα is effective at attenuating the GC-induced gluconeogenic programme. In agreement with a published report by Nader et al co-treatment of DEX with T0901317 (an LXR agonist) was able to attenuate Pepck expression in WT and but not in Lxra-/- hepatocytes (Supplementary Figure 3.f A) (Nader et al. 2012). Moreover, glucose production in WT hepatocytes was decreased following T0901317 co-treatment with DEX (Supplementary Figure 3.f B). These results suggest both LXRα and LXRβ are functionally important in mouse hepatocytes for the regulation of GC-induced gluconeogenesis. In light of this, we were surprised to find that GSK2033 co-treatment with DEX attenuated both Pepck expression and glucose production in WT mouse primary hepatocytes, despite GSK2033 being a dual LXRα/LXRβ antagonist.

Transient co-transfection studies showed that antagonism of LXRβ caused decreased activation of the Pepck promoter whereas antagonism of LXRα caused enhanced activation of the Pepck promoter. To circumvent the actions of LXRα, which have been shown to oppose that of LXRβ in the liver, we used Lxra-/- mice for the proof of principle studies shown herein. Liver ChIP studies found that GSK2033 co-perfusion with DEX decreased GR and RNA Pol II recruitment to the Pepck proximal promoter without changing the recruitment of other co-regulators to the promoter (i.e., SRC-1, P300, C/EBPβ, Torc2 or RXR). Furthermore, via ChIP studies, we did not observe any recruitment of LXRβ to the Pepck proximal promoter. We have not been able to identify the exact mechanism via which LXRβ antagonism decreases GR recruitment to the Pepck promoter. Taken together, results presented in this study indicated that direct or indirect interactions of LXRβ with GC-induced co-regulators/ transcription factors
interacting within 484 bp (from the TSS) of the \textit{Pepck} promoter are crucial for GSK2033 mediated downregulation of Pepck.

Additionally, gene expression studies in the mouse primary macrophages and spleen atrophy measurements in the GSK2033 / DEX co-treatment \textit{in vivo} study indicated that GR-mediated immune suppression (a desired effect of GC-therapy) is not affected by LXR antagonism. This is consistent with our published finding, showing LXR\textsubscript{s} are dispensable for GC-mediated immune suppression (Patel et al. 2011).

Each year 10 million new prescriptions are written for oral glucocorticoids in the United States, and the odds ratio for new-onset diabetes in glucocorticoid-treated patients ranges from 1.5-2.5 (Diapedia 2013). The pharmaceutical industry has long been interested in developing GC therapeutics devoid of metabolic side-effects. However, the development of selective GR modulators has been problematic largely because the traditional view that the immunosuppressive actions of GCs are mediated by transrepression of inflammatory genes (II-1\textbeta, II-6) and the metabolic side effects are mediated by transactivation of metabolic genes (Pepck, G6Pc); it turns out that the potent immunosuppressive actions of GCs require both repression of inflammatory genes (IL-1\textbeta, IL-6) and activation of anti-inflammatory genes (GILZ, DUSP14) (De Bosscher 2010). Combined administration of GCs with another therapeutic (not targeting GR) that can mitigate the gluconeogenic effects of GCs is a possible approach to retain the therapeutic effects of existing GCs drugs.

Results from our group and others suggest that both agonism of LXR\textalpha and antagonism of LXR\textbeta are effective at attenuating the GC-induced gluconeogenic program. A major pitfall associated with use of LXR\textalpha activation to control hyperglycemia is the induction of hepatic \textit{de novo} lipogenesis via activation of Srebp-1c, FAS and SCD-1. LXR\textalpha is highly expressed in liver (Repa and Mangelsdorf 2000) and is the dominant LXR isoform influencing hepatic lipogenesis (Quinet et al. 2006) (Figure 3.2 C) (Repa et al. 2000, Quinet et al. 2006). Thus, long term use of LXR\textalpha activation to attenuate GC-
induced gluconeogenesis would aggravate hepatosteatosis (another detrimental side-effect of GC administration). In contrast, use of LXRβ antagonism to attenuate the glycemic effects of GCs would not cause lipogenic effects associated with LXR activation. Additionally, GC-mediated hepatosteatosis might actually be lowered by LXRβ antagonism, since Lxrβ−/− mice are protected from GC-induced lipid accumulation in the liver (Patel et al. 2011).

Another potential caveat to the use of LXRβ antagonists is the atheroprotective role that both LXR isoforms share. LXRα and LXRβ are equally important for cholesterol reverse transport (via activation of ABCA1) in peripheral organs (Repa et al. 2000, Schwartz et al. 2000). One potential strategy to overcome this potential liability is to develop an LXRβ-specific antagonist that is primarily taken up by the liver (high first-pass metabolism) where GC-induced gluconeogenesis occurs. Recently, Griffett et al. (2013) described a molecule termed SR9238 (an ester derivative of GSK2033) as a liver selective LXR dual inverse agonist, and showed that SR9238 treatment protected diet-induced obese mice from hepatosteatosis by decreasing the expression of lipogenic genes in the liver. The authors showed that the expression of the LXR target gene ABCA1 in the periphery was not affected by SR9238 treatment (Griffett et al. 2013).

At present, potent LXR isoform-specific antagonists have not yet been generated. The development of such small molecules has great potential to provide therapeutic benefit to the millions of patients currently taking oral GCs for the treatment of severe inflammatory diseases. In conclusion, this proof of principle study provides evidence that the gluconeogenic and immunosuppressive actions of GR activation can be mechanistically separated by pharmacological antagonism of LXRβ.
3.5 Supplementary Figures

Supplementary Figure 3.a  GSK2033 co-treatment decreased DEX-induced Pepck expression in Lxrα−/− mouse primary hepatocytes independent of new protein synthesis.

Expression of Pepck in Lxrα−/− hepatocytes following 4 hr treatment with vehicle (V), 10 µM GSK2033 (G), 100 nM DEX (D) or 5 µM cycloheximide (CHX) alone or in combination. Data show a representative experiment Avg ± SD, N= 3, repeated 2 times. *P<0.05 Veh vs. DEX and #P<0.05 DEX vs. DEX + GSK2033, by One way ANOVA, Student-Newman-Keuls.
Supplementary Figure 3.b  Co-treatment of LXR antagonist with GCs attenuates Pepck and G6Pc expression in Lxrα-/- mouse primary hepatocytes.

Expression of gluconeogenic genes (A) Pepck and (B) G6Pc in Lxrα-/- hepatocytes following 18 hr treatment with vehicle (V), 10 μM GSK2033 (G), 1 μM cortisol, 1 μM triamcinolone acetonide (Tri), 1 μM prednisone (Pre), and 100 nM DEX (D) alone or in combination. Expression of gluconeogenic genes (C) Pepck and (D) G6Pc in Lxrα-/- hepatocytes following 18 hr treatment with vehicle (V), 10 μM DMHCL (CL), 10 μM Amgen54 (AM), 10 μM GSK2033 (G), and 100 nM DEX (D) alone or in combination. Gene expression was measured using QPCR. Data show a representative experiment Avg ± SD, N= 3, repeated 3 times. *P<0.05 relative to Veh control; #P<0.05 DEX vs. DEX + LXR antagonist by One way ANOVA, Student-Newman-Keuls.
Supplementary Figure 3.c Dose dependent repression of liver LXR target genes ABCG5 and ABCG8 with T09 ± GSK2033 co-treatment.

Lxra-/− mice were injected i.p. with T09 (5 mg/kg) followed by i.p. injection of 20 or 40 mg/kg GSK2033. Data represent (N=1) mouse per treatment. Data represent Avg ± SD of triplicates from QPCR analysis.
**Supplementary Figure 3.d**  
LXRβ is not recruited to the rat *Pepck* promoter in *Lxra/-* mouse primary hepatocytes.

