Mechanisms of Diet-Induced Dyslipidemia and Insulin Resistance: Role of Chronic LXR Activation

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Biochemistry
University of Toronto

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Masters of Science  
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2008

Abstract

The metabolic syndrome is a collection of pathologies including dyslipidemia, obesity and insulin resistance. A thorough understanding of the mechanisms behind metabolic syndrome development would help in the development of treatment and prevention strategies. Our lab has previously shown that cholesterol feeding exacerbates features of the metabolic syndrome in high fat-, high fructose-fed mice. The nuclear receptor Liver X Receptor (LXR), a master transcriptional regulator of cholesterol, fat and carbohydrate metabolism in the liver, is endogenously activated by oxysterols, metabolic derivatives of cholesterol. In order to determine whether cholesterol exerts its metabolic effects via LXR activation, parallel studies were conducted comparing chronic cholesterol supplementation with LXR activation in the hamster. Results showed that cholesterol feeding and LXR activation caused similar dyslipidemia, glucose intolerance and upregulation of target mRNA and proteins. These data support the hypothesis that the dyslipidemic effects of dietary cholesterol are mediated at least in part by LXR.
Acknowledgments

I would like to acknowledge all of my colleagues, my friends and family who supported me during my graduate degree. I really appreciate all the help, both technical and advisory, that I received from my colleagues, my supervisor and my committee in all aspects of my work. My friends were always there to share a laugh and lighten the mood. Most of all, I would like to acknowledge my family for their love, support and inspiration. My family has lent me their support in so many ways, from general encouragement to putting up with my cranky moods when things didn’t go well. Above all, I would like to thank my family for being such wonderful people. Each of my family members is an example of a person who works hard and takes risks to overcome any obstacle in their path to success. Their examples continue to give me inspiration as I move along my own path.
The work and ideas presented in this thesis were produced as the result of a collaborative approach involving a number of people. All metabolic pulse-chase labeling experiments, in vitro labeling of newly synthesized lipids, hepatic lipid mass analysis, plasma glucose measurements, FPLC separation and analysis, glucose and insulin tolerance tests, hamster drug dosing and data analysis were performed by myself. The majority of Western blots were performed by myself, with assistance from Reyhaneh Emami and Jessica Aiello. All mRNA measurements were performed by Mark Naples. Plasma lipid and enzyme measurements were performed by the Clinical Diagnostic Laboratory staff at the Hospital for Sick Children. General hamster care, including fasting and diet surveillance were performed by myself in cooperation with Chris Baker, Mark Naples, Joanna Nelken, Denise Taylor, Elaine Xu and Rianna Zhang. Cell culture was done by Elaine Xu and Rianna Zhang. Hamster surgeries were performed by Chris Baker, Mark Naples, Joanna Nelken, Denise Taylor and Elaine Xu with my assistance.
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<th>Description</th>
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<tbody>
<tr>
<td>αMEM</td>
<td>Alpha minimal essential medium</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-coA carboxylase</td>
</tr>
<tr>
<td>ACO</td>
<td>Acyl coA oxidase</td>
</tr>
<tr>
<td>apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt export protein</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate response element binding protein</td>
</tr>
<tr>
<td>CLXR</td>
<td>Chow diet supplemented with LXR agonist TO901317</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine palmitoyl transferase</td>
</tr>
<tr>
<td>CYP7A</td>
<td>Cholesterol 7alpha-hydroxylase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethylether)-N,N,N’N’-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
</tbody>
</table>
ER Endoplasmic reticulum
FAS Fatty acid synthase
FABP Fatty acid binding protein
FATP Fatty acid transfer protein
FBS Fetal bovine serum
FF High fat and high fructose hamster diet
FFA Free fatty acids
FFC FF diet supplemented with 0.25% cholesterol
FFLXR FF diet with LXR agonist TO901317
FPLC Fast protein liquid chromatography
FXR Farnesoid X receptor
FXRE FXR response element
G6Pase Glucose-6-phosphatase
GK Glucokinase
GLUT Glucose transporter
GR Glucocorticoid receptor
HDL High density lipoprotein
HL Hepatic lipase
HMG-coA 3-hydroxy-3-methylglutaryl-coenzyme A
HNF Hepatic nuclear factor
HRP Horseradish peroxidase
HSL Hormone-sensitive lipase
IBABP Ileal bile acid binding protein
IDF International Diabetes Federation
IL Interleukin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>LXRE</td>
<td>LXR response element</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>N-CoR</td>
<td>Nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI(3)K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B (also known as AKT)</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase with sequence homology to protein-tyrosine phosphatases and the cytoskeleton protein tensin</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid and thyroid-hormone receptors</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol response element binding protein</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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</table>
1 :: Introduction

1.1 The Metabolic Syndrome: Pathophysiology and Epidemiology

A significant health epidemic is currently affecting populations of developed and developing countries worldwide. This epidemic, called the metabolic syndrome, is a constellation of pathologies including obesity, insulin resistance, dyslipidemia, glucose intolerance and hypertension (Day 2007). The concept of the metabolic syndrome was introduced in 1988 by Gerald Reaven after recognition that several metabolic features associated with insulin resistance indicated higher risk for cardiovascular disease (Kim and Reaven 2004). The precise definition of the metabolic syndrome has been controversial, with different organizations presenting alternate definitions (Day 2007). The International Diabetes Federation (IDF) recently provided a consensus definition of the syndrome. The consensus definition includes the core components of obesity, insulin resistance, dyslipidemia and hypertension (Alberti et al. 2006). The clinical definition includes a requirement for both central obesity and two of the following: raised plasma triglycerides (TG), reduced plasma high density lipoproteins (HDL), raised blood pressure, and raised fasting plasma glucose, each relative to specified standard levels.

Some of the major complications associated with the metabolic syndrome include fatty liver (Aguilera et al. 2008, Lewis et al. 2002), cardiovascular disease (Alberti et al. 2006, Skalicky et al. 2008), and type 2 diabetes (Alberti et al. 2006, Grundy et al. 2005). People who develop the metabolic syndrome have up to five-fold greater risk of developing diabetes compared with people without metabolic syndrome (Stern et al. 2004). Diabetes, in turn, leads to a much higher risk of cardiovascular disease (Roper et al. 2002, Saydah et al. 2002). Indeed, metabolic syndrome patients show an increased risk of cardiovascular disease, whether or not they also have diabetes (Isomaa et al. 2001).

The metabolic syndrome is of relevant concern because of its increasing prevalence throughout the world. It is estimated that between 20% and 30% of adults can be diagnosed with the metabolic syndrome, although this number varies between specific populations (Grundy 2008). Its incidence increases with age and seems to be higher in certain ethnic populations, including
African Americans (Day 2007). It is widely recognized, however, that without careful examination of environmental contributions to the metabolic syndrome and better understanding of its development and pathophysiology, the incidence will not decrease and may continue to rise.

1.2 Mechanisms of Insulin Resistance in the Metabolic Syndrome

Insulin resistance occurs when tissues do not respond appropriately to normal concentrations of circulating insulin. This is thought to occur because of dysregulation of the intracellular molecular insulin signaling pathways (Capeau 2003). When tissues do not respond properly to normal concentrations of insulin, the pancreas compensates by secreting even more of the hormone. Type 2 diabetes eventually occurs when this compensation is not sufficient to regulate plasma glucose.

Insulin is a peptide hormone produced by pancreatic beta cells. It is secreted post-prandially, and serves to both promote glucose uptake and inhibit lipid catabolism in peripheral tissues including adipose, liver and muscle in order to maintain plasma glucose concentrations within a normal range (Capeau 2003). Insulin first encounters the insulin receptor, a tetrameric membrane-bound receptor present on the surface of the insulin sensitive tissues (Capeau 2003). After binding, the receptor undergoes auto-tyrosine phosphorylation (Ramachandran and Kennedy 2003), and tyrosine phosphorylation of its substrates, which then triggers downstream signaling cascades. The insulin receptor substrates (IRS) 1 and 2 are the major early intermediates between the insulin receptor and downstream signaling molecules (Gual et al. 2005). Once phosphorylated at tyrosine residues, IRS-1 and IRS-2 activate the two main downstream pathways of insulin signaling: the mitogen activated protein kinase (MAPK) pathway, and the phosphoinositol 3-kinase (PI(3)K) pathway, which activates Akt/protein kinase B (PKB). These signaling pathways then have effects over control of proliferation, differentiation and metabolism (Capeau 2003).

Negative regulation of the insulin signaling pathway components may be responsible for insulin resistance; several enzymes including protein tyrosine phosphatase (PTP)-1B dephosphorylate the insulin receptor (IR) and IRS, resulting in a weaker signal (Goldstein et al. 1998). Additionally, serine and threonine phosphorylation impair insulin signaling and can occur as a result of TNF-alpha signaling or FFA exposure (Capeau 2003).
Dysregulated fat metabolism can also directly affect the pancreas and influence insulin regulation. First, free fatty acids (FFA) have an insulinotropic effect, promoting insulin release from the pancreas even during fasting, when plasma glucose is low (Raz et al. 2005). Because of this, increased plasma FFA, a component of metabolic syndrome, can contribute to hyperinsulinemia. Additionally, several studies have shown that lipid accumulation in the pancreatic beta-cells can induce apoptosis, which, accumulated over time, can lead to beta-cell dysfunction and type 2 diabetes (Raz et al. 2005).

1.3 Metabolic Dyslipidemia: A Major Complication of the Metabolic Syndrome

Dyslipidemia is recognized as one of the core components of the metabolic syndrome (Alberti et al. 2006, Duran-Sandoval et al. 2005a). Dyslipidemia associated with the metabolic syndrome is a potential harbinger of further, more serious health consequences for the affected patient. Metabolic dyslipidemia can lead to steatohepatitis, atherosclerosis and type 2 diabetes (Hitsumoto et al. 2007). Metabolic dyslipidemia is defined as dysregulated plasma lipids, including elevated plasma triglycerides and apolipoprotein (apo)B-containing lipoproteins as well as decreased HDL (Grundy 2008). Each of these components is an independent risk factor for cardiovascular disease (Krauss 2004). To underline the danger of dyslipidemia, many studies have shown that the pharmacological reduction of plasma lipids, either through 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-coA) reductase inhibitors (statins) or peroxisome proliferator-activated receptor (PPAR)-alpha agonists (fibrates) results in a decrease in the progression to cardiovascular disease (Avramoglu et al. 2006).^ Insulin resistance is known to drive metabolic dyslipidemia from several angles. To understand the role of insulin resistance in the progression of dyslipidemia, we must first introduce the role of insulin under normal conditions.

In the normal (non-insulin resistant) state, insulin suppresses hormone-sensitive lipase (HSL), which hydrolyses TG stored in visceral fat, allowing non-esterified fatty acids (NEFA) to enter the circulation. Insulin also directly inhibits very low density lipoproteins (VLDL) secretion from the liver primarily through the trigger of post-translational apoB degradation (Sparks and Sparks 1994). In addition, insulin stimulates the enzyme lipoprotein lipase (LPL), which hydrolyses TG present in VLDL. This generates remnant particles, which are then further hydrolysed by insulin-
activated hepatic lipase (HL) to form low density lipoproteins (LDL) particles (Aguilera et al. 2008). Each of these insulin-mediated effects regulates VLDL metabolism, keeping its level within acceptable limits. These effects are depicted in figure 1.5.1.

**Figure 1.5.1. The effects of insulin during the insulin-sensitive state.** Insulin exerts effects over several factors involved in lipid and lipoprotein metabolism. During the insulin-sensitive state, insulin carefully regulates plasma and liver lipid levels. During the insulin resistant state, however, these controls are removed.

When insulin resistance sets in, however, these controls are lifted. Uninhibited HSL activity allows increased NEFA flow to the liver, increasing lipid availability for VLDL assembly (Aguilera et al. 2008, Yki-Jarvinen 2005). Insulin-mediated inhibition of VLDL secretion is removed, allowing these newly-assembled VLDL to flow unregulated into the plasma. Once in the plasma, LPL is not stimulated to hydrolyse them, and the level of plasma VLDL does not decrease (Aguilera et al. 2008). Hepatic insulin resistance has in fact recently been shown to be sufficient to produce dyslipidemia and atherosclerosis (Biddinger et al. 2008).

In addition to an increase in the amount of plasma VLDL, the amount of small, dense LDL particles is a recognized component of metabolic dyslipidemia (Rizzo and Berneis 2007). This type of LDL is more potently atherogenic, because of its increased ability to infiltrate the vascular endothelium (Bjornheden et al. 1996) and because it is more susceptible to oxidation.
Increased small, dense LDL has been associated with a three- to seven-fold increase in coronary artery disease risk (Rizzo and Berneis 2007). The size of LDL particles is positively correlated with the amount of plasma HDL, meaning that smaller LDL particles are associated with lower HDL abundance (Austin et al. 1990). The formation of small, dense LDL is favoured with increased activity of HL and decreased activity of LPL (Rizzo and Berneis 2007). Since this situation is stimulated during insulin resistance, these potently atherogenic lipoprotein particles are produced more readily, contributing to the progression of atherosclerosis.

The presence of the enzyme Cholesteryl ester transfer protein (CETP) has also been shown to affect the balance between different types of lipoproteins. CETP has been shown to preferentially transfer cholesteryl esters from HDL to VLDL and dense LDL (Guerin et al. 2001). This is particularly notable in this discussion because humans and hamsters express CETP, while mice and rats do not (Groot et al. 2005). This difference highlights an advantage to using hamsters as an animal model to study the metabolic syndrome and associated dyslipidemia.

### 1.4 Hepatic Lipid Metabolism and Fatty Liver in the Metabolic Syndrome

Metabolic dyslipidemia is intimately linked with hepatic lipid accumulation or fatty liver. Fatty liver, or non-alcoholic fatty liver disease (NAFLD), has the potential to develop into steatohepatitis, fibrosis or even cirrhosis. Indeed, the risk of further developments is increased when fatty liver is associated with other components of the metabolic syndrome such as obesity (Rector et al. 2008). Although fatty liver is dangerous on its own, the condition can further exacerbate existing dyslipidemia. Lewis et al (Lewis et al. 2002) clearly outline the process by which hepatic lipid accumulation can lead to increased plasma lipids through increased production and secretion of VLDL from the liver. Apolipoprotein B, the main protein component of VLDL, is translated into the endoplasmic reticulum (ER) of the hepatocyte, where it is lipidated and eventually formed into a VLDL particle. If lipids are not abundant at the time of apoB translocation into the ER, the protein is degraded. The result of this is that particle lipidation is in fact the rate-limiting step to VLDL production; therefore, increased lipid availability in the liver because of metabolic dysregulation would lead to increased VLDL production. Several studies have shown that increased lipid availability leads to increased VLDL
production in both the human hepatic cell line HepG2 (Dashti and Wolfbauer 1987), in rat primary hepatocytes (White et al. 1992), and in humans (Adiels et al. 2005).

Although fatty liver has been shown to be an initiating factor in the metabolic syndrome, obesity itself is not perfectly correlated with insulin resistance and the metabolic syndrome. Obese people do not always develop metabolic syndrome, and non-obese people sometimes do. The common factor for developing the metabolic syndrome seems to be the amount of fat accumulated in the liver (Kotronen and Yki-Jarvinen 2008, Mensink et al. 2008). This has been realized with the recognition that waist circumference is a better predictor of metabolic problems than raw weight (Manco et al. 2008). This is because waist circumference is a rough but useful indication of abdominal fat accumulation (Manco et al. 2008). While the reasons for different fat distribution among individuals are unknown, fat accumulation in the liver seems to be a trigger for other components of the metabolic syndrome, including insulin resistance (Adiels et al. 2007), glucose overproduction (Seppala-Lindroos et al. 2002) and VLDL overproduction (Adiels et al. 2006).

Fat accumulates in the liver both as a result of increased flux of fatty acids from fat depots, and from increased post-prandial delivery from chylomicrons (Yki-Jarvinen 2005). The ‘portal hypothesis’ elaborates the idea that there is increased NEFA flux from adipose tissue to the liver via the portal vein (Aguilera et al. 2008, Raz et al. 2005, Yki-Jarvinen 2005). This increased flux can often be attributed to insulin resistance, since during this condition, insulin is unable to suppress HSL, allowing more lipolysis of NEFA from visceral fat (Aguilera et al. 2008, Bergman 2000).

The accumulation of fat in the liver is often associated with inflammation, which involves an additional set of complications. As hepatic steatosis progresses to steatohepatitis, which involves inflammatory progression, further metabolic consequences can ensue. The acute inflammatory response normally serves as a response to injury or infection, but an unregulated response can develop into a chronic inflammatory state that influences the progression of metabolic complications. Dyslipidemia, particularly increased circulating FFA, has been shown to be associated with increased circulating cytokines, particularly tumour necrosis factor (TNF)-alpha and interleukin-6 (IL-6) (Warnberg and Marcos 2008). This metabolically-stimulated inflammation has been referred to as “metaflammation” (Hotamisligil 2006), a suggested term.
to differentiate this set of responses from the classical inflammatory pathway. These inflammatory changes are not only circumstantially associated with the metabolic syndrome; adipose tissue has been shown to produce TNF-alpha in obese mice (Hotamisligil et al. 1993), demonstrating a direct link between obesity and inflammation. This TNF-alpha release in turn interferes with insulin signaling by preventing IR and IRS tyrosine phosphorylation (Feinstein et al. 1993). This increased inflammation may also be due, in part, to decreased adiponectin levels seen in the metabolic syndrome. Adiponectin is a protein hormone secreted by the adipose tissue, and whose level is inversely correlated with body weight. Adiponectin has anti-inflammatory properties; therefore, its low levels seen during the metabolic syndrome may contribute to increased inflammation. This appears to be a two-sided relationship, since increased TNF-alpha levels are also known to decrease adiponectin expression (Bruun et al. 2003).

Fat accumulation in the liver is therefore very strongly linked with metabolic dyslipidemia. Increased fat hydrolysis in adipose tissue initiated by insulin resistance leads to greater NEFA flux back to the liver. This, in turn, leads to greater VLDL secretion, which can trigger inflammatory responses, leading to more severe metabolic problems, including atherosclerosis, diabetes and chronic liver disease.

1.5 Nuclear Receptors as Master Regulators of Lipid Metabolism

Nuclear receptors are among the most numerous of transcription factors. They are master regulators of gene transcription, often regulating several different metabolic pathways and feedback loops. Several nuclear receptors have been implicated in the modulation of insulin signaling, lipid and carbohydrate metabolism, and the progression towards the metabolic syndrome. Among these are the Liver X Receptor (LXR), Farnesoid X Receptor (FXR) and PPARs, which will be highlighted here.

1.5.1 Liver X Receptor

The Liver X Receptor (LXR) is a master regulator of lipid and carbohydrate metabolism. It exists as two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). LXRβ is expressed throughout the body whereas LXRα expression is restricted to tissues involved in lipid metabolism, including the
liver, spleen, kidney, macrophages, small intestine, and adipose tissue (Willy et al. 1995). LXR was first discovered as an orphan nuclear receptor, since its ligands were unknown upon initial identification. LXR remained in this family until its natural ligands were discovered and it was shifted to the ‘adopted orphan’ family of nuclear receptors. LXR is endogenously activated by oxysterols, particularly 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol (Lehmann et al. 1997) (Figure 1.5.2). LXR has also recently been shown to be activated endogenously by glucose (Mitro et al. 2007). It is likely that glucose competes with oxysterols for a common binding pocket, and also binds at a separate location (Mitro et al. 2007). Synthetic ligands have been developed and are commonly used for experimental manipulation of LXR. Two currently used non-steroidal agonists are T0901317 (Schultz et al. 2000) and GW3965 (Collins et al. 2002), structures of which are presented in Figure 1.5.3. The synthetic agonist T0901317 was used for many of the experiments reported in this thesis.

Figure 1.5.2. Endogenous LXR ligands.

LXR is activated endogenously by hydroxylated versions of cholesterol, called oxysterols. Three of the most potent LXR activators are shown here.
Figure 1.5.3. Synthetic LXR ligands.

Two synthetic LXR agonists, T0901317 and GW3965, are regularly used in experiments to selectively and potently activate LXR.

LXR’s importance as a cholesterol sensor was highlighted with studies by Peet et al (1998), who discovered that LXRα-/- mice were particularly sensitive to cholesterol feeding (Peet et al. 1998). The knockout mice were similar to wild type mice when fed a normal diet, but accumulated large amounts of hepatic cholesterol and experienced impaired hepatic function upon cholesterol feeding (Peet et al. 1998). The group identified cholesterol 7-alpha hydroxylase (CYP7A1), an enzyme that catalyzes the rate limiting step in the conversion of cholesterol to bile acids, as a transcriptional target of LXR. This seminal discovery was important for identifying LXR as a critical cholesterol sensor. Subsequent studies identified other targets of LXR. Many are involved in cholesterol metabolism and transport, but more diverse targets were discovered as well, including a variety of genes involved in cholesterol, carbohydrate and lipid metabolism. LXR has been shown to activate genes such as members of the ATP-binding cassette (ABC) family of cholesterol transporters (Repa et al. 2002), phosphoenolpyruvate carboxykinase (PEPCK) (Laffitte et al. 2003) (Cao et al. 2003), fatty acid synthase (FAS) (Joseph et al. 2002) and stearoyl-coA desaturase (SCD-1) (Schultz et al. 2000). A summary of some of the main LXR targets is presented in Figure 1.5.4. The implications of activation of these targets will be discussed in a subsequent section.
Figure 1.5.4. Selected metabolic targets of the Liver X Receptor.

LXR exerts effects over genes involved in fat, cholesterol and carbohydrate metabolism. The list presented here depicts targets both activated (sharp arrows) and inhibited (blunt arrows) by LXR.

LXR binds constitutively to its response element as a heterodimer with another nuclear receptor, the Retinoid X Receptor (RXR). The LXR response element (LXRE) is a direct repeat-4 (DR-4), composed of two AGGTCA sequences separated by four nucleotides (Kalaany and Mangelsdorf 2006). The RXR/LXR heterodimer forms a complex with a corepressor, such as silencing mediator for retinoid and thyroid-hormone receptors (SMRT) (Chen and Evans 1995) or nuclear receptor co-repressor (N-CoR) (Horlein et al. 1995), preventing initiation of gene transcription (Kalaany and Mangelsdorf 2006). After binding of the appropriate ligand, LXR undergoes a conformational change and the corepressor is replaced by a coactivator (Unno et al. 2005), allowing gene transcription to begin (Wojcicka et al. 2007).

In order to understand LXR’s role within the context of cholesterol metabolism, a short discussion of oxysterol metabolism is in order. Oxysterols are oxygenated derivatives of cholesterol. They are produced as a way to signal cholesterol molecules for secretion by marking them for conversion to water-soluble bile acids (Bjorkhem 2002). The presence of oxysterols generally signals an excess concentration of cholesterol (Olkkonen 2004). The first oxidation, catalyzed by the previously mentioned enzyme CYP7A1, introduces a hydroxyl group to the 7α position (Bjorkhem 2002). Alternate pathways hydroxylate cholesterol at other positions,
including carbons 22R, 24S, 25 and 27 (Javitt 2002). In addition to synthetic pathways, oxysterols can come from dietary sources. It is estimated that approximately 1% of cholesterol in a Western diet is in an oxidized form (Valenzuela et al. 2003). Oxysterols then have the potential to interact with LXR, further promoting their conversion to bile acids and eventual secretion.

1.5.2 Farnesoid X Receptor

The Farnesoid X Receptor (FXR) is a companion nuclear receptor to LXR. The two receptors act in concert, coordinating cholesterol metabolism in a complementary way to the actions of LXR. While LXR’s natural ligands are oxysterols and it upregulates bile acid synthesis, FXR is activated by bile acids and downregulates the same process. Farnesol compounds were originally considered to be FXR’s endogenous ligands, leading to the receptor’s name (Forman et al. 1995). Farnesyl-pyrophosphate is the last common precursor to all branches of the mevalonate pathway. This metabolic pathway begins with acetyl coA, and eventually branches to produce bile acids, cholesterol, steroid hormones, farnesylated proteins and many other products (Forman et al. 1995). It was subsequently discovered that bile acids, specifically chenodeoxycholic acid, cholic acid, deoxycholic acid and lithocholic acid, are more potent FXR agonists than farnesol compounds are in vivo (Wang et al. 1999a).

FXR works via a similar mechanism to LXR. That is, it is constitutively bound to RXR, and remains bound to its response element (the FXRE) along with corepressors under unstimulated conditions. The FXRE is composed of two inverted repeats of AGGTCA separated by one nucleotide (IR-1). After ligand binding, coactivators replace the corepressors, and transcription begins. FXR’s primary expression in liver, kidney and intestine (Ma et al. 2006) gives it ideal distribution for control of metabolic processes. Two of FXR’s direct targets include the bile salt export protein (BSEP) (Ananthanarayanan et al. 2001) and phospholipid transfer protein (PLTP) (Urizar et al. 2000). One main difference between FXR and LXR is that FXR exerts its control over many of its targets indirectly. FXR directly upregulates transcription of another nuclear receptor called the small heterodimer partner (SHP). SHP, which has a ligand-binding domain but lacks a DNA-binding domain, also forms a complex with RXR to initiate transcription. FXR indirectly affects transcription of targets including downregulation of the previously mentioned CYP7A1 (Lu et al. 2000), microsomal triglyceride transfer protein (MTP) (Hirokane et al. 2004) and steroid response element binding protein (SREBP)-1c (Watanabe et al. 2004). This indirect
control can occur through either SHP or other intermediate transcription factors including hepatic nuclear factor (HNF)-4 (Hirokane et al. 2004). A summary of FXR’s metabolic targets can be seen in Figure 1.5.5.

**Figure 1.5.5. Selected targets of the Farnesoid X Receptor.**

FXR regulates many gene targets involved in both cholesterol and fat metabolism. Many of FXR’s effects are mediated by intermediate factors like the small heterodimer partner, SHP.

FXR was originally identified as a potential mediator of the metabolic syndrome because of the coincidence of gall bladder disease with the metabolic syndrome (Fiorucci et al. 2004). FXR activation by both synthetic and endogenous ligands has been shown to reduce plasma triglycerides and VLDL production (Bilz et al. 2006). FXR activation was also shown to improve cirrhosis and fibrosis, leading to the hypothesis that an FXR agonist may help to counteract metabolic dyslipidemia and hepatic lipid accumulation.

**1.5.3 Peroxisome Proliferator Activated Receptor alpha**

The nuclear receptor PPAR-alpha acts in a similar way to LXR; that is, it forms a heterodimer with RXR in order to activate transcription. PPAR-alpha is activated in response to a variety of long chain fatty acids, eicosanoids and synthetic compounds (Desvergne et al. 1998). As with the two previously discussed nuclear receptors, PPAR alpha exerts control over several aspects of lipid and glucose metabolism and can even modulate inflammation (Blanquart et al. 2003).
PPAR alpha is known to increase fatty acid oxidation, therefore acting in opposition to LXR’s lipogenic role. PPAR alpha is slightly different from other nuclear receptors because PPAR alpha itself is stabilized by its ligands, which have been shown to protect it from proteasomal degradation (Blanquart et al. 2002, Blanquart et al. 2004). There is therefore double regulation of PPAR alpha-activated genes; ligand presence triggers coactivator recruitment, and the absence of the ligand allows proteasomal degradation of the receptor (Blanquart et al. 2002). PPAR alpha also undergoes post-translational modification. In the presence of insulin, PPAR-alpha is phosphorylated and its activity increases (Shalev et al. 1996).

Upon activation, PPAR-alpha targets genes involved in both lipid metabolism and inflammation. PPAR alpha is known to repress transcription of cytokines including nuclear factor-kappa B (NF-kB) (Chinetti et al. 2000), and to activate transcription of LPL (Chinetti et al. 2000). PPAR alpha also increases transcription of genes involved in fatty acid transport and oxidation, including fatty acid transport protein (FATP), fatty acid binding protein (FABP), acyl-coA oxidase (ACO) and carnitine palmitoyl transferase (CPT)-1 (Chinetti et al. 2000, Issemann and Green 1990, Louet et al. 2001, Osumi et al. 1991). Each of these effects contributes to PPAR alpha’s ability to ameliorate hypertriglyceridemia. Indeed, the fibrate class of pharmacological compounds act as PPAR alpha agonists to take advantage of this effect. A recent study, however, has shown that activation of LXR reduces the heterodimerization of PPAR-alpha with RXR and tempers its lipid-reducing effects (Ide et al. 2003).

### 1.6 The Role of Nuclear Receptors in Metabolic Control

LXR is called a master regulator for good reason; it exerts control over many genes involved in fat, carbohydrate and cholesterol metabolism. LXR potently modulates genes involved in cholesterol metabolism and transport, glucose metabolism, and lipid metabolism.

1.6.1 Regulation of cholesterol metabolism by nuclear receptors

LXR is primarily known as a regulator of cholesterol metabolism. LXR acts a sensor for intracellular cholesterol; as cholesterol levels increase and the molecule is oxidized to form oxysterols, LXR is activated and initiates transcription of cholesterol-metabolizing genes. LXR directly activates the gene CYP7a1, which encodes the protein that catalyzes the rate-limiting
step in the conversion of cholesterol to bile acids (Javitt 2002). This effect, however, has been demonstrated to be much stronger in mice and rats than in humans or hamsters (Chiang et al. 2001), perhaps indicating why hamsters and humans develop hypercholesterolemia more readily in response to dietary challenges.

LXR also exerts substantial control over cholesterol transport (Rader 2007). LXR has been shown to coordinate reverse cholesterol transport, increasing the level of circulating HDL and promoting net cholesterol secretion through activation of the ABC family of transporters (Zelcer and Tontonoz 2006), mediated by LXR that exists in macrophages (Levin et al. 2005). LXR’s ability to increase reverse cholesterol transport has made it an attractive drug target for combating atherosclerosis and other cholesterol-related diseases (Joseph et al. 2002).

![Reciprocal control of bile acid production by LXR and FXR.](image)

**Figure 1.5.6. Reciprocal control of bile acid production by LXR and FXR.**

LXR and FXR each exert complementary control over bile acid production from oxysterols. While LXR is activated by oxysterols and promotes their conversion into bile acids, FXR is activated by bile acids and retards their production from oxysterols.