*Lxra/-* primary hepatocytes were co-transfected with the rat Pepck (484 bp) promoter along with GR, LXRβ and RXRa receptors. Following 3 hrs of ligand treatment, ChIP assays were performed using LXRβ polyclonal antibody (SC-N20, Santa-Cruz), LXRβ monoclonal antibody (PP-K8917-10, PPMX) and GR polyclonal antibody (SC-M20, Santa-Cruz). Specific rat *Pepck* promoter primers were used to amplify the template DNA. The results are expressed relative to 3% input and normalized to GR recruitment to the Pepck GRE for each protein. Data represent one experiment as the Avg ± SD of triplicates from QPCR analysis.
Supplementary Figure 3.e  LXRβ is not recruited to the *Pepck* promoter in H4IIE cells stably over-expressing HA-tagged LXRβ.

GR and HA recruitment to (A) *Pepck* promoter and (B) SREBP-1c LXRE. The results are expressed relative to 3% input and normalized to GR recruitment to the *Pepck* GRE for each protein and genomic locations. Data represent one experiment as the Avg ± SD of triplicates from QPCR analysis, repeated twice.
Supplementary Figure 3.f  Co-treatment of LXR agonist with GCs attenuates Pepck expression and glucose production in WT mouse primary hepatocytes.

Expression of Pepck (A) in WT, Lxrα-/- and Lxrβ-/- mouse primary hepatocytes following 18 hr treatment with Veh, 250 nM T0901317 (T), 100 nM DEX alone or in combination. (B) Glucose output over 8 hrs from WT hepatocytes following DEX 500 nM ± 250 nM T0901317 treatment. Data show one experiment Avg ± SD, N= 3. *P<0.05 relative to Veh control; #P<0.05 DEX vs. DEX + T09 by One way ANOVA, Student-Newman-Keuls.
Chapter 4  Glucocorticoid receptor directly regulates hepatic expression of fibroblast growth factor 21 (FGF21).

This chapter was included as part of the following manuscript:


**Author contributions**

**Rucha Patel:** Conducted the in-vivo studies presented in Figure 4.1, the primary hepatocyte studies in Figure 4.3 and ChIP studies in Figure 4.4. I also conducted FGF21 proximal promoter transient co-transfection reporter studies (data not shown). I conducted in-silico analysis of the genomic region near the FGF21 gene to find putative for GR binding sites.

**Angie L. Bookout:** Conducted *PPARα/-* DEX treatment studies presented in Figure 4.2.

**Lilia Magomedova:** Cloned the truncation and mutation reporter constructs surrounding the putative GR binding region near the FGF21 gene and conducted transient co-transfection reporter studies presented in Figure 4.5.
4.1 Introduction

Fibroblast growth factor 21 (FGF21) is an atypical member of the fibroblast growth factor family that acts in an endocrine manner in contrast to other members of the family that have autocrine or paracrine actions. FGF21 is predominantly secreted by the liver and unlike many other family members, FGF21 does not bind heparin, allowing its circulation in the bloodstream (Nishimura et al. 2000, Kharitonenkov et al. 2005, Badman et al. 2007). To exert a physiologic response via the FGF receptors (FGFR1-4), FGF21 requires β-klotho as a co-receptor. The limited tissue expression pattern of β-klotho dictates tissue specific FGF21 responsiveness (Goetz et al. 2007, Kharitonenkov et al. 2008, Fisher et al. 2011, Ding et al. 2012). Unlike the classical members of the FGF family, FGF21 does not exhibit mitogenic activity, but acts as an endocrine factor involved in the regulation of energy homeostasis, insulin sensitivity, and glucose and lipid metabolism (Kharitonenkov et al. 2005, Domouzoglou and Maratos-Flier 2011). In rodents, elevating circulating FGF21, by either systemic infusion of the recombinant protein or transgenic expression, counteracts obesity-related metabolic disorders, including hyperglycemia, dyslipidemia, insulin resistance, and fatty liver disease (Kharitonenkov et al. 2005, Domouzoglou and Maratos-Flier 2011).

Similar to many other metabolic hormones, FGF21 levels are regulated by circadian rhythm and nutritional status in both rodents and humans (Tong et al. 2010, Wang et al. 2010, Yu et al. 2011). During prolonged fasting and in response to a ketogenic (high fat, low carbohydrate) diet, hepatic FGF21 expression is markedly induced through a mechanism that involves the activation of peroxisome proliferator-activated receptor α (PPARα) by elevated levels of free fatty acids (Badman et al. 2007, Inagaki et al. 2007). Previous studies have demonstrated a critical role of FGF21 in the regulation of gluconeogenesis and ketogenesis during the adaptive response to starvation (Potthoff et al. 2009). A number of studies have demonstrated that PPARα is necessary for the basal expression of FGF21 and pharmacological activation of PPARα further induces FGF21 expression in the liver (Badman et al. 2007, Inagaki et al. 2007, Muise et al. 2008, Oishi et al. 2008). Several other signaling molecules including insulin, glucagon,
thyroid hormone, growth hormone and bile acids have also been shown to regulate FGF21 expression (Adams et al. 2010, Berglund et al. 2010, Tong et al. 2010, Chen et al. 2011, Cyphert et al. 2012).

In normal physiology, stress-triggered activation of the hypothalamic-pituitary-adrenal (HPA) axis induces GC synthesis and secretion from the adrenal cortex, which is tightly regulated by feedback inhibition (Vegiopoulos and Herzig 2007). The essential role of GCs is to supply enough glucose into the circulation to fuel the brain and ensure survival of the organism under conditions of acute stress or reduced food intake. In response to elevated GCs, hepatic glucose production is augmented and breakdown of fat and muscle is initiated to provide additional substrates for glucose production (Vegiopoulos and Herzig 2007). In Cushing’s patients and patients taking GC drugs to manage severe inflammatory conditions, chronic activation of GC-GR signalling causes insulin resistance, central obesity, hepatosteatosis and diabetes (Rockall et al. 2003, Shibli-Rahhal et al. 2006).

Glucocorticoids and FGF21 are both endogenous catabolic hormones. There are several lines of evidence suggesting that the FGF21 and GC signaling pathways are interconnected. First, the early morning rise in serum cortisol has a strikingly similar pattern to that of serum FGF21 in healthy humans (Yu et al. 2011). Second, both circulating FGF21 and endogenous GCs are elevated in mouse models of obesity and type 2 diabetes (db/db and ob/ob) (Ohshima et al. 1984, Shimomura et al. 1987, Lundasen et al. 2007, Zhang et al. 2008, Badman et al. 2009, Livingstone et al. 2009, Hale et al. 2012). Third, osteoporosis is a detrimental side effect of pharmacological administration of GCs as well as FGF21 (Lukert and Raisz 1994, Wei et al. 2012). Fourth, hepatic gluconeogenic genes induced by GCs (i.e., PGC1α, Pepck, G6Pc, Igfbp1) are also induced by FGF21 treatment (Badman et al. 2009, Potthoff et al. 2009). Additionally, several studies have demonstrated that deletion of PPARα does not completely suppress the induction of FGF21 by a ketogenic diet or by starvation, suggesting that additional signaling pathways are involved in modulating the nutritional regulation of FGF21 expression (Badman et al. 2007, Inagaki et al. 2007). Experiments
using pharmacologic doses of FGF21 have shown strong anti-inflammatory, anti-diabetic and hypolipidemic effects of FGF21 administration in both rodents and primates, thus, there has been significant interest in understanding the endogenous regulators of FGF21 expression (Kharitonenkov et al. 2005, Wente et al. 2006, Kharitonenkov et al. 2007). While analyzing microarray data from our laboratory, we discovered that FGF21 expression in the liver was induced in wildtype mice by 5 day treatment with dexamethasone (a synthetic GC); and along the same time Laskewitz et al showed that 7 days Prednisolone (a synthetic GC) treatment increases hepatic FGF21 expression in wildtype mice during both fed and fasted state (Laskewitz et al. 2010).