As shown in Figure 1.5.6, FXR’s role in sterol metabolism is placed later in the pathway, in the control of bile acid synthesis and transport. To prevent hepatic bile acid toxicity, FXR responds to excess bile acids by downregulating transcription of *Cyp7a1* (Chen et al. 2001, Rader 2007). In the event that both LXR and FXR are active and competing for control of the *Cyp7a1* promoter, FXR has been found to have overriding control (Shang et al. 2007). FXR additionally
controls bile acid trafficking, increasing expression of such proteins as BSEP (Ananthanarayanan et al. 2001) and ileal bile acid binding protein (IBABP) (Grober et al. 1999).

^1.6.2 Regulation of triglyceride metabolism by nuclear receptors

Although LXR has positive effects on glucose metabolism and insulin state, it also has very serious effects on lipid metabolism. Treatment of both mice and hamsters with the synthetic LXR ligand T0901317 resulted in a serious increase in triglyceride production (Schultz et al. 2000). Results from experiments with LXR knockout mice further demonstrate the role of LXR in lipid metabolism. LXR -/- mice have approximately four-fold lower plasma triglycerides than wild type mice (Wojcicka et al. 2007). In 2005, Kalaany et al showed that LXR -/- mice are resistant to diet induced obesity and insulin resistance (Kalaany et al. 2005).

After further investigation, LXR was found to exert control over several components of the lipogenic program, including SCD-1 (Schultz et al. 2000) and FAS (Joseph et al. 2002). LXR also directly upregulates the transcription factors SREBP-1c (Repa et al. 2000, Yoshikawa et al. 2001) and carbohydrate response element binding protein (ChREBP) (Cha and Repa 2007). SREBP1c in turn activates transcription of lipogenic genes including FAS, acetyl-coA carboxylase (ACC) and SCD1 (Brown and Goldstein 1997, Schultz et al. 2000). ChREBP is normally activated by carbohydrates as a signal to convert excess sugar to lipids. Some of its targets include ACC, SCD1 and FAS (Postic et al. 2007). There are several theories to explain the function of LXR’s induction of lipogenesis. Since an important role for LXR is to prevent cholesterol toxicity in cells, increasing the amount of lipid available might increase the production of cholesterol esters, preventing free cholesterol accumulation (Yoshikawa et al. 2001). A schematic representation of LXR and its lipogenic effects can be seen in Figure 1.5.7.
After activation of LXR by its natural ligand, oxysterols, LXR activates both the lipogenic enzymes FAS and SCD, as well as lipogenesis-inducing transcription factors ChREBP and SREBP-1c. This increased lipogenesis leads to accumulation of both cholesterol esters and triglycerides, which, when combined with the protein ApoB, form VLDL particles, which are then secreted into circulation.

LXR’s induction of SCD1 is of particular interest because of SCD1’s role in the development of diabetes. SCD1 catalyzes the desaturation reaction to convert stearoyl coA into oleoyl coA. A recent study has found that the absence of SCD1 in LDL receptor (LDLR) knockout mice improves the observed insulin resistance and symptoms of the metabolic syndrome (Macdonald et al. 2008). The SCD1 knockout model was also resistant to hypertriglyceridemia after treatment with the LXR agonist (Macdonald et al. 2008). Further studies have shown that SCD1 is required for the development of diet-induced insulin resistance (Gutierrez-Juarez et al. 2006), as hepatic knockdown of SCD1 using antisense oligonucleotide (ASO) treatment corrects high-fat diet induced insulin resistance in rats without an associated change in food intake or body weight.
SCD1 knockout was also shown to protect against LXR-mediated increases in plasma TG and decreases in plasma HDL (Chu et al. 2006).

### 1.6.3 Regulation of carbohydrate metabolism by nuclear receptors

In vivo treatment of animals with a synthetic LXR agonist has been shown to decrease hyperglycemia in several models (Cao et al. 2003, Laffitte et al. 2003). LXR downregulates several genes involved in gluconeogenesis, including glucose-6-phosphatase (G6Pase) (Laffitte et al. 2003) and PEPCK (Cao et al. 2003). Glucokinase, one of the first enzymes involved in glycolysis, is upregulated in the liver after LXR activation (Laffitte et al. 2003), as is the glucose transporter GLUT4 (Dalen et al. 2003). Each of these modulations contributes to a net decrease in circulating glucose.

LXR activation has also been shown to increase insulin sensitivity in high fat-fed rats. After five weeks of high fat feeding to induce insulin resistance and a subsequent three weeks of treatment with the LXR agonist GW3965, Commerford et al showed that LXR agonist-treated animals had higher glucose infusion rates in a euglycemic-hyperinsulinemic clamp experiment (Commerford et al. 2007). They further demonstrated that this improvement occurred because of changes in gluconeogenic enzymes outlined above, including PEPCK and G6Pase (Commerford et al. 2007). Some of the apparent anti-diabetic action of LXR may also be attributed to its negative regulation of the transcription factor glucocorticoid receptor (GR), as seen in the leptin receptor-deficient db/db mice (Liu et al. 2006). Increased expression of GR is associated with hyperglycemia and insulin resistance (Rizza et al. 1982).

Whatever the mechanisms, LXR seems to have positive effects on glucose metabolism and insulin sensitivity in mice and rats. This would make it an attractive pharmacological target to combat diabetes and insulin resistance. Indeed, much effort is being spent towards that end. However, LXR’s effects on lipid metabolism may reduce the shine from this attractive target.
1.7 Dietary Animal Models of the Metabolic Syndrome

The metabolic syndrome doesn’t happen spontaneously. It is mainly caused by lifestyle factors, particularly dietary and exercise habits. The three dietary components that have been strongly linked to components of the metabolic syndrome are fructose, fat and cholesterol.

A substantial amount of research has shown that excessive fructose consumption leads to increased circulating TG (Elliott et al. 2002, Stanhope and Havel 2008), hyperinsulinemia and insulin resistance (Adeli et al. 2001, Taghibiglou et al. 2000). When glucose enters the liver, its conversion into acetyl-coA, and eventually to fat, is controlled by the rate-limiting enzyme phosphofructokinase. Fructose bypasses this step and enters the glycolytic chain downstream of this enzyme, allowing unlimited flow of carbon atoms into the lipogenic pathway (Voet and Voet 2000). Fructose feeding in rats for as little as two weeks has been shown to cause alterations in IR, IRS-1 and IRS-2 signaling (Bezerra et al. 2000, Catena et al. 2003). Hamster studies have further showed that fructose-induced insulin resistance causes an increase in assembly and secretion of apoB containing lipoproteins from the liver and the intestine (Adeli et al. 2001, Haidari et al. 2002). Fructose diets also upregulate hepatic SREBP-1, SCD1 and FAS expression in both the human HepG2 cell line and in mice, which increases the level of lipogenesis in the liver. This in turn increases the synthesis and secretion of VLDL and apoB-containing lipoproteins (Bennett et al. 1995, Biddinger et al. 2008, Brown and Goldstein 1997).

High fat feeding has also been strongly implicated with metabolic complications (Buettner et al. 2007). High fat feeding in C57-Bl6 mice has been shown to cause signs of early metabolic syndrome in muscle tissue (de Wilde et al. 2007), including decreased mitochondrial function. High fat feeding clearly induces several features of the metabolic syndrome in numerous models. After about four weeks of feeding in rodents, animals develop obesity, moderate hyperglycemia, and insulin resistance (Buettner et al. 2007). It is important to differentiate the source of fat when discussing its effects, however. Unsaturated fats in the diet have in fact shown to decrease plasma TG, while saturated fats increase it (Buettner et al. 2007). Indeed, the effects of fat feeding are generally much more pronounced when dietary fat is derived from lard or beef tallow, rather than from fish or plant oils (Buettner et al. 2007). High fat diets derived from lard were also found to cause hepatic steatosis, and upregulated genes involved in fatty acid
synthesis, including those regulated by SREBP-1c (Buettner et al. 2006). A meta-analysis of high-fat diet feeding has identified the optimal diet for studying the detrimental effects of dietary fat. Buettner et al. identified diets containing more than 40% of their fat from an animal source as being ideal for studying dyslipidemia and insulin resistance. As 87% of the fat in the diet used in these experiments (and 26% of the entire FF diet) is composed of beef tallow, the FF diet is ideal for examining the dyslipidemic effects of dietary fat. Fatty acids have also been shown to directly upregulate $Lxr$ gene expression, providing another avenue for cross-talk between the pathways (Tobin et al. 2000).

These dietary additives do not act in isolation. The combination of fat and fructose has been shown to increase plasma TG and cholesterol in both hamster and gerbil models after only one week of feeding (Sullivan et al. 1993). The addition of cholesterol to a high fat diet in mice has also been shown to cause further insulin resistance, as well as increased liver inflammation and macrophage infiltration (Subramanian et al. 2008). The high fat/high cholesterol diet was also shown to increase plasma cholesterol and triglycerides in the hamster model (Sullivan et al. 1993). In hamsters, the addition of 3% cholesterol to a 15% fat diet caused a four-fold increase in plasma cholesterol after four weeks of feeding (Nistor et al. 1987). Cholesterol alone as a supplement to the chow diet can also dose-dependently increase the severity of atherosclerosis (Kleemann et al. 2007) in mice.

One advantage of using animals, especially mice, is that they can be genetically manipulated to determine the effect of particular genes. Two popular mouse models, the $ob/ob$ (obese) and $db/db$ (diabetic) mice, take advantage of mutations in the leptin signaling pathway. Disease progression in these models resembles that in humans, with early hyperinsulinemia, followed by obesity and finally pancreatic beta-cell failure (Chung et al. 1996, Neubauer and Kulkarni 2006, Zhang et al. 1994). The Zucker diabetic fatty rat (ZDF) is similar to these mice models in that it possesses mutations in the leptin receptor and also becomes obese and hyperglycemic early in its life (Cefalu 2006). This model develops hypertriglyceridemia and hyperinsulinemia, but does not exhibit excess plasma glucose (Sparks and Sparks 1994). These animals’ adipocytes are resistant to insulin, causing them to release excess free fatty acids, which increases hepatic VLDL production and secretion (Sparks and Sparks 1994).
1.8 Conclusion

The metabolic syndrome is a serious condition affecting people in the developed world and increasingly in the developing world. The consequences of this syndrome can be very diverse and include such outcomes as atherosclerosis, steatohepatitis and type 2 diabetes. The metabolic syndrome is most often initiated by diets high in fructose, fat and cholesterol. The serious population health consequences associated with metabolic syndrome should prompt more study into the mechanisms of its development and progression.

Modulation of nuclear receptors to control the metabolic syndrome is an ever-expanding area of biochemical research. New discoveries are being uncovered continually, expanding the understanding of this interesting field. Perhaps the next decade will clarify the mechanisms by which these nuclear receptors, particularly LXR, exert their effects.

1.9 Rationale for the Current Study

A trend towards increased fat, sugar and cholesterol intake, as well as increased sedentary activity, has prompted a shift in metabolic state in the Western world toward a situation of obesity, hypertension and insulin resistance. This cluster of pathologies has been collectively called the metabolic syndrome, and is of increasing concern worldwide.

Animal models have demonstrated the contributions of fructose, fat and cholesterol to development of components of the metabolic syndrome including dyslipidemia and insulin resistance. The hamster model has been of particular value because of its similarity to humans in lipoprotein metabolism (Hoang et al. 1993). Previous studies have shown that fructose feeding in the hamster model induces insulin resistance and metabolic dyslipidemia (Taghibiglou et al. 2000). Preliminary studies in our laboratory have shown that the addition of fat and cholesterol to the fructose diet further exacerbates this phenotype, causing even greater dyslipidemia. Cholesterol in particular has a potent effect, with experiments showing that cholesterol dose-dependently exaggerates the effects of the high fat/high fructose diet.

The nuclear receptor LXR has broad metabolic control, affecting transcription of genes involved in cholesterol, carbohydrate and fatty acid metabolism (Kalaany and Mangelsdorf 2006). Although LXR is known to improve reverse cholesterol transport and reduce hyperglycemia, it is
known to increase lipogenesis through upregulation of certain lipogenic genes. LXR’s endogenous ligands are oxysterols, a natural derivative of cholesterol.

The effect of LXR activation have been studied mainly in acute, short-term studies. Since LXR exerts such broad metabolic control, some of the effects may change after long-term activation.

1.10 Overall Hypothesis

Since cholesterol feeding and LXR activation are known to have similar manifestations, and since LXR is naturally activated by a derivative of cholesterol, cholesterol may exert its metabolic effects via LXR activation. LXR may also exacerbate the effects of dietary fat and fructose, as cholesterol is known to do. These effects may also increase in intensity with chronic, long term treatment.

1.11 Specific Objectives

1. Assess the effects of acute LXR activation with a lower LXR agonist dose than that which was previously used.

2. Determine the effects of the interaction between LXR activation and fat/fructose feeding over several time points.

3. Determine the chronic, long-term effects of LXR activation with either chow or fat/fructose diet feeding.

4. Directly compare the effects of dietary cholesterol with LXR activation on both the chow and fat/fructose diet background to determine whether cholesterol exerts its effects via LXR activation.
2. Materials and Methods

2.1 Materials

2.1.1 Animals

Male Syrian golden hamsters (*mesocricetus auratus*) were supplied by Charles River (Montreal, QC). Hamsters were maintained on either a chow diet or custom-produced combination of starch, fat, cholesterol and fructose (Dyets Inc, Bethlehem, PA). Since a portion of the fat in the high-fat diet is derived from beef tallow, there is some cholesterol in all of the diets containing high fat. The initial level of cholesterol in these diets is estimated to be 0.025%. The concentration listed in the other diets is exogenous cholesterol added to the diet. A diet listed as containing 0.25% cholesterol therefore in fact contains 0.275% cholesterol in total, and a high-fat diet with no additional cholesterol in fact carries the basic level of 0.025%. A detailed outline of each diet composition is available in Table 2.1.1. Isoflurane used as anaesthetic during all surgical procedures was from Baxter (Toronto, ON).

**Table 2.1.1. Diet composition.** Each dietary component is presented as a percentage of the total diet by weight. Diet composition information was provided by the manufacturer, Dyets Inc. FF is the high fat, high fructose diet; FFC is the FF diet supplemented with 0.5% cholesterol.

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</tbody>
</table>
Table 2.1.2. Detailed breakdown of dietary fat. Each type of fat is presented as a percentage of the total fat in the respective diet, by weight. The manufacturer, Dyets Inc, provided diet composition information.

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>FF</th>
<th>FFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>0.90</td>
<td>2.56</td>
<td>2.54</td>
</tr>
<tr>
<td>Myristoleic acid</td>
<td>0.011</td>
<td>0.495</td>
<td>0.491</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>21.40</td>
<td>24.00</td>
<td>23.82</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>1.62</td>
<td>3.71</td>
<td>3.68</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>8.99</td>
<td>20.91</td>
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<td>Oleic acid</td>
<td>30.20</td>
<td>35.58</td>
<td>35.30</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>27.50</td>
<td>12.65</td>
<td>12.55</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>1.85</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Trace</td>
<td>0.083</td>
<td>0.847</td>
</tr>
<tr>
<td>Other</td>
<td>7.44</td>
<td>Trace</td>
<td>Trace</td>
</tr>
</tbody>
</table>

2.1.2 Chemicals and reagents

Alpha minimal essential medium (αMEM) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Wisent (Montreal, QC). DMEM and αMEM without methionine, cysteine or glutamine were bought from Sigma (Oakville, ON). Certified grade fetal bovine serum (FBS), perfusion media, digest, collagenase and hepatocyte wash medium were supplied by Invitrogen Life Technologies (Burlington ON). Fibronectin pre-coated 6-well plates were obtained from BD Biosciences (Mississauga, ON).

The LXR agonist T0901317 was obtained from Cayman Chemical (Ann Arbor, MI). [35S]methionine protein labeling mixtures were purchased from PerkinElmer Life Sciences (Woodbridge, ON). Amplify was from Amersham (Baie d’Urfé, QC). All chemicals used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from BioRad (Mississauga, ON). Zysorbin used for immunoprecipitation was from Zymed Laboratories (Markham, ON). Polyvinylidene difluoride (PVDF) membrane was obtained from PerkinElmer Life Science (Boston, MA). SuperSCRIPTII RNase H- reverse transcriptase and primers were from Invitrogen Life Technologies (Burlington, ON). Eppendorf Mastercycler and Taq DNA polymerase were from Eppendorf Scientific, Inc. (Mississauga, ON). Bovine Serum Albumin (BSA) was from EMD Biosciences (Mississauga, ON). β-mercaptoethanol, bromophenol blue, glycerol, polyoxyethylene sorbitan monolaurate (Tween 20), sucrose, potassium bromide and all other chemicals of analytical grade were purchased from Sigma Aldrich (St. Louis, MO).
Trasylo (aprotinin) was acquired from Bayer (Etobicoke, ON). Protein inhibitor cocktail tablets were supplied by Roche Diagnostics (Laval, QC).

The blood glucose analysis kit, including disposable measurement strips, was obtained from Roche Diagnostics (Laval, QC). Lithium-heparin blood collection tubes were obtained from BD Biosciences (Mississauga, ON).

Kodak X-Omat Blue XB-1 film was from Eastman Kodak (Rochester, NY). Western Lightening enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Science (Boston, MA). Pre-stained molecular markers were obtained from New England Biolabs (Ipswich, MA) or from Fermentas (Burlington, ON). Mouse and rabbit horseradish peroxidase (HRP)-linked secondary antibodies were from Amersham (Buckinghamshire, UK), and goat HRP-linked secondary antibodies were from Sigma (St. Louis, MO). Rabbit anti-hamster apoB antibody was commercially prepared for our laboratory. Anti-MTP was a generous gift from Dr Andre Theriault. Remaining antibodies were obtained commercially: SREBP-1c, ChREBP from Santa Cruz (Santa Cruz, CA); FAS from Novus Biologicals (Littleton, CO); PPARα from Research Diagnostics Inc (Concord, MA); AKT, phospho-AKT (Ser473) and PTP-1B from Upstate Cell Signaling (Lake Placid, NY); Beta-actin from Sigma (St. Louis, MO). Polyclonal antibodies were used to augment inter-species reactivity when hamster-specific antibodies were not available.

2.1.3 Lab equipment and apparatuses

The Kinematica homogenizer used was from Brinkmann Instruments (Mississauga, ON). The Gel Doc Gel Documentation System was from Bio-Rad Laboratories, Inc. (Hercules, USA). The clinical chemistry analyzer utilized was a VITROS 950 from Ortho-Chemical Diagnostics (Rochester, NY). The Fast protein liquid chromatography (FPLC) system used was from Amersham Pharmacia Biotechnology (Uppsala, Sweden). Superose 6 prep grade cross-linked agarose gels were from Pharmacia (Piscataway, NY). The Personal Lab fraction autoanalyzer was manufactured by Roche Diagnostics (Laval, QC).
2.2 Methods

2.2.1 Animal protocols

Male Golden Syrian hamsters (100-110 g) (Mesocricetus auratus) were housed individually on alternating 12-hour light, 12-hour dark cycle with free access to food and water. After about a week of acclimatization and blood collection, animals were placed on either a control diet (normal chow), or on a diet enriched with fat and fructose (FF) (hamster diet with 40% fructose and 30% fat) (Table 2.2.1), supplemented with either nothing or 0.25% cholesterol (FFC). Selected animal groups, fed either the chow or the FF diet, were also administered the chemical LXR agonist TO901317 (10 – 50 mg/kg) by oral gavage. The agonist was first dissolved in a small amount of dimethylsulfoxide (DMSO), usually about 60 µl, to obtain a final concentration of 0.5 mg/ml. The appropriate amount of stock agonist mixture was added to 400 µl of 1% carboxymethylcellulose (CMC) to give a dose proportional to each hamsters’ body weight. Animals not receiving the LXR agonist treatment were given 400 µl of 1% CMC with the appropriate amount of DMSO added. The diet and agonist treatments were continued for between 4 and 30 days. For the FXR agonist study, a randomly selected group of hamsters were given the chemical FXR agonist GW4064, which was kindly supplied by Dr Tim Willson (GlaxoSmithKline). The agonist was dissolved in the vehicle, 2-hydroxypropyl-β-cyclodextrin, and was administered at a dose of 30 mg/kg twice per day for five days.

For all agonist studies, hamster weights were monitored at baseline, at intermediate points throughout their treatment protocol and at the endpoint. Blood was collected at baseline, at intermediate points, and at the endpoint of each study. Blood collected for glucose measurement was taken by pricking the saphenous vein and collecting a drop of blood directly into a hand-held glucose meter. For baseline and intermediate measurements, plasma was collected via retro-orbital bleed into lithium heparin-coated tubes. For endpoint measurement, plasma was collected by cardiac puncture. Blood was centrifuged at 4°C for 15 minutes at 6,000 rpm, and plasma was then removed by micropipette. All lipid, enzyme and FPLC measurements were conducted within 48 hours on fresh plasma. Protease inhibitors were added to remaining plasma, which was then frozen at -80°C for subsequent protein analysis.
All surgical procedures were conducted under isoflurane (4% in 100% oxygen by mask). Euthanasia was conducted under isoflurane gas via a thoracotomy as to create negative pressure. Before collection of blood and tissues, hamsters were fasted for 6-8 hours. Tissues including liver, muscle, intestine, pancreas and adipose were snap-frozen in cryo-tubes in liquid nitrogen and stored at -80°C for subsequent RNA isolation, protein mass analysis, and TG/cholesterol mass analysis. Liver and adipose tissue (gonadal fat pads) were first weighed using an analytical balance and the weights were recorded. Surgery and liver perfusions were performed under anesthesia, on a heating pad to maintain body temperature. Livers were perfused by isolation from the circulatory system; the thoracic aorta, the caudal vena cava, the abdominal aorta, and the abdominal vena cava were blocked using sutures. The portal vein was then severed, allowing the flow of 50 ml of 39 °C liver perfusion medium out of the liver. After this, 50 ml of 39 °C liver digest medium containing collagenase was circulated through the liver. The liver was excised and minced in hepatocyte wash medium. Digested liver tissue was filtered through a cell strainer (100 µm) and the released hepatocytes were pelleted by centrifugation (60 g, 3 min), washed 3 times in hepatocyte wash medium, and resuspended in attachment media (Williams E-containing 5% fetal bovine serum, 1 µg/ml insulin, 0.1% penicillin–streptomycin). Cells were seeded in fibronectin-coated dishes (1–1.5 million ~ 106 cells/35 mm dish) and incubated for approximately 3 h (37 °C, 5% CO2) to facilitate attachment. All subsequent experiments were performed on attached cells. Viability of hepatocytes isolated in this manner ranged from 75 to 90% as measured by trypan blue dye exclusion. Isolated hepatocytes were not used for experiments if the viability was less than 70%. All animal experiments were approved by the Animal Ethics Committee of the Hospital for Sick Children and were conducted according to national guidelines.

2.2.2 Intraperitoneal glucose and insulin tolerance tests

Prior to glucose injection, baseline blood glucose levels were determined using the Roche Diagnostics kit, as described above. A volume of 30% glucose solution amounting to 1.5 g/kg total glucose per animal was injected into the peritoneum of the hamsters. Blood glucose was then measured at 15, 30, 60 and 120 minutes after initial glucose administration. Hamsters from each diet/treatment group were measured concurrently to avoid any confounding effects due to
fasting time or hormonal changes during the day. Insulin tolerance tests were conducted the same way, with each hamster receiving 5 units of insulin per kg of body weight.

2.2.3  Plasma analyses

Blood samples were withdrawn by either retro-orbital bleed at baseline points, or by cardiac puncture at endpoints, under anesthesia using isoflurane. Blood was collected in a lithium heparin-coated tube and centrifuged at 6000 rpm for 15 minutes at 4°C to obtain plasma. Cholesterol, alanine transaminase (ALT), aspartate transaminase (AST), and TG levels were determined on a clinical chemistry analyzer from freshly collected plasma.

2.2.4  Hepatic triglyceride and cholesterol mass measurements

Hepatic lipid extraction method was based on the Folch method (Folch et al. 1957). The weights of liver tissue pieces were recorded. Tissue pieces were then soaked for at least two days in 20 volumes of a 2:1 chloroform:methanol mixture. Tissue pieces were removed from the solvent using forceps. Saline solution (0.9% NaCl, 0.2 volumes) was added to the solvent to wash. The mixture was vortexed for several seconds, and the tubes were centrifuged at 2000 rpm for three minutes. The upper aqueous layer was removed using suction, as well as any remaining small tissue pieces. The tubes were then left uncapped in a fume hood for several days until all solvent had evaporated. Lipids were then reconstituted in 2 ml of 100% ethanol and agitated, capped, for at least four hours to ensure all lipids had dissolved. Lipid concentration was measured in each sample using the Randox analytical kit (Crumlin, Co. Antrim, UK) and read at 500 nm on a microplate reader.

2.2.5  Analysis of lipoproteins by fast-pressure liquid chromatography

At the end of each designated treatment period, animals were killed and the blood was collected by cardiac puncture. Lipoproteins from the plasma were separated by automated size exclusion gel filtration chromatography, on an FPLC system. Plasma samples were loaded onto a Waters 715 Ultra WISP Sample Processor, where 100-200 uL were injected into an Amersham Biosciences Tricorn Superose 6 10/200 GL column. The flow rate was set at 0.5 ml/minute. Fifty one ml fractions were collected with a BioRad Model 2110 Fraction Collector, and fractions 10
to 45 were assayed for cholesterol and TG. Cholesterol and TG reagents were purchased from Roche Diagnostics (Indianapolis, IN) and adapted onto the Personal Lab enzyme linked immunosorbant assay (ELISA) plate analyzer. A series of standards were prepared from human samples with known cholesterol and TG concentrations, and the optical density readings from the fractions were compared to these standards.

2.2.6 Immunoblot analysis

For whole-cell protein mass analysis, whole liver was placed in Solubilizing Buffer A (Phosphate-buffered saline [PBS: 0.137 mol/l NaCl, 27 mmol/l KCl, 0.01 mol/l Na2HPO4*7H2O, 18 mmol/l KH2PO4, pH 7.4] containing 1% Nonidet P-40 (NP-40), 24 mmol/l sodium deoxycholate, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l ethylene glycol-bis(β-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), 2 mmol/l phenylmethylsulfonylfluoride (PMSF), 100 kallikrein-inactivating units/ml aprotinin, pH 7.4). Tissues were then homogenized using either a Kinematica mechanical electric homogenizer, or the Ultrasonic Processor XL sonicator (Heat Systems, Inc, Farmingdale, NY) set at level 4. Sonicated samples were subjected to three sonication bursts lasting 5 seconds each, while the tubes were immersed in an ice bath to avoid overheating.

For subcellular isolation, whole liver was homogenized in Solubilizing Buffer B (3 mmol/L imidazole, 250 mmol/L sucrose at pH 7.4 with 1 mmol/L PMSF, 100 kallikrein-inactivating units/ml aprotinin) using a homogenizer, and centrifuged (2200 g) to separate the nuclear pellet and the cytosol. Nuclear pellets were then lysed using Buffer A. Hepatocyte cytosolic and nuclear lysates containing equivalent amounts of total protein were separated by SDS-PAGE on mini-gels (8 x 5 cm) that were composed of 3% stacking and 5-10% resolving gels, using prepared running buffer (25 mmol/l Tris, 192 mmol/l glycine, 0.1% SDS, pH 8.4). Following electrophoresis, proteins were transferred electrophoretically onto PVDF membranes using a BioRad Wet Transfer System, using prepared transfer buffer (70% methanol, 0.192 mol/l glycine, 25 mmol/l Tris, pH 8.3). The membranes were blocked with 5% fat-free dry milk powder solution and then incubated with the selected primary antibodies. Membranes were washed three times for 10 minutes each with 10 ml PBS-T (PBS containing 0.5% Tween-20), and then the membranes were incubated with a secondary antibody conjugated to horseradish
peroxidase. After another round of washing, membranes were incubated in an ECL detection reagent for 1 minute and exposed to Kodak X-Omat Blue XB-1 film. Films were developed and quantitative analysis was performed using Imaging Densitometer and FluorChem software densitometry. Determination of the molecular weight of electrophoretically separated proteins was based on comparison with prestained markers of known molecular weight (Fermentas, Burlington ON).

To detect phosphorylation level of hepatic AKT, hamsters were treated in vivo with 5 U/kg of insulin for two minutes via the vena cava. Livers were then homogenized in Solubilizing Buffer C containing a phosphatase inhibitor mixture (150 mmol/l NaCl, 10 mmol/l Tris (pH 7.4), 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 1% NP-40, 2 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, and 2 mmol/l sodium orthovanadate). Hepatic homogenates were then subjected to SDS-PAGE as described above and proteins transferred to a PVDF membrane. Immunoblotting was first carried out using the anti-phospho-AKT antibody (Ser473) to determine the level of phosphorylation. Membranes were then stripped using stripping buffer (2% SDS, 62.5 mmol/l Tris, 1% beta-mercaptoethanol, pH 6.8) for 20 minutes at 60°C, and then 10 minutes at room temperature. Membranes were then rinsed three times for 10 minutes each with PBS-T, and finally blocked for either one hour at room temperature, or overnight at 4°C in PBS-T with 5% dry milk. A second immunoprecipitation was conducted using the AKT mass antibody, in order to normalize the phosphorylation to the level of total AKT protein expressed.

To measure the amount of apoB in the plasma, plasma was first diluted by 200x in ddH2O. Seven parts diluted plasma were then mixed with 2.5 parts 4X sample preparation buffer (8% SDS, 0.25 mol/l Tris, 40% glycerol) and 0.5 parts beta-mercaptoethanol with 5% bromophenol blue. Equal volumes of this sample mixture were loaded to a 6% SDS-PAGE gel and separated by electrophoresis for four hours at 100V. Protein was then transferred to a PVDF membrane using a wet transfer system and the membranes were blotted for apoB as described above.