Therefore, we investigated whether FGF21 was directly regulated by GCs. Herein, we show that GCs indeed regulate hepatic FGF21 expression in a GR-dependent and cycloheximide-independent manner. We further demonstrate that GR’s induction of FGF21 is not dependent on, but instead additive with, PPARα-mediated activation.
4.2 Methods

4.2.1 Animal experiments

All procedures and use of animals were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center in Dallas or the Faculty of Medicine and Pharmacy Animal Care Committee at the University of Toronto. Mice were maintained in a temperature and light-controlled environment. Unless otherwise stated in specific dietary studies, all animals were maintained on 2016/2916 Global Diet (Harlan Teklad). Male mice were used in all studies. Animals were sacrificed by rapid decapitation to avoid activation of the HPA axis via the stress response. Wildtype (WT) (129SvEv strain) mice were intraperitoneally injected with dexamethasone (DEX) 5 mg/kg and WY14643 20 mg/kg (10% EtOH in saline) alone or in combination at 20 hrs and 6 hrs prior to sacrifice. WT and Ppara\(^{-/-}\) mice (C57Bl/6) were subcutaneously injected with DEX (5% EtOH in sesame oil) at 2.5 mg/kg bid for 5 days. Tissues were collected, snap-frozen in liquid nitrogen, and stored at -80°C until assayed.

4.2.2 Plasma analyses

Trunk blood was collected into K\(^+\)EDTA tubes on ice, and plasma was separated and stored at -80°C until assayed. Plasma FGF21 levels were measured using an ELISA kit (Biovendor) according to the manufacturer’s instructions.

4.2.3 Mouse primary hepatocyte studies

Primary hepatocytes were isolated from 7 to 10 week old WT mice. Mice were anesthetized with isoflurane and the portal vein cannulated with a 24G angiocatheter (BD science). Using a peristaltic pump, the liver was perfused at a rate of 4 mL/min with 30 mL perfusion buffer (Invitrogen) and 30 mL collagenase (Invitrogen) digestion.
buffer. Thereafter, the liver surface capsule was peeled off with forceps and the cells were dispersed in a low glucose DMEM wash medium. The hepatocytes were separated from other cell types via low gravity (50xg) centrifugation for 3 minutes. The cells were washed three times, thereafter, counted, checked for viability, and plated on collagen I–coated plates in William’s E medium supplemented with 10% ssFBS, 10 nM insulin and 100 U/mL penicillin/streptomycin. After 2-3 hours, cells were washed with PBS twice and the medium was changed to M199 supplemented with 10% ssFBS, 1 nM insulin and 100 U/mL penicillin/streptomycin. The cells were allowed to grow overnight in a 37°C incubator, all the experiments were started the next day. For gene expression analysis cells were plated in 6-well plates at a density of 4 x 10⁵ cells/well. For luciferase reporter assays cells were plated in 48 well plates at a density of 8 x 10⁴ cells/well. Ligands were added to the cells in M199 medium without FBS and cells were harvested 9 hrs after ligand treatment for RNA extraction.

4.2.4 Liver chromatin immunoprecipitation analysis

ChIP was carried out using the EZ ChIP kit (Millipore). Livers were perfused in situ for 30 min via the portal vein with either vehicle or 10 nM DEX in perfusion buffer. Whole liver was minced and cross-linked in 1% formaldehyde containing PBS, 1 mM DTT and 1 mM PMSF for 10 min at RT. Cross-linking was stopped by addition of glycine to a final concentration of 125 mM for 5 min at RT, followed by centrifugation and washing the pellet twice in ice-cold PBS containing 1 mM DTT and protease inhibitors (Complete, Roche). Liver nuclei were recovered by dounce homogenization in a hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2% Nonidet P-40, 0.2 mM sodium orthovanadate, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose, 1 mM DTT and protease inhibitors) and layered onto a cushion buffer (10 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, 1 mM DTT and protease inhibitor)
followed by centrifugation. The nuclear pellet was washed with cold PBS and resuspended in 2 mL sonication buffer (0.75% SDS, 2 mM EDTA and 50 mM Tris-HCl, pH 8.0). The chromatin was sheared to 200-1000 bp by sonication. The sonicated chromatin was diluted 7.5 fold in dilution buffer (Millipore) and 800 μL of diluted sample per immunoprecipitation was used. After 1 hr pre-clearing with protein G-agarose beads (100 μL/IP), 10 μg of GR (M-20) antibody (Santa Cruz Biotechnology Inc.), 1 μg of H3K27Ac (abcam) or 1 μg H3 (Millipore) were added for overnight incubation. Protein G agarose (50 μL) was used to recover the immune complexes (2 hrs at 4°C). Washes and elutions were performed in accordance with the ChIP kit. DNA was reverse-crosslinked overnight at 65°C, RNase treated for 30 min at 37°C, proteinase K treated for 2 h at 45°C and purified using a spin column to a final volume of 50 μL. The eluate was diluted 5-fold with water and QPCR was performed using 1.2 μL of template DNA with the FGF21 gene primers listed in Table 4.1. Quantitation was performed by QPCR (standard curve method) using serial dilutions of the input as standards (Bookout et al. 2006).
Table 4.1  ChIP FGF21 gene walking QPCR primers

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4.2.5 RNA isolation, cDNA synthesis, and real-time QPCR analysis

Total RNA was extracted from cells and tissues using RNA STAT-60 (Tel-Test Inc., Friendswood, TX), treated with DNase I (RNase-free; Roche), and reverse transcribed into cDNA with random hexamers using the High Capacity Reverse Transcription System (ABI; Applied Biosystems). Primers used were: cyclophilin (NM_000942.4) forward: 5’ggagatggcacaggaggaa3’ reverse: 5’gcccgtagtgcttcagttt’3 FGF21 (NM_020013.4) forward: 5’cctctaggtttctttgccaacag3’ reverse 5’aagctgcaggcctcaggat3’. Real-time quantitative PCR (QPCR) reactions were performed on an ABI 7900HT in 384-well plates containing 12.5 ng cDNA, 150 nM of each primer, and 5 µl 2X SYBR Green PCR Master Mix (ABI) in a 10 µl total volume. Relative mRNA levels were calculated using the comparative Ct method (Bookout et al. 2006).

4.2.6 FGF21 reporter constructs and primary hepatocyte luciferase reporter assays

The pCMX and pCMX-mGR were from Dr. David Manglesdorf (University of Texas Southwestern Medical Center, Dallas, TX), pSL9-renilla was from Dr. Stephane Angers (University of Toronto, Toronto, Canada), pGL4.10-E4TATA was from Dr. Eric Bolton (University of Illinois at Urbana-Champaign, Urbana, IL). Specific mouse FGF21 promoter regions were amplified from genomic mouse DNA using Platinum Taq High-Fidelity DNA Polymerase (Invitrogen) and cloned into the pGL4.10-E4TATA vector using Kpn I and Xho I sites. Promoter deletion and mutant constructs were generated using Quik Change Site-Directed Mutagenesis Kit (Agilent). Oligonucleotides with three copies of the putative GR binding sites (GBSs) and the corresponding mutants were synthesized with Kpn I - Xho I sites (see Table 4.2 for oligonucleotide sequences). Annealed oligonucleotides were then cloned into the pGL4.10-E4TATA vector.
For FGF21 gene reporter activation assays the primary hepatocytes were transfected following Lipofectamine 2000 (Invitrogen) protocol in OPTI-MEM medium. The total amount of plasmid DNA (250 ng/well) included 125 ng - FGF21 gene fragment reporter plasmids, 50 ng *Renilla* plasmid, and 25 ng of pCMX-mGR plasmid, 50 ng of filler CMX plasmid. Six hours after transfection, medium was changed to M199 medium supplemented with 10% ssFBS, 1 nM insulin and 100 U/mL penicillin/streptomycin. The next evening, ligands were added in M199 (without FBS). Cells were harvested 20 hours later in passive lysis buffer (Promega) and luciferase and *Renilla* activities were measured using a dual luciferase kit (Promega). Luciferase values were normalized to *Renilla* to control for transfection efficiency and expressed as Relative Luciferase Units (RLU).