2.2.7 Metabolic labeling and immunoprecipitation

All pulse-chase labeling experiments were performed in triplicate. For metabolic labeling and immunoprecipitation experiments, cells were pre-incubated in methionine-free media at 37°C for
1 hour and pulsed with 100 μCi/ml of [35S]methionine for 45 minutes. At various time points, the media were collected and the cells were washed twice with PBS. Chase medium (20% FBS-DMEM) was added at this time for pulse-chase experiments and incubated for 2 hours. The medium was then collected and cells were lysed in cell solubilization buffer (PBS containing 1% NP-40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, 10 μg/ml aprotinin). The lysates were clarified by centrifugation and supernatants were collected for immunoprecipitation. 500 μL of solubilizing buffer and 5 μL of rabbit anti-hamster apoB antibody were added to each sample. Samples were incubated at 4°C for 18 hours with gentle shaking. After incubation with primary antibody, 50 μL of a 50% Zysorbin slurry (protein A) was added, and samples were mixed at room temperature for 1 hour. The samples were centrifuged at 13 000 rpm for 3 minutes, and the supernatant was removed. The pellet was washed 3 times with 1 ml of cold PBS resuspending the pellet for 10 minutes between each wash. Samples were prepared for electrophoresis by resuspending in 100 μL SDS-PAGE Laemmli sample buffer and boiling for 5 minutes. ApoB was resolved by 5% SDS-PAGE at 70 V, for 16 hours. After electrophoresis, the gels were fixed and incubated in Amplify before being dried and exposed to Kodak X-Omat Blue XB-1 film at -80°C for 72 to 86 hours. The radioactivity in the apoB100 band was quantified by digestion of the gel slices by addition of 200 μL 60% perchloric acid and 400 μL hydrogen peroxide, and 18-hour incubation at 60°C. Radioactivity was quantified by liquid scintillation counting and results were normalized according to total radiolabeled protein levels as measured by trichloroacetic acid (TCA) precipitation, to control for total radioactivity.

2.2.8 In vitro labeling of newly synthesized lipids

Primary hamster hepatocytes were pulsed for 18 h with 5 μCi/ml [3H]acetate to assess the rates of synthesis and secretion of cholesterol. Cholesteryl ester and TG synthesis and secretion were analyzed by labeling hamster hepatocytes for 18 h with 5 μCi/ml [9,10-3H]oleic acid bound to BSA. Following labeling, cells and media were extracted with hexane/isopropanol (3:2) and the total lipid extract was dried, dissolved in hexane, and applied to a thin layer chromatogram (TLC). The TLC plates were developed using a solvent system to separate neutral lipids with petroleum ether/diethyl ether/acetic acid (90:10:1). The lipids were stained with iodine vapour
and identified based on comparison to lipid standards. The spots identified on the TLC plates were excised and quantified by scintillation counting.

2.2.9 Tissue collection, RNA extraction and cDNA synthesis

Total RNA was extracted following homogenization of frozen liver samples using a commercially available RNA isolation kit (RNeasy Mini kit; Qiagen). All samples were treated with DNase (RNase-Free DNase; Qiagen) during the extraction protocol to preclude the presence of any contaminating DNA in subsequent procedures. Prior to cDNA synthesis, total RNA was quantified by ultraviolet (UV) spectrophotometry. To ensure the quality of RNA, aliquots of extracted RNA were denatured and separated on MOPS-buffered 1% agarose gels. RNA was deemed to be of acceptable quality if both the 28S rRNA and 18S rRNA were visible as clear, sharp bands under UV light after staining with ethidium bromide. Following quantification, 0.4 µg of total RNA was converted to single-stranded cDNA using TaqMan Reverse Transcription (RT) Reagents (ABI). Random hexamers were used to prime the RT reaction, allowing the expression of 18S rRNA to be used as an internal reference during subsequent polymerase chain reactions (PCR). The RT reaction was carried out in a MiniCycler (MJ Research) as follows: samples were pre-incubated at 25°C for 10 min, RT performed at 37°C for 60 min and, finally, the reverse transcriptase was inactivated by heating the samples at 95°C for 5 min. Following RT, cDNA was stored at -20°C.

2.2.10 Real-time quantitative PCR analysis

mRNA levels of a number of genes (see Table 2.2.2) were assessed by real-time quantitative RT-PCR using an ABI Prism 7700 sequence detector. All PCR reactions were performed in a total volume of 50 µl and included the following components: cDNA derived from 10 ng of total RNA, 400 nmol/l of each primer, RNase-free water, and 25 µl of SYBR Green PCR Master Mix (ABI), an optimized buffer system containing AmpliTaq Gold DNA polymerase and dNTPs. All PCR reactions were performed in duplicate and cycling parameters were as follows: after an initial denaturation step for 10 min at 95°C, 40 subsequent cycles were performed in which samples were denatured for 15 s at 95°C followed by primer annealing and elongation at 60°C.
for 1 min. Relative quantities of mRNA were calculated from CT values using the comparative
CT method (Livak and Schmittgen 2001) using 18S rRNA as an internal reference.

Primer pairs for real-time PCR were designed using Primer3 software (NIH) and sequence
information obtained from GenBank. Hamster-specific sequences were unavailable for many of
the genes measured in this study. In these cases, primers were constructed to highly conserved
regions of individual genes identified through multiple sequence alignments between other
closely related species (e.g. rat, mouse, or human).

To ensure specificity of amplification during real-time PCR (essential when using SYBR Green
given that this dye binds to all double-stranded DNA), amplified products were subjected to
agarose gel electrophoresis to visually confirm the presence of a single amplicon of the expected
size.

Table 2.2.1. Sequence information for genes measured by real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'-sense-3'</th>
<th>5'-antisense-3'</th>
<th>Product size (bp)</th>
<th>Reference or accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srebp-1c</td>
<td>5'-GGGGGACGCAGTCTGGG-3'</td>
<td>5'-ATGAGCTGGAGCATGTCTTTCAAA-3'</td>
<td>95</td>
<td>(Shimomura et al. 1997)</td>
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<tr>
<td>Srebp-2</td>
<td>5'-AGCTGGGAAATCAGAAAGACAAG-3'</td>
<td>5'-GATTAAAGTCTTTCAATCTTTCAAGTCCAC-3'</td>
<td>93</td>
<td>(Field et al. 2003)</td>
</tr>
<tr>
<td>Fas</td>
<td>5'-AGCCCCCTCAAGTGACAGATG-3'</td>
<td>5'-TGCCAATGTGTTTTCCCTGA-3'</td>
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<td>(Field et al. 2003)</td>
</tr>
<tr>
<td>Mtp</td>
<td>5'-GTCAGGAAGCTGTGTCAGA-3'</td>
<td>5'-CTCCTTTTTTCTTGCTTTCCA-3'</td>
<td>99</td>
<td>U14995*</td>
</tr>
<tr>
<td>Scd-1</td>
<td>5'-ACATGTCTGACCTGAAAGCTGA-3'</td>
<td>5'-GTCCCTTCTGGAAACATCAC-3'</td>
<td>50</td>
<td>NM_139192*</td>
</tr>
<tr>
<td>Chrebp</td>
<td>5'-GTGTAGACAAACAAAGATGGA-3'</td>
<td>5'-TAATATTGAAACGCCTTCTTC-3'</td>
<td>76</td>
<td>AB074517*</td>
</tr>
<tr>
<td>Bsep</td>
<td>5'-TCAAGGCAATGTGTTACCACAAGATG-3'</td>
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<td>AF186585*</td>
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<td>5'-TTGTCTTTATTGGAGCTTCTTC-3'</td>
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<td>NM_013454*</td>
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<td>18S</td>
<td>5'-TAAGTCCTGAGGCGCTTTTGACACA-3'</td>
<td>5'-GATCCGAGGGCCTCTACA-3'</td>
<td>71</td>
<td>(Field et al. 2003)</td>
</tr>
</tbody>
</table>

*Primers based on sequence homology; no hamster sequence available
2.2.11 Protein analysis

Protein content was measured according to the Detergent Compatible (DC) Protein Assay from BioRad using BSA as the standard.

2.2.12 Statistical analysis

Statistical differences were analyzed by using Student’s t test with the level of significance set at 0.05.
3 :: Results

3.1 Acute LXR Activation

Previous work in our lab focused on the effect of acute LXR activation on overall lipid metabolism in the hamster model. These experiments showed that LXR activation at an agonist dose of 50 mg/kg caused increased plasma triglycerides, changes in apoB regulation, and dysregulated insulin signaling. In this thesis, several of the previously acquired results were repeated as confirmation, and additional experiments were performed using a lower agonist dose, in order to verify the agonist’s effects at a weaker concentration.

3.1.1 Confirmation of experiments at 50 mg/kg

In order to confirm previous results showing no difference in lipid synthesis and secretion between LXR-treated hamsters and those given the vehicle, hamster hepatocytes were treated ex-vivo with radioactive-labelled acetate and oleate to measure their incorporation into cellular lipids. Results showed that there was no difference between the LXR agonist-treated group and vehicle-treated groups with respect to triglyceride and cholesterol synthesis rates (Figure 3.1.1). Hepatic lipid accumulation, measured in preceding experiments by a former graduate student, were re-analyzed using a new method. The LXR-treated and vehicle-treated groups did not differ in their hepatic triglyceride and cholesterol accumulation (Figure 3.1.1). This was surprising as metabolic dyslipidemia had been observed in the LXR agonist-treated hamsters, with respect to plasma lipid levels and VLDL production and secretion (Basciano et al. 2008, in submission).

3.1.2 Confirmation of dyslipidemic effects using a lower agonist dose

To determine if the previously obtained results were a result of a super-physiological dose of LXR agonist, the experiments were repeated using a dose of 10 mg/kg, rather than 50 mg/kg, as was used in previous experiments. Published studies have shown that doses as low as 1 mg/kg in mice are effective in eliciting increases in known LXR targets including Scd and Srebp-1 (Schultz et al. 2000).
Figure 3.1.1. No apparent change in lipid synthesis levels after treatment with the LXR agonist, T0901317.

Hepatocytes were isolated from vehicle and TO901317 treated, chow-fed hamsters and incubated in $[^3\text{H}]$ acetate- or $[^3\text{H}]$ oleate-labeled media for 18 hours. Lipids were extracted from both the cells and media, dissolved in hexane, and applied to a thin layer chromatogram. The TLC plates were developed using a solvent system to separate neutral lipids and stained with iodine vapour. Samples were identified based on comparison to lipid standards and excised and quantified by scintillation counting. Data (mean + SD) are representative of three independent experiments (n=3).

In hamsters, a dose of 10 mg/kg T0901317 administered for four days resulted in a slight but insignificant 15% increase in plasma triglycerides (Figure 3.1.2). There was also no significant change in plasma cholesterol or alanine transaminase (ALT), a marker of liver damage. There
was, however, an increase in the TG content of the VLDL plasma fraction, as determined by Fast Protein Liquid Chromatography (FPLC) analysis (Figure 3.1.3). This was corroborated by a parallel increase in the amount of plasma apoB measured by Western blot (Figure 3.1.2). This perhaps means that LXR activation causes a shift in plasma TG toward the VLDL fraction.

**Figure 3.1.2. Low dose T0901317 increases plasma apoB but not triglycerides.**

Blood samples were drawn from all hamsters by cardiac puncture under anaesthesia using isoflurane. Plasma was isolated by centrifugation. A) Cholesterol, TG and ALT measurements were conducted using a clinical chemistry analyzer. Data (mean + SD) are representative of between six and nine independent measurements (n=6-9). B) Plasma apoB mass was analyzed by immunoblotting using an anti-apoB antibody.

Previously, alterations in synthesis and secretion of apoB from the liver were seen at a dose of 50 mg/kg. In order to determine whether these alterations were maintained at a lower dose of 10 mg/kg, a pulse-chase experiment was conducted using primary hepatocytes from hamsters treated with either the LXR agonist or vehicle control. LXR agonist-treated hamsters showed a two-fold increase in total hepatic apoB after one hour, and this was maintained after two hours
(Figure 3.1.4). This was significantly higher than the level observed in cells from the vehicle-treated hamsters (p<0.03), whose apoB levels dropped by 23% after one hour, and remained at this level after 2 hours. ApoB secretion was also significantly higher in hepatocytes from LXR agonist-treated hamsters. While the control group showed a drop in apoB secretion to slightly less than half, LXR agonist-treated hepatocytes exhibited a 13% increase after two hours (p<0.03 compared to vehicle). This result is similar to that seen with the 50 mg/kg dose, showing that the lower dose of 10 mg/kg T0901317 is as effective in bringing about dyslipidemic changes.

![Figure 3.1.3. Low dose T0901317 increases TG in the VLDL fraction.](image)

Blood samples were collected at the end of the four-day treatment period by cardiac puncture. Lipoproteins were separated by density using automated size exclusion gel filtration chromatography, on a fast pressure liquid chromatography (FPLC) system. Fractions 11 to 45 were then assayed for cholesterol and TG.
Figure 3.1.4. Low dose T0901317 increases apoB synthesis and secretion.

Hepatocytes were isolated from both vehicle and T0901317-treated, chow-fed hamsters. The cells were incubated in methionine-free medium for 45 minutes, and then subjected to a 60-minute pulse with [35S]-labelled methionine. Following the pulse, cells were rinsed and allowed to recover for 0, 60 or 120 minutes. Cells and media were collected and solubilized and apoB was immunoprecipitated from each sample overnight. ApoB was then solubilized in SDS sample buffer and subjected to SDS-PAGE. ApoB100 was quantified by physically cutting each band and measuring radioactivity by liquid scintillation counting. Results were normalized according to total radiolabeled protein as measured by TCA precipitation. Data (mean ± SD) are representative of three experiments (n=3).
3.1.3 Conclusion

The LXR agonist T0901317 can be administered in a dose of 10 mg/kg to hamsters and have metabolic effects comparable to those observed at 50 mg/kg, particularly when observing hepatic apoB synthesis and secretion. While there was not a significant effect on plasma TG, neither was there a measurable increase in ALT, an indicator of hepatic damage. Perhaps an intermediate dose of T0901317 would be most appropriate, to optimize the dyslipidemic effects while minimizing hepatic injury.

3.2 Effect of LXR Activation With the Fat/Fructose Diet

The lipogenic effects of LXR activation have been documented in previous work (Chisholm et al. 2003, Greghorst et al. 2002, Schultz et al. 2000, Yoshikawa et al. 2001). There are other dietary factors that can have lipogenic effects as well, however, including fat and carbohydrates, particularly fructose (Basciano et al. 2005). Since previous work has shown that cholesterol exacerbates the metabolic effects of a high fat/high fructose diet (Basciano 2008, in submission), and since the nuclear receptor LXR is activated by oxysterol derivatives of cholesterol, it is possible that LXR activation may also augment the effects of these dietary additives. To test this hypothesis, LXR was administered to hamsters being fed a combination of 30% fat and 40% fructose (the FF diet). In addition, previous studies on LXR activation in hamsters have looked primarily at an acute activation period of four days. In order to determine the chronic effects of LXR activation on the FF diet background, these studies lasted between four and 16 days in duration, using a dose of 20 mg/kg.

3.2.1 LXR activation increases plasma lipids

A time-course study of LXR activation with either a chow or fat/fructose diet background was conducted to determine the progression of plasma lipid changes over time points up to 16 days (Figure 3.2.1). Plasma triglycerides were shown to increase by over nine-fold above its baseline values after 16 days of FF diet feeding and T0901317 administration (p<0.0001). By comparison, the hamsters fed the FF diet alone experienced a more modest three-fold increase above its baseline level (p<0.0001). This increase appeared to manifest itself quickly, with a
significant increase in both groups above their baseline levels after just four days (p<0.05 for both). The group given the LXR agonist also showed a significantly greater increase than the group fed the FF diet alone after eight days, increasing by eight-fold compared to two-fold, respectively (p=0.02).

Figure 3.2.1. LXR activation increases plasma lipid levels in FF diet-consuming hamsters.
Blood samples were drawn from all hamsters by retro-orbital bleed at intermediate points, or by cardiac puncture at final collection. Plasma was isolated by centrifugation. A-D) Cholesterol, TG, AST and ALT were measured using a clinical chemistry analyzer. Data (mean ± SD) are representative of between four and six independent measurements (n=4-6). E) Hamster body weights measured during experiment progression using an analytical scale. F) Plasma apoB mass was analyzed by immunoblotting using an anti-apoB antibody. Representative blots are shown here.

The effect of LXR activation on plasma cholesterol levels was noticeable but less marked. Both groups showed increased plasma cholesterol levels compared with their respective starting levels after as little as four days of treatment (p<0.006 for all comparisons). Activation of LXR caused a significantly greater increase than the FF diet alone after four and eight days. The FFLXR group increased by 44% compared with 13% in the FF group after four days (p=0.025), and 52% compared with 35% after eight days (p=0.045), but cholesterol levels in both groups converged at a similar increase of approximately 35% above baseline after 16 days.

The enzymes ALT and aspartate transaminase (AST) are known to increase in plasma during a state of liver damage (Fraser et al. 2008). These enzymes were measured in the plasma of the two groups as a measure of hepatic function. Neither the FF diet alone, nor the diet in combination with the LXR agonist caused a striking elevation in either of the enzymes when compared to their baseline levels. This indicates that neither diet significantly injured the liver by these measures.

LXR activation had no effect on body weight when compared with the FF diet alone (Figure 3.2.1 E)

3.2.2 Analysis of lipid density fractions in hamster plasma

In order to examine the effect of different diets and treatments on lipoprotein density distribution, plasma samples were separated according to density using FPLC. This method separates the different lipoprotein components by density, allowing analysis of the TG and cholesterol content of each lipoprotein fraction.
Measuring the lipid content of the different density fractions of the hamster plasma gives a good indication of whether the distribution of lipids shifts in response to different treatments. In order to best observe this shift in lipid distribution, the amount of lipid in each fraction was plotted as a percentage of total lipid collected from that animal. In this way, the change in distribution can be seen regardless of any changes in raw lipid amount.

Figure 3.2.2. LXR activation shifts plasma lipids to the VLDL fraction.

Blood samples were collected at the end of the treatment period by cardiac puncture under isoflurane anaesthetic. Plasma lipoproteins were separated by density using automated size exclusion gel filtration chromatography, on a fast pressure liquid chromatography (FPLC) system. Fractions 11 to 45 were then assayed for cholesterol and TG. Density divisions were identified by peaks in cholesterol and TG. VLDL was defined as fractions 11-20, LDL as fractions 21-28, and HDL as fractions 29-45. The percentage of the lipid in each fraction as a proportion of the total lipid collected was calculated and plotted as shown. Data (mean + SD) represent the average measurements of four independent animals (n=4).
The effect of LXR activation on the fat/fructose dietary background was measured over different time periods. After four and eight days of treatment, the LXR agonist-fed group showed a non-significant shift of both TG and cholesterol towards the VLDL fraction (Figure 3.2.2). After 16 days, however, this shift achieved significance, with the T0901317-fed group showing 67% of their cholesterol and 75% of their plasma TG in the VLDL fraction, compared to 18% and 53% for the vehicle-fed control group, respectively (p<0.001 for both). In all cases, the increase in VLDL lipids seemed to be at the expense of both the LDL and HDL fractions.

The changes in VLDL composition are corroborated by measurements of plasma apoB level by Western blot. Since apoB is the main protein component of VLDL, its level should correlate with that of the lipoprotein. In fact, the apoB level does not change significantly with the addition of the LXR agonist to the FF diet after four or eight days of treatment, but does achieve a significant increase after 16 days. This is in agreement with the shifts in VLDL composition described above.^

### 3.2.3 Hepatic lipid accumulation was not affected by LXR agonist feeding

The amount of lipid stored in the liver is another good indication of metabolic dyslipidemia. Severe lipid accumulation indicates hepatic steatosis, which may progress to the more serious and inflammatory steatohepatitis (Rector et al. 2008). Measurements of hepatic cholesterol and TG mass in the FF diet-fed groups showed that the addition of the LXR agonist had no significant effect on the accumulation of either lipid in the liver (Figure 3.2.3). The LXR agonist seemed to show a slight increase in hepatic cholesterol over the FF diet group after 16 days, but this effect was not significant (p=0.17). The change in both TG and cholesterol levels over time was also not significant for either group.
3.2.4 LXR activation causes an increase in liver mass but not adipose tissue mass

While the LXR agonist did not induce an increase in hepatic lipid accumulation, it did affect the mass of the liver. After four days the LXR agonist-treated group showed a non-significant increase of 16% above the group only receiving the FF diet \((p=0.065)\) (Figure 3.2.4). After eight days, this increase reached a significant level, rising to 24% above the FF group \((p=0.037)\) and remaining at that level after 16 days \((p=0.00046)\). It is interesting and slightly puzzling that the FFLXR group did not show a parallel increase in lipid accumulation to explain this gain in liver mass. However, since the liver weight is taken as a percentage of their total body mass, it is possible that they are losing weight elsewhere in their bodies, increasing the proportional weight of their livers.
The weight of the gonadal fat pads was also measured and is plotted as a percentage of total body weight in Figure 3.2.4. Adipose tissue mass was not affected by LXR activation in animals being fed the FF diet. The weight of the fat pads as a percentage of total body mass also did not appear to change over time. This indicates that if the animals are storing excess fat after LXR activation, it is being deposited elsewhere in the body.

3.2.5 Changes in mRNA expression upon LXR activation

Since LXR is a transcription factor, the primary result of its activation is initiation of gene transcription. In order to measure the effects of this activation, mRNA levels of several LXR targets were measured by real-time RT-PCR.

One surprising effect was the lack of substantial change in \textit{Abca1} mRNA expression in the FFLXR group compared to the FF-fed group after 4, 8 or 16 days (Figure 3.2.5). There was a significant 89% increase after eight days (p=0.0004), but this was not maintained after 16 days (50% decrease, p=0.052). \textit{Abca1} is a known LXR target, and so it is surprising that the addition...
of the LXR agonist to the FF diet did not augment its gene expression level. Since other factors, including PPAR-alpha, are known to modulate Abca1 expression, the fat in the FF diet may already activate this gene’s expression. The addition of the LXR agonist may not have had a further effect in activating this expression. Another known LXR target, the lipogenic factor Scd, was in fact increased by over seven-fold after four days of activation (p=0.0002), by almost ten-fold after eight days (p=0.000008), and by three-fold after 16 days (p=0.0026), when compared with the FF diet alone. The level of Fas expression increased strongly after four days of treatment with T0901317 above the FF diet alone (2.5-fold, p=0.0036). This increase was maintained after eight days of feeding (2.7-fold, p=0.0055), but not after 16 days (15% increase, p=0.31). The level of Mtp was measured to determine a possible avenue by which LXR activation may affect VLDL assembly. The pattern of Mtp expression over time is similar to that of Fas. That is, there was an initial increase in Mtp with LXR activation on the background of the FF diet (2.3-fold increase after four days, p=0.0008), which was reduced to a still significant 30% increase after eight days (p=0.008). After 16 days, however, the level of Mtp expression fell to 44% below the control group level (p=0.005).

The expression levels of several other transcription factors were also measured to determine more widespread effects of LXR activation. Srebp-1 and Chrebp are transcription factors that control lipogenic programs in liver cells. Srebp-1 showed an initial 80% increase with T0901317 administration after four days (p=0.047) that increased even more to 2.6 times the FF-fed level after eight days (p=0.0002). Although there appears to be a decrease in Srebp-1 expression relative to the FF-fed animals after 16 days, the changes in the mRNA levels were not significant. The 50% increase in Chrebp mRNA after 4 days was not significant (p=0.056), although the Chrebp level rose to 2.2 times the control level with LXR activation after eight days (p=0.0048). These increases faded after 16 days of feeding, however, with no apparent difference between the two different treatment groups. Srebp-2 is a transcription factor that controls genes involved in cholesterol biosynthesis. Its level is known to increase in LXR -/- animals (Wojcicka et al. 2007), and so it is curious to see a decrease in the mRNA level of this factor after both four days (114% increase, p=0.048) and eight days (50% increase, p=0.015) with LXR activation on the FF diet background, compared to FF diet-feeding alone. The 70% decrease in Srebp-2 mRNA after 16 days (p=0.0052) is in better line with expectations. LXR is not, however, known to regulate Srebp-2 expression either positively or negatively, so the effect seen in LXR -/- mice
may be more strongly related to cholesterol depletion in these animals, rather than the lack of LXR activation (Sato et al. 1996).

**Figure 3.2.5. Changes in hepatic mRNA levels after treatment of FF-fed hamsters with T0901317.**

Total RNA was isolated from snap-frozen hamster liver tissue using the Qiagen RNeasy Mini kit according to manufacturer’s protocol. RNA concentrations were determined using spectrophotometry. After reverse transcription, cDNA levels were assessed by real-time quantitative RT-PCR, using 18S rRNA as an internal control. Data (mean + SD) are expressed
relative to the FF-fed vehicle control, and represent between four and six independent measurements (n=4-6).

FXR is a nuclear receptor that acts in a complementary way to LXR. FXR is activated by bile acids and downregulates the bile-acid producing gene *Cyp7a1* (Cariou and Staels 2007). *Bsep*, a known target of FXR, was measured to determine the extent to which FXR was also activated under the previous conditions. *Bsep* was in fact found to be upregulated by two-fold after four days of LXR activation (p=0.013) when compared with FF diet feeding alone, and by 93% after eight days (p=0.004). This indicates that LXR activation interacts with the activation state of FXR. This may happen either through direct activation of FXR by T0901317 (Houck et al. 2004), or because of LXR-mediated increases in FXR’s natural agonists, bile acids. FXR activation is not maintained after 16 days of feeding, however, as *Bsep* expression levels drop to 65% of FF-fed levels (p=0.0024) with LXR activation. It may be misleading to compare the effect of the LXR agonist only to the FF diet, since the diet may have substantial effects on its own. The effect of LXR activation will be directly compared with both the FF diet and the chow diet in a subsequent section.

### 3.2.6 LXR activation induces expression of lipogenic proteins

Hepatic protein expression was measured by Western blot on a variety of different factors, including signaling proteins, lipogenic enzymes and transcription factors (Figure 3.2.6). The most notable change was the increase in the two lipogenic proteins in the LXR-treated group, stearoyl-coA desaturase (SCD) and fatty acid synthase (FAS) after both four and eight days of treatment, compared to the group fed the FF diet alone. Expression of SCD was 89% (p=0.0001) and 130% (p=0.00003) higher in the LXR agonist-treated group after four and eight days, respectively, while FAS was increased by 97% (p=0.029) and 75% (p=0.047) at the same time points. A very interesting point is that expression of each of these factors seemed to decrease over time. After 16 days, expression of SCD was a non-significant 41% above FF-fed levels in the LXR-treated animals (p=0.13), and FAS a mere (but still significant) 33% elevated above controls (p=0.024). This initial increase followed by subsequent decrease may indicate some type of feedback mechanism controlling the expression of these enzymes.
Other protein changes after LXR activation include an initial increase in MTP level (30% after four days, \( p=0.034 \)), followed by normalization. The level of the nuclear receptor PPAR-alpha was similar to that of control after four and eight days, but decreased to 60% of control level after 16 days (\( p=0.0006 \)). Expression of another transcription factor that controls lipogenesis, ChREBP, followed a similar pattern to that of PPAR-alpha. Its levels were not significantly different from control until 16 days, when its level dropped by 40% compared with the FF diet group (\( p=0.0026 \)). Interestingly, the level of the immature form of the transcription factor SREBP-1c, which is involved in regulation of lipogenic genes, was initially decreased by 40% (\( p=0.03 \)), and later climbed back to the level of the FF-fed group. Despite this change in the level of the immature form, the level of SREBP-1c’s mature form was not significantly affected during the time course. The levels of two negative regulators of insulin signaling, PTP-1B and Phosphatase with sequence homogy to protein tyrosine phosphatases and the cytoskeleton protein tensin (PTEN) were also measured to determine if there were perturbations to the insulin signaling pathway. Neither of these proteins was significantly affected. The levels of both proteins remained within 12% of control level at all three time points. As another measure of insulin signaling, the amounts of both AKT and its phosphorylated form were measured.

During activation of the insulin signaling pathway, AKT becomes activated by phosphorylation. The ratio of phosphorylated AKT to total protein mass after insulin stimulation can therefore be used as an indicator of the integrity of the insulin signaling pathway. After treatment with the LXR agonist, there was an initial significant rise in the AKT phosphorylation-to-mass ratio by approximately 2.4 fold (\( p=0.038 \)) (Figure 3.2.7). This increase was moderated over the remaining time points measured during this experiment. The difference between the two groups was not significantly different after 8 or 16 days (\( p=0.19 \) and 0.52 respectively). AKT mass did not differ significantly between the two groups over the course of the experiment.
Protein levels after four days

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<td>Immature SREBP-1c</td>
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B

% of FF-fed group

* p<0.05 compared to FF, n = 4
Protein levels after eight days

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**D**

![Bar graph showing protein levels after eight days](image)

* p<0.05 compared to FF, n = 4
Figure 3.2.6. Changes in hepatic protein mass levels after treatment of FF-fed hamsters with T0901317.

Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide). Proteins were then transferred to a PVDF membrane and immunoblotted with the respective antibodies. Data (mean ± SD) are expressed as a percentage of the FF-fed control group level. A-B) Western blots and protein level quantification after 4 days of treatment. C-D) Western blots and protein level quantification after 8 days of treatment. E-F) Western blots and protein level quantification after 16 days of treatment.
Figure 3.2.7. Changes in AKT mass and phosphorylation levels after treatment of FF-fed hamsters for 4, 8 or 16 days.

Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide) and then transferred to a PVDF membrane. Membranes were first blotted for phospho-AKT using an antibody specific for phosphorylation at Serine 473. Membranes were then stripped and re-blotted to measure total AKT mass. Data (mean ± SD) are expressed as a percentage of the FF-fed control group level.

3.2.7 Conclusion

Activation of LXR in hamsters being fed the FF diet seems to augment any dyslipidemia that the hamsters experience, specifically by increasing plasma TG and cholesterol. The effect also appears to change over time. After LXR activation, the animals’ plasma TG increases and shifts toward the VLDL density fraction, an effect which becomes more pronounced as time passes. This effect appears to be explained, at least at the early time points, by an increase in the
lipogenic proteins SCD and FAS. However, since this increase is less pronounced later in the time course, there may be other mechanisms involved.

### 3.3 Long-term LXR Activation in the Hamster Model on Chow and FF Diets

LXR activation on the FF dietary background over a 2 week period showed an increase in plasma lipids that continued to rise over time. In order to extend these measurements, and to measure more long-term effects of LXR activation on the normal chow diet, an experiment was designed whereby the LXR agonist was administered to animals fed either the chow diet or the FF diet, and compared with vehicle-fed controls for each diet. These groups were maintained on their respective treatments for 30 days, with plasma measurements taken at 10 and 20 days to show progression of changes. Drug-treated animals were given a lower T0901317 dose of 10 mg/kg in order to avoid potential toxicity over the long term.