**Table 4.2   WT and mutant GBS of the FGF21 gene locus**

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<td>GBS&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>GGAACACTCAGGACAGTCGACGGAACACTCAGGACAGTCGACGGAACACTCAGGACA</td>
</tr>
<tr>
<td>GBS&lt;sub&gt;mu1&lt;/sub&gt;</td>
<td>GGTTCACTCAGGACAGTCGACGGAACACTCAGGACAGTCGACGGAACACTCAGGACA</td>
</tr>
<tr>
<td>GBS&lt;sub&gt;mu2&lt;/sub&gt;</td>
<td>GGAACACTCACCACAGTCGACGGAACACTCACCACAGTCGACGGAACACTCACCACA</td>
</tr>
<tr>
<td>GBS&lt;sub&gt;mu3&lt;/sub&gt;</td>
<td>GGTTCACTCACCACAGTCGACGGAACACTCACCACAGTCGACGGAACACTCACCACA</td>
</tr>
</tbody>
</table>

4.2.7 Statistical analyses

Data are presented as mean ± SEM unless otherwise indicated. GraphPad Prism was used for ANOVA followed by multiple comparisons testing as appropriate; or by two-tailed Student’s *t*-test. *P*<0.05 was considered significant.
4.3 Results

4.3.1 GCs induce FGF21 in vivo

Endogenous GCs are increased during the adaptive phase of fasting (Makimura et al. 2003, Kim et al. 2008), and given the important role of FGF21 during starvation (prolonged fasting), we assessed whether GCs themselves could contribute to increased FGF21 expression. Because fasting also induces the levels of glucagon and GH, we decided to explicitly test the role of GCs on FGF21 expression in vivo using synthetic agonists of GR and PPARα. Wildtype mice were injected acutely with DEX or the PPARα agonist WY 14643 (WY) alone or in combination and analyzed the following day for FGF21 gene and protein expression. Hepatic FGF21 expression was induced 7-fold by DEX or WY treatment alone. Notably, FGF21 expression increased in an additive (15-fold) manner when the mice were co-treated with DEX and WY (Figure 4.1A). Moreover, in parallel with gene expression, plasma FGF21 levels were elevated in an additive manner following DEX and WY co-treatment (Figure 4.1B).
Wild-type mice (WT) were administered with Vehicle (Veh), DEX (5 mg/kg) and WY14643 (WY) 20mg/kg alone or in combination twice at 20 hrs and 6 hrs prior to sacrifice. (A) Liver FGF21 gene expression measured by RT-QPCR. (B) Plasma FGF21 levels were measured by an ELISA kit (Biovendor). (A-B) Data represent the Avg ± SEM, N=4-6. * Comparison of Veh vs. DEX or WY groups <0.05 and # comparison of DEX vs. DEX + WY groups P<0.05, by One way ANOVA, Student-Newman-Keuls.
To assess whether GR required the presence of PPARα to mediate GC-induced FGF21 induction, WT and Ppara-/- mice were treated with DEX for 5 days. Ppara-/- mice were included in this experiment due to the prominent role of PPARα in basal and fasting induced regulation of FGF21 (Inagaki et al. 2007). Notably, liver FGF21 levels were increased 7-fold with DEX compared to vehicle in WT mice. Similarly, Ppara-/- mice showed a significant induction with DEX (12-fold) but the absolute level of FGF21 remained below that of the WT vehicle treated mice (Figure 4.2A). The significantly different baseline levels of FGF21 in WT vs Ppara-/- mice are in agreement with previously published data that support the idea that PPARα is required for maintaining constitutive FGF21 expression (Figure 4.2A). In agreement with the gene expression, circulating FGF21 levels were increased by 15-fold in DEX-treated WT mice (Figure 4.2B). Consistent with the lower gene expression levels of FGF21 in the Ppara-/- mice, we were unable to detect FGF21 in the plasma of Ppara-/- vehicle-treated animals, but FGF21 was detectable in DEX-treated Ppara-/- mice (Figure 4.2B). Taken together, these results indicate that in addition to PPARα, GR activation significantly induces FGF21 expression in mice.
Figure 4.2  FGF21 expression is induced by DEX in PPARα-/− mice.

WT and PPARα-/− mice were treated with DEX 2.5mg/kg bid for 5 days (A) Liver FGF21 gene expression measured by RT-QPCR. (B) Plasma FGF21 levels were measured by an ELISA kit (Biovendor). (A-B) Data represent the Avg ± SEM, N=4-6. * Comparison of Veh vs. DEX groups <0.05 and # comparison of WT vs. PPARα -/- groups P<0.05, by One way ANOVA, Student-Newman-Keuls.
4.3.2 GCs induce FGF21 in primary hepatocytes

To assess whether GCs mediate their effects on FGF21 directly at the level of the liver, and not through altered signaling from other metabolic tissues, we performed experiments in mouse primary hepatocytes. Treatment of hepatocytes for 9 hrs with 100 nM DEX increased FGF21 levels 5-fold (Figure 4.3 A). This response was completely attenuated by co-treatment of DEX with the GR antagonist RU486 (Figure 4.3 A). Consistent with a direct role for GR in mediating FGF21 induction, treatment with the protein synthesis inhibitor cycloheximide did not influence the magnitude of GC-mediated FGF21 induction (Figure 4.3 A). Additionally, we treated mouse primary hepatocytes with either DEX or the synthetic PPARα agonist WY-14643 (WY). Treatment with saturating doses of DEX (100 nM) or WY (1 μM) alone resulted in a similar level of FGF21 induction (4 24 to 5-fold); however, co-treatment with DEX + WY resulted in a 20-fold induction of FGF21 (Figure 4.3 B). Thus, our studies in primary hepatocytes suggest GR is directly contributing to the induction of hepatic FGF21 in a cell autonomous manner.
Figure 4.3  FGF21 induction by DEX is GR dependent and liver autonomous.

(A-B) Primary hepatocytes were isolated from wildtype mice, plated on 6-well collagen-coated plates and treated with indicated ligands for 9 hrs. FGF-21 gene expression in the hepatocytes was measured by real-time QPCR. Data show a representative experiment Avg ± SD, N=2-3, repeated at least 3 times. * compared to Veh group P<0.05 by One way ANOVA, Student-Newman-Keuls.
4.3.3 The glucocorticoid receptor binds and activates FGF21 via an atypical GRE downstream of the FGF21 transcription start site

To confirm that GR was indeed acting directly at the FGF21 gene locus we performed a gene promoter walking analysis using ChIP. Livers of WT mice were perfused in situ with either vehicle or 10 nM DEX for 30 mins. After perfusion, the livers were fixed and processed for ChIP with lysates incubated either with no antibody or an antibody selective for GR. By designing primers at approximately 1kb intervals along the length of the FGF21 gene (from -10kb to +10kb surrounding the TSS) we determined that GR was being selectively recruited in a ligand dependent manner to at least two regions that are approximately -1kb and +5kb from the transcription start site (TSS) (Figure 4.4A). Recent liver ChIP-seq studies have shown that nuclear receptors tend to bind to open chromatin regions in the genome. Given that GR binding was detected at +5kb, outside the FGF21 promoter, we next examined whether this region was in an open chromatin conformation by performing ChIP against H3K27 acetylation and normalizing these results to the total histone 3 (H3) content. There was significantly more H3K27 acetylation at -1kb and +5kb compared to the non-GR binding site (negative control region) at -2.9kb (Figure 4.4B) demonstrating that these regions are in an open conformation.
Figure 4.4  GR binds to two regions of open chromatin surrounding the FGF21 gene locus.

(A) Chromatin immunoprecipitation assay demonstrating GR recruitment to the FGF21 gene locus at -1kb and +5kb relative to the transcription start site (TSS, 0 kb) after DEX treatment. *P<0.05 compared to GR vehicle. (B) Acetylation of H3K27 relative to total H3 content at -2.9 kb (negative control region), -1 kb and +5 kb from the TSS of mFgf21 gene was assessed by real-time QPCR. Data are from a representative experiment (Avg ± SD, N=3) repeated in independent sample sets at least 3 times. *P<0.05 compared to the level of H3K27Ac/H3 at -2.9kb. One way ANOVA followed by Student-Newman-Keuls.
To assess whether the putative GR binding site in the FGF21 promoter was responsible for DEX induction, we performed co-transfection assays in CV-1 cells using a -1.4kb FGF21 promoter luciferase construct that was previously reported to be PPARα responsive (Inagaki et al. 2007). We confirmed PPARα-mediated activation of this reporter; however, we saw no significant change in reporter activation when cells were co-transfected with GR and treated with DEX (data not shown). Using nuclear receptor binding site prediction software (NUBIscan (Podvinec et al. 2002)), we found that the region between +4kb and +5kb of the FGF21 gene contained two high scoring putative nuclear receptor binding sites. We hypothesized that either the direct repeat 3 (DR3) element (GGAACActcAGGACA) located between +4388 bp and +4374 bp or the inverted repeat 4 (IR4) element (TGTACAaatgTGTACA) located between +4721 bp and +4705 bp could be atypical glucocorticoid receptor binding sites (GBS). We subcloned this genomic region (+4.8kb to +4.3 kb) into an enhancer trap pGL4.10-E4TATA vector plasmid. In transient transfections of mouse primary hepatocytes, deletion or mutation of the DR3, but not the IR4, markedly reduced the ability of DEX to stimulate FGF21 gene reporter activity (Figure 4.5A). To establish whether this novel GBS was sufficient to induce GR activation, we cloned three repeating units of the DR3 GBS upstream of the E4TATA promoter. DEX-treatment of transiently transfected hepatocytes with the WT 3xGBS construct showed a 51-fold induction in luciferase activity compared to Veh-treated cells (Figure 4.5B). In contrast, all of the mutated 3xGBS constructs had significantly attenuated responses to DEX (Figure 4.5B). These findings indicate that ligand-bound GR activates FGF21 gene transcription by binding to an atypical DR3 binding site located ~4.4kb downstream of the transcription start site.
Figure 4.5  GR activates transcription via an atypical GR binding site 4.4kb downstream of the FGF21 TSS.

(A) Mouse primary hepatocytes were transiently transfected with a series of plasmids containing fragments of the mouse FGF21 gene harboring putative GR binding site (GBS), deleted GBS or mutated GBS near the FGF21 gene locus linked to a luciferase reporter along with mouse GR. 24 hrs after transfection, cells were treated with 100 nM DEX for 18 hrs and FGF21 GBS activity was measured using the dual luciferase reporter kit (Promega). Left, the constructs used in these experiments. In truncation studies of the FGF21 gene fragment reporter, black boxes represent potential GBS sites, striped box represents mutated GBS (sequence shown) and ——^— denotes the construct with putative GBS deleted. (B) In mutation studies of 3x GBS reporters, mu1, mu2 and mu3 are different site mutations (sequences shown) of the FGF21 gene GBS between (+4388 bp and +4374 bp). Data are from a representative experiment Avg ± SD, N=3, repeated at least 3 times. *P<0.05 Comparison of Veh vs. DEX groups and #P<0.05 comparison of WT vs. mutant or deleted GBS by One way ANOVA followed by Student-Newman-Keuls.
4.4 Discussion

In the present study we identify a new signaling pathway that induces hepatic FGF21 expression. We show that ligand activation of GR directly regulates FGF21 gene expression and that this induction is additive when combined with the basal and inducible regulation of FGF21 by PPARα. We observed GR binding to the open chromatin region near the FGF21 gene using ChIP from mouse livers perfused with DEX. GR binds directly to the FGF21 gene at a site distinct from that of PPARα and results in an additive effect on FGF21 expression when the ligands for both receptors are present. Co-transfection studies of truncated and mutated FGF21 gene reporters showed that GR binds to the nucleotide sequence GGAACActcAGGACA located +4.4kb downstream of the FGF21 transcription start site. This GR regulatory sequence is atypical, in that it is not an IR-3, but is instead a DR-3. However, the key central nucleotides in the response element ACAxxxAGG have previously been shown to be over-represented in ChIP-seq studies performed with GR (Grontved et al. 2013). Additionally, the androgen receptor, another steroid hormone receptor, has been shown to bind to a non-consensus DR3 site (Shaffer et al. 2004).

The levels of numerous endocrine hormones are altered in response to prolonged fasting in order to effectively manage the crucial transition between the fed and fasted state (e.g., insulin, leptin, ghrelin, glucagon, glucocorticoids, GH and FGF21). There is a high degree of overlap in the functions of these hormones, which we perceive as a natural ‘built-in’ redundancy to ensure survival of the organism through prolonged periods of nutrient deprivation (Suemaru et al. 1986, van den Berghe 1991, Lemberger et al. 1996, Kersten et al. 1999, Makino et al. 2001). With this objective in mind, it makes intuitive sense that GCs and FGF21, which are both elevated in the long-term response to fasting and positively regulate gluconeogenesis, feed-forward regulate each other’s synthesis. Hormones that are elevated earlier in the fasting response, such as glucagon and growth hormone (GH), have also been shown to increase FGF21 expression. While glucagon induces FGF21 via activation of AMPK and PPARα (Berglund et al. 2010), GH has been shown to regulate hepatic FGF21 expression both
directly by activating STAT5 in liver (Yu et al. 2012) and indirectly by inducing lipolysis in the adipose (Chen et al. 2011). The apparent cooperativity in the continuum of endocrine hormones involved in the upregulation of FGF21 supports a pivotal role for this factor in a broad physiologic context. Indeed, FGF21 has recently been implicated in modulating growth and reproduction, two key physiologic functions that are repressed with starvation. While GH normally activates STAT5 in the liver to increase the levels of IGF-1 in circulation, under conditions of prolonged fasting, IGF-1 levels are decreased because FGF21 signaling interferes with STAT5 activation (Inagaki et al. 2008). In reproduction, FGF21 was shown to inhibit the luteinizing hormone surge required for ovulation by inhibiting the vasopressing-kisspeptin axis in the suprachiasmatic nucleus (Owen et al. 2013). Upon nutrient availability, a post-prandial increase in insulin promotes the recruitment of the transcriptional repressor E4-binding protein 4 (E4BP4) to the FGF21 promoter and represses FGF21 gene expression (Tong et al. 2010).

In addition to the data reported in our study, there are several lines of evidence that support a role of GCs in the regulation of FGF21. A clinical study of patients with Cushing’s syndrome, a condition of elevated endogenous glucocorticoids, had elevated FGF21 levels; however, FGF21 levels were not linearly correlated with circulating cortisol levels in these patients (Durovcova et al. 2010). Mice treated with the synthetic GC prednisolone for 7 days were found to have a 2-fold increase in FGF21 levels compared to vehicle treated mice (Laskewitz et al. 2010). Consistent with a role for glucocorticoids in modulating the inducible levels of FGF21, human circadian studies have demonstrated that FGF21 is temporally regulated, with the highest levels appearing in the early morning (Andersen et al. 2011, Yu et al. 2011). This pattern correlates tightly with the 24 hr oscillatory pattern of free fatty acids and cortisol (Wildenhoff et al. 1974, Yu et al. 2011). Based on our primary hepatocyte and in vivo mouse experiments, the co-incident increase in these metabolic regulators of PPARα and GR are likely to both contribute to diurnal FGF21 expression.
In this study, we have identified a novel mechanism where, in response to increased GC signalling, GR directly binds to the FGF21 gene locus and regulates hepatic FGF21 expression in mice. Given this finding, we hypothesize that one explanation for the paradoxical finding that genetically obese mice have high circulating FGF21 levels may be their high circulating and/or intra-tissue corticosterone levels (Ohshima et al. 1984, Shimomura et al. 1987, Friedman et al. 1997, Liu et al. 2005). Since humans with insulin resistance and fatty liver are also thought to have enhanced intra-tissue GC signaling (Andrews et al. 2003, Torrecilla et al. 2012), we further speculate that this novel regulatory mechanism may be contributing to the elevated FGF21 levels in these patients (Zhang et al. 2008, Mraz et al. 2009, Li et al. 2010).
Chapter 5  Limitations and future directions

Model systems used in biomedical research to probe human diseases are not perfect; therefore, it is important to address a few limitations of the studies presented, and possible areas of improvement.