#### 3.3.1 Plasma alterations after chronic LXR activation

Plasma lipids were measured to gain insight into the development of metabolic dyslipidemia under the experimental conditions. After 10 days of treatment, there was a marked increase in plasma cholesterol in both groups consuming the FF diet, regardless of whether they were also receiving the LXR agonist (Figure 3.3.1). The FF and FFLXR groups’ plasma cholesterol increased by 46% and 52% above their respective baseline levels (p<0.001). These changes were significantly higher than the 10% and 16% increases seen in the chow and CLXR groups, respectively (p<0.02 for all comparisons). These differences were maintained after 20 days but were less pronounced after 30 days. At the end of the time course, both the FF and FFLXR groups showed significantly higher increases above their respective baseline levels than the chow group did (p=0.011 and 0.004, respectively), but were not significantly different from the CLXR group.

The differences in plasma TG levels were not quite as striking as those observed in the previous time course. Both groups of hamsters being fed the FF diet experienced an approximately two-fold increase in plasma TG after 10 days (p<0.02). These two groups did not differ significantly from each other at this or at any other time points. The plasma TG of the FF diet-fed group continued to increase over the 30-day experiment, reaching a high of 2.6 times over its baseline
level (p=0.019). The FFLXR group did not show a similar increase; in fact, the plasma TG level in this group finished with a non-significant 64% above its baseline level (p=0.15). LXR

Figure 3.3.1. Comparison of plasma lipids and lipoproteins after either FF diet feeding or T0901317 administration for 30 days.

Blood samples were drawn from all hamster subjects by retro-orbital bleed at intermediate points, or by cardiac puncture at final collection under anaesthesia using isoflurane. Plasma was isolated by centrifugation. A-D) Cholesterol, TG, AST and ALT measurements were conducted using a clinical chemistry analyzer. Data (mean + SD) are representative of four independent measurements (n=4). E) Plasma apoB mass was analyzed by immunoblotting using an anti-apoB antibody. A representative blot is shown here.
activation with the chow diet did promote increased plasma TG levels after 30 days, however, achieving an 86% increase above its baseline level. This was significantly higher than the 2% change seen in the chow-fed animals (p=0.007). The FF diet appears to have a stronger effect on plasma TG accumulation than the effect of the LXR agonist alone or even the combination of the two diets. This is generally confirmed by Western blot for plasma apoB levels. The FFLXR group shows a greater increase in plasma apoB compared with the level seen in other groups, including the chow group (Figure 3.3.1).

Plasma levels of the hepatic enzymes ALT and AST were again measured as an indicator of hepatic function. Neither of the lipid levels increased dramatically during the time course of the experiment, demonstrating that there is not excessive liver injury as a result of any of the diet or drug treatments. The level of AST in fact decreased significantly by about 30% from baseline levels in all experimental groups (p<0.02 for all).

Dietary fat and fructose therefore appear to play a more important role in regulation of plasma lipid levels than LXR activation does. One very important consideration, however, is that the dose of LXR agonist that the animals were receiving was lower than in the previously conducted time course. The lower dose of 10 mg/kg was used in part to prevent toxicity in the hamsters, and in part to avoid using a super-physiological agonist dose. This may have led to lower LXR activation and thus less pronounced metabolic perturbations.

\[ \text{3.3.2 Alterations in plasma lipoprotein profile following chronic LXR activation} \]

To measure any shift in lipids in lipoprotein profile, the plasma lipids were again separated by FPLC as described above. Each of the three experimental groups (CLXR, FF and FFLXR) prompted a shift in plasma cholesterol to the VLDL fraction (Figure 3.3.2). These three groups carried approximately 25% of their plasma cholesterol in the VLDL fraction, compared to only 9% in the chow-fed animals. This difference was significant when comparing the chow group with the CLXR group (p=0.008) and FFLXR group (p=0.016), and approached significance with the FF group (p=0.10). This increase appeared to be almost completely at the expense of LDL cholesterol. LDL carried between 5% and 13% of total cholesterol in each of the three treatment groups compared to 22% in the chow-fed group. This difference was significant in the FF group (p=0.0002) and approached significance with the CLXR (p=0.21) and FFLXR (0.051) groups.
The shift in distribution of plasma TG was almost identical to that of plasma cholesterol. Each of the three treatment groups demonstrated a nearly significant shift in TG from the LDL density group to the VLDL fractions. When comparing each of these treatment groups to the chow values, the p-values ranged from 0.072 to 0.093.

Overall, the observed shifts in lipoprotein distribution indicate that both the FF diet and LXR activation can induce dyslipidemia over the long term.

The FF diet induces hepatic TG accumulation

After 30 days of feeding, the FF diet caused significant hepatic TG accumulation when compared with each of the three other treatment groups (p<0.02 for all comparisons) (Figure 3.3.3). The level of TG stored in the liver of the FF animals was 2.4 times that in the chow-fed animals (p=0.006). Treatment with the LXR agonist, with or without the FF diet, produced no change in the amount of TG accumulated in the liver compared with the chow group. It is curious that the FF diet did not induce hepatic TG storage while being co-administered with the LXR agonist, especially when, at 16 days, the two treatment regimes resulted in similar TG storage. The LXR agonist, may, however, preferably stimulate increased VLDL production over the longer term (30 days), favouring TG export rather than storage.

None of the experimental manipulations affected the amount of cholesterol stored in the liver. This is not entirely surprising since the LXR agonist didn’t have a significant effect on cholesterol storage on the FF diet background at 16 days either. The FFLXR group had a tendency to increase hepatic cholesterol storage when compared with the chow group, but this 26% increase was not significant (p=0.076).
Figure 3.3.2. LXR activation and FF diet feeding shift plasma cholesterol to the VLDL fraction.

Blood samples were collected at the end of the treatment period by cardiac puncture under isoflurane anaesthetic. Plasma lipoproteins were separated by density using automated size exclusion gel filtration chromatography, on a fast pressure liquid chromatography (FPLC) system. Fractions 11 to 45 were then assayed for cholesterol and TG. Density divisions were identified by peaks in cholesterol and TG. VLDL was defined as fractions 11-20, LDL as fractions 21-28, and HDL as fractions 29-45. The percentage of the lipid in each fraction as a proportion of the total lipid collected was calculated and plotted as shown. Data (mean + SD) represent the average measurements of four independent animals (n=4).
Figure 3.3.3. FF diet feeding causes increased hepatic TG accumulation.

Lipids were extracted from liver tissue using the Folch method. Lipids samples were measured against standards using TG and cholesterol RANDOX kits. Data (mean ± SD) are representative of four independent animals (n=4).

Both the FF diet and LXR activation increase liver mass

After 30 days of treatment, both the FF diet and LXR agonist, either alone or in combination, triggered an increase in liver mass as a proportion of total body mass (Figure 3.3.4). The
combination of FF diet and LXR agonist showed a significant 23% increase in liver mass (p=0.0009) compared to the chow group. This was larger but not significantly different from the 14% and 16% increases above the chow control seen with the CLXR and FF groups, respectively (p=0.013 and 0.008). There were no significant differences among the three treatment groups with respect to liver mass. Since the increases in hepatic lipid accumulation do not completely explain the changes in organ mass, this increase in liver mass is perhaps an indication of hepatic dysfunction or inflammation.

The mass of the gonadal fat pads was also measured as a percentage of total body weight. None of the diet treatments caused a significant change in fat pad mass after 30 days of treatment, compared to any of the other experimental groups.

^3.3.5 Glucose and insulin tolerance

In order to determine the effect of the FF diet and the LXR agonist on insulin sensitivity, glucose tolerance and insulin tolerance tests were performed. These tests were performed at 23 days and 24 days, respectively, so as not to severely disturb the hamsters’ metabolic response too soon before sacrifice and organ collection.

The results from the glucose tolerance test were plotted first as a time-course curve representing the level of plasma glucose over the two hours following glucose injection. The area under each curve was then calculated and plotted in a separate graph (Figure 3.3.5). The glucose tolerance tests showed that those animals fed the FF diet were less able to compensate for the glucose challenge than the two groups fed the chow diet. When comparing the areas under the curve, these differences were not significant. The comparison between the FF group and the chow and CLXR groups approached significance however, with p-values of 0.06 and 0.097, respectively.
Figure 3.3.4. LXR activation and FF diet feeding stimulate increased liver mass.

Following surgery and organ harvest, livers and gonadal fat pads from each animal were weighed using a digital scale. The weight of each organ as a percentage of each animal’s total body weight is plotted here. Data (mean + SD) represent four independent measurements per group (n=4).

The lack of significance may be due to a weakness in statistical power; since both insulin tolerance and glucose tolerance tests were to be conducted, only half of the animals in each
group (n=3) were used for each type of test. The FF diet may therefore reduce the hamsters’ ability to dispose of glucose, signifying possible insulin resistance.

The insulin tolerance test showed even less difference between the different groups. The FF diet group and the LXR agonist on the chow diet (CLXR) showed the least response to insulin stimulus, but these differences were again not significant when compared to the chow group.

Figure 3.3.5. FF diet feeding may initiate glucose intolerance.

A) A glucose tolerance test was conducted after 23 days of feeding. Animals were injected intraperitoneally with 1.5 mg/kg of glucose, in the form of a 30% glucose solution. Blood glucose levels were measured using a hand-held glucometer immediately before injection (time=0) and at regular time intervals thereafter (t=15, 30, 60 and 120 minutes). B) An insulin tolerance test was conducted after 25 days of feeding. Animals were injected intraperitoneally with 5 U/kg of insulin. Blood glucose levels were measured immediately before injection (time=0) and at regular time intervals thereafter (t=15, 30, 60, and 120 minutes). Results for each
test are plotted as curves of blood glucose concentration over time, and as area under each curve for better comparison. Both tests were conducted on a set of three animals (n=3), and each animal was used for only one test.

^ 3.3.6  Protein expression of lipogenic and signaling proteins

Hepatic levels of two lipogenic proteins, SCD and FAS, were measured in each of the four treatment groups to try to explain the observed metabolic changes. Administration of T0901317 along with chow diet feeding caused a trend towards a 35% increase in SCD expression when compared with chow diet feeding alone (p=0.054) (Figure 3.3.6). The FF diet by itself didn’t cause a significant change in the hepatic level of the enzyme with or without the LXR agonist, when compared with the chow diet. These two groups did, however, show significantly lower SCD expression level than the CLXR group (p<0.05 for both comparisons). The hepatic level of FAS protein was not significantly affected by any of the different diet combinations over the 30-day time period.

Figure 3.3.6. Changes in hepatic protein levels after FF diet feeding and T0901317 administration.
Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide). Proteins were then transferred to a PVDF membrane and immunoblotted with the respective antibodies. For AKT measurements, phospho-AKT was measured first, and membranes were subsequently stripped and re-blotted for total AKT mass. Data (mean + SD) are expressed as a percentage of the chow-fed control group level.

The phosphorylation level of the insulin signaling pathway component AKT was also measured, by taking a ratio of the level of phosphorylated AKT to total AKT mass, as measured by Western blot. The ratio of AKT phosphorylation to its mass increased significantly with the FFLXR diet compared to either the chow or CLXR diet treatments (p<0.04 for both).

### 3.4 Comparison of LXR Activation with Dietary Cholesterol Supplementation

Interestingly, the metabolic perturbations induced by chronic LXR activation appeared to be similar to metabolic effects seen previously with cholesterol added to the FF diet (Basciano et al 2008). In order to determine whether LXR activation mediates the lipogenic and diabetogenic effects of dietary cholesterol, several studies were designed to directly compare the effect of dietary cholesterol with the effects of chronic LXR activation on the FF diet background. In the first, the LXR agonist or cholesterol were added to the FF diet and compared to the FF diet alone. Two sets of animal groups were maintained on their respective treatments for 4 days (T0901317 dose: 50 mg/kg) and 14 days (T0901317 dose: 25 mg/kg). In the second experiment, both the FF and chow diets were each supplemented with either 20 mg/kg LXR agonist or vehicle control for 16 days, and these were compared with the FF diet supplemented with cholesterol (the FFC diet).

#### ^3.4.1^ LXR activation and cholesterol feeding influence plasma lipid profile

After four days of treatment, those hamsters whose FF diet was supplemented with the LXR agonist showed a significant three-fold increase in plasma TG from their baseline level (p=0.010) (Figure 3.4.1). The FFC group showed a non-significant 75% increase from its baseline level (p=0.059), which was comparable to the 54% increase seen in the FF-alone group (p=0.06). This LXR-stimulated increase in plasma TG was augmented with time, in spite of a lower drug dose.
Figure 3.4.1. Comparison of the metabolic effects of T0901317 and dietary cholesterol on the FF diet background.

Blood samples were drawn from all hamsters by cardiac puncture at final collection under anaesthesia using isoflurane. Plasma was isolated by centrifugation. A-C) Cholesterol, TG, AST and ALT measurements were conducted using a clinical chemistry analyzer. D) Body weight was measured using an analytical balance. E) Plasma ApoB level was determined using Western blot. Data (mean + SD) are representative of between four and six independent measurements (n=4-6).

After 14 days, the increase above baseline level jumped to 6-fold and to 7.5-fold after 16 days (p<0.0001 for both). This substantial effect of LXR activation was potentiated by the FF diet, since after 16 days, the addition of the LXR agonist to a chow diet alone caused only a four-fold increase above its baseline level (p<0.02 compared to chow and to baseline). After 14 and 16 days, the addition of cholesterol to the FF diet caused a significantly greater increase in plasma TG than the FF diet alone did (p<0.003 for both), showing that cholesterol feeding does impact de novo lipogenesis beyond the effects of the fat-rich diet. Since VLDL is the major TG carrier in the blood, and apoB is the main protein component of this lipoprotein particle, it follows that a rise in plasma TG should be accompanied by a rise in plasma apoB. This is in fact what was
observed, as the increase in plasma TG as a result of both the FFLXR and FFC diets is parallel to a similar increase in plasma apoB levels, as seen by Western blot (Figure 3.4.1).

The effect on plasma cholesterol was less pronounced than the effect on TG, and was more strongly affected by the addition of dietary cholesterol, as would be expected. After 4 days, both the FFC and FFLXR groups showed a 30% increase above their baseline levels (p=0.001 for both). This was significantly higher than the 15% increase observed in the FF diet-alone group (p<0.03 for both). As with plasma TG, the effect on plasma cholesterol increased over time. After 14 days, the increase in plasma cholesterol in the FFLXR and FFC groups climbed to 74% and 100% above their respective baseline levels (p<0.001 for both). These changes were significantly greater than the changes seen with the FF group, showing that both the addition of dietary cholesterol and LXR activation significantly increase plasma cholesterol in the hamster model. After 16 days, a similar effect was observed, although less pronounced in the FFLXR group, which showed a more modest but still significant 42% increase in plasma cholesterol (p=0.0003) above its baseline level. The addition of the LXR agonist to the chow diet did not significantly affect plasma cholesterol, causing only a non-significant 16% increase from its baseline level.

Plasma levels of ALT and AST were again measured in order to gauge the effect of each treatment on liver function. After 4 and 14 days, plasma levels of AST increased significantly by between two- and three-fold for all three groups (p<0.03). Plasma levels of ALT remained constant or in fact decreased in both of these groups. After 16 days, there were no significant differences in either enzyme when compared to either their respective baseline levels or between groups.