5.1  Glucocorticoid effects on peripheral tissues

Glucocorticoid therapy causes insulin resistance in the liver, muscle, adipose and bone, which collectively contribute to GC-mediated hyperglycemia. Studies presented in this thesis were focused almost exclusively on the liver contribution to GC-mediated hyperglycemia; thus, the role of LXRβ in GC-induced insulin resistance in other tissues should be investigated. Throughout the different in vivo experiments, I collected other GC-affected metabolic tissues including adipose, muscle, and bone. These tissues will be used for future studies investigating this question.

There are several circulating factors secreted from adipose such as adiponectin and leptin; bone (i.e., osteocalcin) that can also promote insulin sensitivity of peripheral tissues. We have not measured these endocrine hormone levels in vivo following GC administration. Notably, these hormones have been shown to be dysregulated by GC administration and contribute to whole body insulin resistance (Mazziotti et al. 2011, Brennan-Speranza et al. 2012, Sukumaran et al. 2012).

Glyceroneogenesis, the synthesis of the glycerol moiety of triacylglycerol from pyruvate, has been shown to be quantitatively important in both the liver and adipose for fatty acid-triglyceride cycling. In fact, using the tracer chase technique, Kalhan et al showed that in overnight fasted human subjects 50-60% of glyceride-glycerol in triglyceride isolated from VLDL was derived from hepatic glyceroneogenesis (Kalhan et al. 2001). Additionally, another study in which rats were subjected to either a chow
diet, a 48 hr fast, or a high sucrose diet, reported that glyceroneogenesis accounted for 60% of triglyceride glycerol synthesis in the liver under each dietary condition (Nye et al. 2008). The Pepck enzyme catalyses a key step common to both gluconeogenesis and glyceroneogenesis in the liver. GC-mediated lipid metabolism is complex and involves futile cycling of triglycerides among multiple tissues. GCs act in the liver to increase fatty acid synthesis and decrease fatty acid oxidation, which leads to hepatic fat accumulation (Cole et al. 1982). In Chapter 2 we showed that Lxrβ-/- and Lxraβ-/- mice were protected from GC-mediated fatty liver and hyperglycemia. However, we were unable to find the exact mechanism via which the GC-treated Lxrβ-/- and Lxraβ-/- mice were protected from the fatty liver phenotype. Notably, we showed that GC-treatment fails to induce Pepck expression in Lxrβ-/- and Lxraβ-/- hepatocytes. Given the contribution of hepatic glyceroneogenesis to triglyceride synthesis, a plausible hypothesis that could be tested in future studies is that the Lxrβ-/- and Lxraβ-/- mice are protected from fatty liver due to insufficient glyceroneogenesis. An elegant tracer pulse chase study could be designed in DEX-treated WT and Lxr-null mice to test this hypothesis.

5.2 Potential pitfalls of pharmacological agents

A common caveat associated with the use of pharmacological agents to target a protein function is cross-reactivity with other proteins. In my studies, I used the potent synthetic glucocorticoid DEX to assess the glycemic phenotype caused by prolonged GR activation in vivo and in various cell-culture models. In mice and humans, DEX has been shown to activate the pregnane X receptor (PXR) when used at a high concentrations (>1 µM) (Kliewer et al. 1998, Lehmann et al. 1998). Therefore, to avoid PXR activation, we used 10-100 nM of DEX for our in vitro studies. In our in-vivo DEX- administration studies, plasma concentrations of DEX were measured by LC/MS/MS and found to be between 250-500 nM. In transient transfection reporter assays, these concentrations of DEX activate GR but not PXR (Kliewer et al. 1998, Lehmann et al. 1998). Notably, activation of PXR has been shown to suppress gluconeogenesis via nuclear exclusion of FOXO1 and dissociation of PGC1α from the
Pepck gene promoter (Bhalla et al. 2004, Kodama et al. 2004). In contrast, we observe the expected GR-mediated increase in Pepck and gluconeogenesis in WT mice. Moreover, GR has been shown to directly regulate expression of the nuclear receptor PXR (Bhadhprasit et al. 2007); however, based on our liver gene expression analysis, the expression of PXR is similar in WT and LXR-null mice following DEX treatment. These observations indicate that in our DEX-induced metabolic syndrome model, the nuclear receptor PXR is not a major factor contributing to the glycemic phenotype.

In our co-treatment studies with DEX and LXR antagonist we used a dual-LXRα/LXRβ antagonist, GSK2033. To the best of my knowledge at this time, potent LXRβ-specific antagonists have not yet been published. Therefore, to assess the consequences of LXRβ antagonism with DEX co-administration, I conducted in vivo studies in Lxra−/− mice and cell-culture studies in primary cells isolated from WT, Lxra−/− and Lxrβ−/− mice. Recently, our laboratory has established collaborations with Dr. Arturo Orellana (York University, Toronto) and Dr. William Zuercher (GlaxoSmithKline, North Carolina, USA) to develop an LXRβ specific antagonist. In the future, following the discovery of an LXRβ specific antagonist, the proof of principle studies presented in this thesis could be repeated in WT mice.

5.3 Drug administration

The half-life of DEX in the mouse is only 50 minutes after i.p. administration (Lu et al. 2010, Scheer et al. 2010). We elected to administer DEX via subcutaneous injections b.i.d. over multiple days. The twice daily injection of DEX was chosen to try to minimize the large difference in the plasma concentration of the drug at the peak and trough. A better system to administer a continuous steady drug level to the body is subcutaneous implantation of pellets or an osmotic pump. We had conducted a pilot study, where pellets containing three different doses of DEX (Innovative Research of America, Sarasota, FL) were implanted by trochar subcutaneously in the mice. In this study, we observed that the surgical wounds were not healed in the DEX group and we
failed to observe a dose dependent increase in the plasma and liver DEX concentrations in WT mice. Therefore, I continued with subQ b.i.d. injections to administer DEX in my studies.

5.4 Specificity of the LXRβ antibody

The studies presented in this thesis show that the nuclear receptor LXRβ is required to stabilize GR recruitment to the Pepck promoter following DEX treatment in the liver. The genomic knockdown or pharmacological antagonism of LXRβ decreases the DEX-dependent recruitment of GR to the the Pepck promoter, which results in diminished Pepck expression and hepatic glucose production. We showed that co-treatment of DEX with GSK2033 did not change general GR nuclear localization. Furthermore, in transient transfection reporter assays, we showed that GSK2033 co-treatment attenuated DEX-induced Pepck promoter activation when LXRβ but not LXRα was also co-transfected. However, our LXRβ ChIP studies from mouse liver and a hepatic cell-line over-expressing HA-tagged LXRβ indicated that LXRβ was not recruited to the Pepck GRU. Notably, using a gel shift assay, Herzog et al showed that LXRα binds to the Pepck promoter at -342bp from the transcription start site (within the gAF3).

Additionally, a liver ChIP-seq paper that used a non-commercial LXRα/β antibody found that LXR was bound to the Pepck promoter under basal conditions in WT mouse liver (Boergesen et al. 2012). By quantitative real-time PCR, it is estimated that LXRα is expressed at levels two-times higher than LXRβ in WT mouse liver. Therefore, it is possible that only the LXRα isoform is contributing to this reported observation. In my studies, I used an LXRβ-isoform specific antibody. The specificity of the antibody was confirmed in control experiments in which LXR was shown to bind to the SREBP-1c promoter in Lxra/- but not Lxrβ/- mouse liver (Figure 5.1).
LXRβ ChIPs were performed in Vehicle (Veh) or DEX perfused \textit{Lxrα−/−} and \textit{Lxrβ−/−} livers. Data represent one liver per treatment, error bar represents QPCR error (Avg±SD, N=3).

Thus, in my ChIP studies I detected LXRβ recruitment to the SREBP-1c promoter but not the \textit{Pepck} promoter. There are two possible reasons for this observation:

1) LXRβ is not directly recruited to the \textit{Pepck} promoter but is present in a very large protein complex and due to formaldehyde cross-linking, the antibody epitope is masked.

2) LXRβ alters the recruitment of one (or more) of the required proteins at the \textit{Pepck} promoter that are essential for GR interactions at the weak \textit{Pepck} promoter GRE.

As described in the introduction (\textbf{Chapter 1}) proper interactions of many hepatic transcription factors and co-regulatory proteins are essential for full GR recruitment to and activation of the \textit{Pepck} promoter activity. One method that could be used to distinguish between the above mentioned possibilities is unbiased proteomic analysis of GR ChIPs from \textit{Lxra−/−} livers perfused with DEX ± GSK2033. This technique of rapid immunoprecipitation mass spectrometry of endogenous proteins was recently used to identify a novel cofactor associating with estrogen receptor signaling (Mohammed et al. 2013). I employed the standard ChIP technique for my studies, where DNA-protein
interactions within cells or livers are cross-linked by formaldehyde. Recent studies have shown that when a protein of interest is present in a large protein complex on chromatin, formaldehyde fixation is not sufficient to capture long-range protein-protein interactions. Relatively long distance (7-17 Å) protein-protein interactions need to be captured via another chemical crosslinking agent (i.e., DSG or EGS) that cross-links primary amines prior to adding formaldehyde to crosslink protein to DNA (Nowak et al. 2005, Ramadoss et al. 2010). This two-step cross-linking ChIP method could also be used to deduce whether LXRβ is present in a large protein complex.

5.5 Molecular interactions of LXRβ and GR

Over 1500 genes are altered in mouse liver in response to GC treatment (Phuc Le et al. 2005). Therefore, it is likely that the GC-mediated metabolic phenotype is rendered by a combinatorial effect of many gene changes. Recent ChIP-seq and RNA-seq studies in liver and adipose-cells have found that DEX-activated GR binds to intra-genomic regions to regulate many genes involved in glucose and lipid metabolism (Yu et al. 2010, Grontved et al. 2013). The studies in this thesis assessed GR recruitment to two known key target genes Pepck and TAT. To assess if LXRβ antagonism affects GR recruitment to other metabolic target genes that may contribute to GC-metabolic phenotype, ChIP-seq and RNA-seq studies should be performed from the livers of Lxrα-/- mice treated with DEX± GSK2033.

5.6 Pathophysiologival relevance of FGF21 regulation by GCs

Db/db mice and obese diabetic humans have high circulating levels of both endogenous GC and FGF21 hormones. We have shown that DEX-activated GR directly up-regulates the starvation hormone FGF21 via an atypical GBS located +4.4 kb from the TSS in mouse liver. DEX- treatment studies should be performed in liver-specific
Fgf21−/− mice, to assess whether FGF21 is important for mediating some of the negative effects of GCs
Chapter 6  Summary of key findings and significance

6.1  \textit{Lxr\textbeta{-}/-} mice are protected from GC-induced hyperglycemia

Studies in our lab found an unexpected novel crosstalk between GR and LXR\textbeta{} in the regulation of glucose and lipid metabolism by GCs. We showed that the \textit{Lxr\textbeta{-}/-} and \textit{Lxra\textbeta{-}/-} mice are protected against hyperglycemia and hepatosteatosis compared to the WT and \textit{Lxra{-}/-} mice following DEX administration. In contrast, a similar degree of spleen atrophy (a measure of immune suppression) was present in both WT and each of \textit{Lxr-null} mice, suggesting that \textit{Lxr\textbeta{-}/-} and \textit{Lxra/\textbeta{-}/-} mice were selectively resistant to some of the effects of GC administration (\textbf{Chapter 2}).

At the molecular level, the induction of liver gluconeogenic genes, Pepck, FOXO1 and PGC1\textalpha{} after DEX treatment was significantly attenuated in the \textit{Lxra/\textbeta{-}/-} mice compared to WT mice. In addition, \textit{Lxra/\textbeta{-}/-} mice remained more insulin tolerant than WT mice after DEX treatment determined from basal insulin values and an insulin tolerance test, and this effect was specifically due to loss of LXR\textbeta{}. From our experiments in primary hepatocytes of WT, \textit{Lxra{-}/- Lxra\textbeta{-}/-} and \textit{Lxra/\textbeta{-}/-} mice, we found that the selective transcriptional effect of GCs was cell autonomous and required LXR\textbeta{} despite the fact that the enhanced insulin tolerance of the \textit{Lxra/\textbeta{-}/-} mice would also contribute to protection against GC-induced hyperglycemia. Together, these data suggest that LXR\textbeta{} in the liver contributes selectively to the regulation of key gluconeogenic enzymes including Pepck. Notably, we showed that GR recruitment to the \textit{Pepck} promoter is diminished in the absence of LXR\textbeta{}. This effect is not due to a general decrease in recruitment of co-factors to the \textit{Pepck} promoter in the \textit{Lxra/\textbeta{-}/-} mice since two other factors important for Pepck activation (C/EBP\textbeta{} and SRC-1) were similarly recruited. Our studies in primary macrophage cells demonstrated that DEX-mediated suppression of inflammatory genes IL-1\beta{}, IL-6 and TNF\textalpha{} occur independent
of LXRs. These findings have provided a unique novel mechanism by which the desired immunosuppressive and detrimental gluconeogenic actions of GCs can be separated.

6.2 Antagonism of LXRβ suppresses the gluconeogenic side-effects of GCs without affecting their immunosuppressive function

To follow up our findings in the Lxr-null mice, we explored if LXRβ antagonism can mimic the beneficial effect of LXRβ knockdown during GC administration. We utilized a potent LXR antagonist GSK2033 for the proof of principle studies shown in this dissertation. We demonstrated that LXRβ antagonism prevents DEX-induction of gluconeogenesis without affecting the immunosuppressive actions of GR in primary hepatocytes as well as in mice (Chapter 3).

At the molecular level, expression of the gluconeogenic genes, Pepck, FOXO1, Pgc1α and FGF21 was attenuated by GSK2033 co-treatment with DEX in Lxra-/- mouse liver. Conversely, the DEX-induced expression of an anti-inflammatory gene GILZ was unaffected by LXRβ antagonism in-vivo. In agreement with the in vivo results, studies in mouse primary hepatocytes showed that GSK2033 co-treatment with DEX attenuated Pepck gene expression and glucose production in WT and Lxra-/- hepatocytes. We showed that GSK2033 co-treatment with DEX did not change general GR nuclear localization or activation. Using transient transfection reporter assays, we demonstrated that GSK2033 co-treatment attenuates DEX-induced Pepck promoter activation in the LXRβ but not LXRα co-transfected hepatocytes. Notably, using ChIP assays we demonstrated that DEX-mediated GR and RNA polymerase II recruitment to the Pepck promoter is decreased in the Lxra-/- mouse liver co-perfused with GSK2033. These changes were not due to decreased recruitment of the co-factors (p300, Torc2, C/EBPβ, SRC-1 and RXR) or altered chromatin status (H3K9Ac/H3, H3K27Ac/H3 and AcH4/H4) near the Pepck gene. Our ChIP studies showed that the nuclear receptor
LXRβ is not recruited to the same regions that GR is recruited to in the *Pepck* promoter under basal or ligand treated conditions in mouse liver.

Notably, DEX-mediated suppression of inflammatory genes (i.e., IL-1β, IL-6) was not affected by GSK2033 co-treatment in mouse primary macrophages. Accordingly, a similar degree of spleen atrophy (a measure of immune suppression) was present in *Lxra/-* mice administered DEX or DEX + GSK2033 for 5 days. This important proof of principle study demonstrated that gluconeogenic side-effects of GC-administration can be mitigated by LXRβ antagonism, without affecting its immunosuppressive actions.

### 6.3 The starvation hormone FGF21 is directly regulated by GR

In *Chapter 4* we show that ligand activation of GR directly regulates FGF21 gene expression and that this induction is additive when combined with the basal and inducible regulation of FGF21 by PPARα. We observed GR binding to the open chromatin region near the FGF21 gene using ChIP from mouse livers perfused with DEX. GR binds directly to the FGF21 gene at a site distinct from that of PPARα. Cotransfection studies of truncated and mutated FGF21 gene reporters showed that GR binds to the nucleotide sequence GGAACActcAGGACA located +4.4kb downstream of the FGF21 transcription start site. This GR regulatory sequence is atypical, in that it is not an IR-3, but is instead a DR-3. However, the key central nucleotides in the response element ACAxxxAGG have previously been shown to be over-represented in ChIP-seq studies performed with GR and do not differ between the IR-3 and DR-3 sequences (Grøntved et al. 2013).

Our finding that GCs directly regulate FGF21 expression provides a potential explanation for the paradoxical finding that genetically obese mice have high circulating FGF21 levels since these mice also exhibit high circulating and intra-tissue

6.4 Significance

It is estimated that 1.2% of the US population is currently being treated with a prescription GC drug to manage an inflammatory condition (Overman et al. 2013). However, the long-term use of high doses of glucocorticoid drugs is limited due to undesirable side effects that include insulin resistance, hepatosteatosis and type 2 diabetes. The potent induction of hepatic glucose production by GCs is partially responsible for the development of hyperglycemia. The pharmaceutical industry has long been interested in developing GC therapeutics devoid of metabolic side-effects. However, the development of selective GR modulators has been problematic largely because the traditional view that the immunosuppressive actions of GCs are mediated by transrepression of inflammatory genes (IL-1β, IL-6) and that metabolic side effects are mediated by transactivation of metabolic genes (Pepck, G6Pc) is not absolute. It turns out that the potent immunosuppressive actions of GCs require both repression of inflammatory genes (IL-1β, IL-6) and activation of anti-inflammatory genes (GILZ, DUSP14) (De Bosscher 2010). Additionally, a recent genome-wide study of the GR cistrome from LPS-induced macrophages found that following DEX-treatment, GR inhibits the transcription of inflammatory genes via both cis-repression and trans-repression, and simultaneously GR activates multiple anti-inflammatory genes via both cis-activation and transactivation (Uhlenhaut et al. 2013). Combined administration of GCs with another therapeutic (not targeting GR) that can mitigate the gluconeogenic effects of GCs is a possible approach to retain the therapeutic effects of existing GC drugs.

We identified novel molecular modulators that can be targeted to avoid gluconeogenic side effects of GC-therapy (Figure 6.1). We show that LXRβ is required for GC-induced hepatic glucose production, hyperglycemia and hepatosteatosis in mice. We also
showed that activated GR directly regulates the fasting induced hepatokine FGF21 in mouse liver.

### 6.4.1 GC regulation of FGF21

FGF21 is a hepatokine that regulates whole body energy homeostasis, insulin sensitivity, and glucose and lipid metabolism. In normophysiology, FGF21 levels are elevated by long term fasting to coordinate the adaptive starvation response in mice and humans. In Chapter 4 we showed that activated GR directly regulates the fasting induced hepatokine FGF21 in mouse liver.

The levels of numerous endocrine hormones are altered in response to prolonged fasting in order to effectively manage the crucial transition between the fed and fasted state. There is a high degree of overlap in the functions of these hormones, which we perceive as a natural ‘built-in’ redundancy to ensure survival of the organism through prolonged periods of nutrient deprivation. With this in mind, it makes intuitive sense that GCs and FGF21, which are both elevated in the long-term response to fasting and positively regulate gluconeogenesis, feed-forward to regulate each other’s synthesis (Liang et al. 2014).

### 6.4.2 LXRβ and GC-induced gluconeogenesis

Notably, we demonstrated that either genomic knockdown or antagonism of LXRβ decreases GR recruitment to the GRE of a key gluconeogenic gene, Pepck, which decreases gene expression and glucose production following GC-administration in mice (Chapter 2 and 3). LXRβ regulates hepatic GR targets in a gene-specific manner, as GR recruitment to the GRE of another target gene TAT is not affected by either genomic knockdown or antagonism of LXRβ. We also demonstrate that DEX-mediated immune suppression is not affected by LXRβ antagonism in mice. My studies in mouse
primary hepatocytes and macrophages found that during GC administration, the beneficial effects of LXRβ knockdown or LXRβ antagonism is cell autonomous.

Results from other groups suggest that agonism of LXRα may also be effective at attenuating the GC-induced gluconeogenic programme (Nader et al. 2012). A major pitfall associated with use of LXRα activation to control glycemic effects is the induction of hepatic *de novo* lipogenesis via activation of Srebp-1c, FAS and SCD-1. In liver, LXRα is the dominant transcriptional regulator of Srebp-1c (Figure 3.2 C) (Repa et al. 2000, Quinet et al. 2006). Thus, long term use of LXRα activation to attenuate GC-induced gluconeogenesis would aggravate hepatosteatosis (another detrimental side-effect of GC administration). In contrast, the use of LXRβ antagonists to attenuate glycemic effects of GCs would not cause lipogenic effects associated with LXR activation. Additionally, GC-mediated hepatosteatosis might actually be lowered by LXRβ antagonism, as *Lxrβ*-/ mice are protected from GC-induced lipid accumulation in the liver (Patel et al. 2011).

The atheroprotective effects of LXR have been attributed to both isoforms. In agreement, ageing has been shown to increase macrophage foam cell accumulation in the spleen, lung and the arterial wall in *Lxra/β*-/ mice, but not *Lxra*-/ or *Lxrb*-/ mice (Schuster et al. 2002). Both LXRα and LXRβ are equally important for cholesterol reverse transport (via activation of ABCA1) in peripheral organs (Repa et al. 2000, Schwartz et al. 2000), so antagonism of LXRβ may interfere with this process. One potential strategy to overcome this hurdle is to develop an LXRβ- specific antagonist that is primarily taken up by the liver where GC-induced gluconeogenesis occurs. Recently, Griffett et al. (2013) described a molecule termed SR9238 (an ester derivative of GSK2033) as a liver selective LXR dual inverse agonist, and showed that SR9238 treatment protected diet-induced obese mice from hepatosteatosis by decreasing the expression of lipogenic genes in the liver. The authors showed that the expression of the LXR target gene ABCA1 in the periphery was not affected by SR9238 treatment (Griffett et al. 2013).
The proofs of principle studies presented in this thesis provide evidence that the gluconeogenic and immunosuppressive actions of GR activation can be separated by antagonism of LXRβ. These discoveries will likely revive interest by the pharmaceutical industry to develop LXR isoform-specific small molecules to capitalize on these potential beneficial therapeutic outcomes.

Figure 6.1  GR regulation of hepatic glucose production
Chapter 7  References


Li, H., Q. Fang, F. Gao, J. Fan, J. Zhou, X. Wang, H. Zhang, X. Pan, Y. Bao, K. Xiang, A. Xu and W. Jia (2010). "Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride." J Hepatol 53(5): 934-940.


Liu, Y., Y. Nakagawa, Y. Wang, R. Sakurai, P. V. Tripathi, K. Lutfy and T. C. Friedman (2005). "Increased glucocorticoid receptor and 11{beta}-hydroxysteroid dehydrogenase type 1 expression in hepatocytes may contribute to the phenotype of type 2 diabetes in db/db mice." Diabetes 54(1): 32-40.


Ramadoss, P., F. Chiappini, M. Bilban and A. N. Hollenberg (2010). "Regulation of hepatic six transmembrane epithelial antigen of prostate 4 (STEAP4) expression by


Schwabish, M. A. and K. Struhl (2007). "The Swi/Snf complex is important for histone eviction during transcriptional activation and RNA polymerase II elongation in vivo." 


phosphoenolpyruvate carboxykinase gene expression involves the coactivator p300."


Chapter 1

Chapter 2

Chapter 4