Although the hamsters in each diet group gained weight over the course of the study, none of the groups demonstrated different degrees of weight gain from any of the others (Figure 3.4.1 D)

3.4.2 Plasma lipoprotein profiles

The distribution of plasma lipids within different lipoprotein density fractions gives an indication of the size distribution of the particles secreted by the liver. FPLC analysis showed that, after four days of treatment, the proportion of plasma cholesterol in the VLDL fraction is increased in
the two groups administered the LXR agonist or dietary cholesterol, compared with the group only receiving the FF diet. In contrast, with the previously described four-day experiment (see section 3.2.2), the addition of T0901317 or cholesterol to the FF diet triggered a shift away from the VLDL fraction toward the LDL and HDL density fractions (Figure 3.4.2). After 14 days, however, there were no significant differences between TG profiles of any of the three treatment groups. There was a shift of plasma cholesterol towards the VLDL fraction, especially in the FFLXR group, which showed a significant 2.2-fold increase over the FF group (p=0.017). The shift demonstrated by the FFC group was more modest and not quite significant, with a p-value of 0.09.

In the 16 day treatment study groups, the addition of either the LXR agonist or cholesterol to the FF diet resulted in a shift of both plasma cholesterol and TG towards the VLDL fraction. The LXR agonist on its own did induce a significant shift in TG to the VLDL density (p=0.036), and a slight shift in cholesterol in the same direction (p=0.12). The shift in plasma cholesterol distribution was augmented by the addition of the FF diet; the FFLXR group transported almost 70% of its cholesterol in the VLDL fraction. The addition of cholesterol to the FF diet also shifted both plasma cholesterol and TG towards the VLDL fraction in a similar pattern to that of the FFLXR diet. The FFC diet resulted in 46% of plasma cholesterol and 73% of plasma TG being transported in the VLDL fraction, compared to 15% and 58%, respectively, in the chow-fed hamster plasma (p=0.002 and 0.049). It should be noted that since the results from the FPLC analysis are plotted as a proportional distribution of total lipids, the total amount of lipid in each group relative to the others is not reflected here. For this reason, the amount of cholesterol in the VLDL fraction of the FFC group may appear to be lower than that of the FFLXR group, when in fact this is a proportional representation.
Figure 3.4.2. Cholesterol feeding and T0901317 shift plasma lipids to the VLDL fraction.

Blood samples were collected at the end of the treatment period by cardiac puncture under isoflurane anaesthetic. Plasma lipoproteins were separated by density using automated size exclusion gel filtration chromatography, on a fast pressure liquid chromatography (FPLC) system. Fractions 11 to 45 were then assayed for cholesterol and TG. Density divisions were identified by peaks in cholesterol and TG. VLDL was defined as fractions 11-20, LDL as fractions 21-28, and HDL as fractions 29-45. The percentage of the lipid in each fraction as a proportion of the total lipid collected was calculated and plotted as shown. Data (mean ± SD) represent the average measurements of four independent animals (n=4).

3.4.3 Chronic LXR activation and cholesterol feeding both induce hepatic lipid storage

The effects of LXR activation and cholesterol feeding on hepatic lipid accumulation were similar to the effects seen on plasma lipid levels. Activation of LXR had a strong influence on hepatic TG accumulation, especially in the 14 day group. In the 16-day experiment, all of the groups being fed the FF diet demonstrated more TG accumulation compared to the chow group.
The addition of LXR agonist to the chow diet alone caused a trend towards an increase in hepatic TG storage (p=0.15). The FF diet likely plays a prominent role in driving TG accumulation in the liver.

Cholesterol accumulation in the liver, however, was not significantly affected by the FF diet compared with the chow diet. This was accented by the addition of T0901317; the drug increased the amount of hepatic cholesterol in the 14-day group by 87% compared with the FF-diet fed group (p=0.023). When the FF diet was supplemented with cholesterol, hepatic cholesterol accumulation increased by over three-fold (p=0.002) in the 14-day group and by 58% in the 16-day group (p=0.036), compared with the FF diet group. The effect of LXR activation therefore appeared to mimic the effects of cholesterol accumulation with respect to cholesterol accumulation in the liver.

### 3.4.4 LXR activation increases liver mass

The weights of the liver and gonadal fat pads were measured after 16 days of feeding as a rough guide to lipid accumulation and injury. These were calculated as a proportion of the total body weight in order to accurately compare changes between groups. After 16 days, every treatment group had a significantly heavier liver as a fraction of its total body weight, compared to the chow group (p<0.03 for all) (Figure 3.4.4). The group given a combination of the FF diet and the LXR agonist showed the biggest gain in liver weight, with the liver occupying 6.3% of their total body weight, compared with 4.4% for the chow-fed animals. This was significantly higher than the FFC group, whose liver encompassed 5.5% of its total body weight. This value was still significantly higher than both the chow-fed group and the chow group given the LXR agonist (p<0.02 for both comparisons). The similar increase in liver mass in the FFLXR and FFC groups may indicate that both cholesterol feeding and LXR activation may act through similar mechanisms, and may both depend on the involvement of fat and fructose for maximal effects.

Changes in adipose tissue mass were not very dramatic. The FFC-fed group had gonadal adipose stores that occupied 2.9% of their total body mass, which was significantly greater than the proportion of adipose tissue in the chow and CLXR groups (2.4% for both, p=0.04, 0.02). This 0.5% change in adipose tissue mass represents a gain of approximately 0.6 g. The measured increases in hepatic lipid accumulation (see section 3.4.3) do not fully explain the significant increases in proportional liver mass. This indicates
Figure 3.4.3. Changes in hepatic lipid accumulation with FF diet feeding, LXR activation and cholesterol feeding.

Lipids were extracted from liver tissue using the Folch method. Lipids samples were measured against standards using TG and cholesterol RANDOX kits. Data (mean ± SD) are representative of between four and six independent animals (n=4-6).
Figure 3.4.4. FF diet feeding, LXR activation and dietary cholesterol affect liver mass after 16 days of treatment.

During surgery and organ harvest, livers and gonadal fat pads from each animal were weighed using a digital scale. The weight of each organ as a percentage of each animal’s total body weight is plotted here. Data (mean ± SD) represent four independent measurements per group (n=6).
that there is either another contributor to increased liver mass, or that the hamsters were losing weight elsewhere in their bodies, increasing the proportional mass of their livers. The latter possibility is not as likely, after observing no decrease and in fact a slight increase in adipose tissue mass. There was no change in total body weight in any of the study groups at any of the time points examined (data not shown).

3.4.5 Effects of cholesterol feeding and LXR activation on glucose disposal and insulin sensitivity

In order to measure the effect of the different diet treatments on the insulin response, a glucose tolerance test was performed after 15 days of treatment. Glucose was injected intraperitoneally at time 0, and glucose was measured at regular intervals over the following two hours. The curves are presented in Figure 3.4.5, accompanied by a plot of the area under the curve. After careful analysis, it appears that the FF diet alone slightly impairs the hamsters’ ability to dispose of glucose (p=0.07 compared to chow). When either the LXR agonist or 0.25% cholesterol were added to the FF diet, however, the differences between these groups and the two chow-fed groups became significant (p<0.03 for all comparisons). There are two important implications of this observation. The first is that it appears that the combination of fat, fructose and either the LXR agonist or cholesterol is necessary for the impairment of glucose uptake, since LXR agonist alone and the FF diet alone did not induce the same degree of glucose intolerance. The second implication is that both chronic LXR activation and dietary cholesterol supplementation induce a similar response in the hamsters, supporting the hypothesis that dietary cholesterol is exerting some of its effects through activation of this nuclear receptor.
Figure 3.4.5. LXR activation and dietary cholesterol reduce glucose tolerance when combined with the FF diet.

A glucose tolerance test was conducted after 15 days of feeding. Animals were injected intraperitoneally with 1.5 mg/kg of glucose, in the form of a 30% glucose solution. Blood glucose levels were measured using a hand-held glucometer immediately before injection (time=0) and at regular time intervals thereafter (t=15, 30, 60 and 120 minutes). Results for each test are plotted as curves of blood glucose concentration over time, and as area under each curve for better comparison. The glucose tolerance test was conducted on six animals from each treatment group (n=6).
3.4.6 Gene expression profiling

Changes in expression of select target genes were assessed in order to determine some of the molecular pathways that may be responsible for the observed dyslipidemia. Since LXR is a transcription factor, its primary actions would be in transcriptional activation of its target genes including SCD and FAS. After 16 days, SCD mRNA increased dramatically with all four types of treatment, relative to chow (p<0.001 for all comparisons) (Figure 3.4.6). The addition of the LXR agonist to the chow diet elicited an 11-fold increase in SCD expression, and this was augmented to 18-fold when the drug was administered with the FF diet. The FF diet alone increased SCD expression by six-fold, and this level was not increased by the addition of cholesterol to the diet. These results were confirmed by the measurements taken after 14 days as well. The addition of the LXR agonist to the FF diet increased SCD expression by three-fold, but the addition of cholesterol had no relative effect. None of these changes were significant, however, and in fact there were no significant changes in expression levels of other genes examined in the 14-day group. FAS mRNA levels were only significantly affected by the combination of the FF diet and the LXR agonist, achieving a level 2.6 times that of chow-fed animals (p=0.033). The FF and FFC-fed animals showed a nearly significant 2-fold increase in FAS expression relative to chow (p=0.06 and 0.09, respectively).

Activation of LXR not only directly affects the expression of specific enzymes, but can also activate transcription of other transcription factors, and as a result can have a cascading effect on downstream targets. One such target, Srebp-1, is known to control expression of several lipogenic genes, including Fas and Scd. Each of the four diet treatments increased SREBP-1 mRNA relative to chow-fed animals (p<0.007). The FF-fed animals showed the strongest increase, with a level ten times that of the chow-fed animals. This level was surprisingly decreased by the addition of either the LXR agonist or cholesterol, which both caused a roughly four-fold increase in Srebp-1 expression, relative to chow. This is surprising because Srebp-1 is known to be a direct LXR target. An interesting point is that the LXR agonist and cholesterol had similar effects on this SREBP-1 mRNA, indicating that cholesterol may be acting through LXR.

A second transcription factor known to be under LXR’s control is Chrebp. The addition of LXR agonist alone triggered the largest rise in CHREBP mRNA, with a six-fold increase (p=0.004). This effect was slightly tempered by the addition of the FF diet, as the FFLXR group only
Figure 3.4.6. Hepatic mRNA alterations after FF diet feeding, LXR administration and dietary cholesterol after 14 and 16 days.

Total RNA was isolated from snap-frozen hamster liver tissue using the Qiagen RNeasy Mini kit according to manufacturer’s protocol. RNA concentrations were determined using spectrophotometry. After reverse transcription, cDNA levels were assessed by real-time quantitative RT-PCR, using 18S rRNA as an internal control. Data (mean + SD) are expressed relative to the chow or FF-fed vehicle control, and represent between three and six independent measurements (n=3-6).
stimulated a 4.3-fold increase (p=0.03) in CHREBP mRNA. *Chrebp*’s expression was tempered even further with the FFC diet, with that group only achieving a 2.8-fold increase in expression relative to chow (p=0.052). SREBP-2 is a transcription factor related to SREBP-1, but which is encoded by a separate gene. SREBP-2 is responsible for activating genes involved in cholesterol synthesis, including HMG-CoA reductase. SREBP-2 mRNA was shown to be dramatically and significantly upregulated by either LXR activation (4.7-fold) or by FF feeding (6.3-fold), (p<0.0008 for both). This effect was seriously tempered, however, when the two stimuli were combined. Following both FF feeding and LXR activation, *Srebp-2* reached a level only 1.9-fold greater than with chow alone, and this increase was not statistically significant (p=0.09). FFC feeding showed a similar result, with a mere 40% increase above chow (p=0.28). Cholesterol is known to inhibit SREBP-2 processing and this may have a feedback effect on its gene expression (Wojcicka et al. 2007).

The protein MTP is a chaperone and lipid transfer protein that aids in the folding and lipid loading of nascent apoB as it is translated into the endoplasmic reticulum lumen. MTP is necessary for VLDL assembly, as is demonstrated by the fact that *Mtp* knockout mice are unable to produce VLDL at all (Gordon and Jamil 2000). All four of the treatment groups produced a significant increase in MTP mRNA (p<0.05). The FFC group elicited the smallest increase, augmenting MTP mRNA by only 50%. In comparison, the FF-fed group increased MTP mRNA by nearly seven-fold, compared with the chow-fed group. It is interesting that the addition of either cholesterol or the LXR agonist to the FF diet caused a relative decrease in MTP mRNA, compared to the FF diet alone.

Another direct target of LXR, the cholesterol transporter *Abca1* was also measured in order to verify that LXR activation was indeed occurring. Each treatment group showed an increase in ABC-A1 mRNA following 16 days of treatment, compared to the group fed only regular chow (p<0.0001). A similar pattern is seen with the regulation of this gene as was seen with *Srebp-1* and *Mtp*. The FF diet increased ABC-A1 mRNA the most (nearly 20-fold above chow), while the FFLXR and FFC groups were only able to induce an increase in expression of approximately 9-fold.

FXR is another member of the nuclear receptor that acts in a similar way to LXR. FXR is known to act in a complementary way to LXR, by inhibiting certain genes that LXR activates, such as
Cyp7a1. Since the chemical LXR agonist T0901317 is known to activate human FXR in addition to LXR (Houck et al. 2004), the mRNA level of the FXR target Bsep was measured to determine the extent to which this was happening in the hamster model. The three groups CLXR, FF and FFLXR did show a significant increase in BSEP mRNA relative to the chow-fed group (p<0.03). These three treatment groups stimulated increases in BSEP mRNA of 57%, 126% and 46%, respectively. The FFC group in fact caused a non-significant 25% decrease in BSEP mRNA (p=0.16). Although the LXR agonist is a dual agonist, when BSEP mRNA is compared with that of ABC-A1, T0901317 doesn’t appear to activate FXR with the same potency as the stimulation of its intended target, LXR.

3.4.7 Protein expression profiling

In order to determine the effect of the different diet and drug treatment combinations on specific target protein expression, hepatic levels of different proteins were measured by Western blot. Transcription factors other than LXR are involved in stimulating dyslipidemic changes. Such transcription factors include SREBP-1c, ChREBP and PPAR-alpha. SREBP-1c is a transcription factor initially produced in its longer, immature form. It remains in this form in the ER membrane until a series of events causes its activation and transport into the nucleus by specific cleavage events. Both the mature (nuclear) and immature (cytoplasmic) forms of SREBP-1c were measured, by first separating the different subcellular components and then measuring the relative amounts of the appropriately-sized proteins. Although SREBP-1c is recognized as a direct LXR target, the immature level of this protein did not change with any of the different treatment combinations. The level of mature SREBP-1c did increase, albeit non-significantly, with all four treatment types after 16 days of treatment (Figure 3.4.7). The strongest activation was seen with the two groups being given the LXR agonist, the CLXR and FFLXR groups. They elicited an increase in mature SREBP-1c protein level of 40% and 52%, respectively (p=0.059, 0.061 compared to chow). The mechanism whereby LXR activation could increase SREBP-1c maturation is unknown, and if this trend can be confirmed, would be a very interesting avenue to pursue.

ChREBP is another transcription factor known to interact with LXR. Challenging the animals with either the LXR agonist on its own or just the FF diet did not induce a change in protein level, but the addition of either T0901317 or cholesterol to the FF diet decreased ChREBP levels
Figure 3.4.7. Changes in protein expression after 16 days of treatment.

Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide). Proteins were then transferred to a PVDF membrane and immunoblotted with the respective antibodies. Data (mean ± SD) are expressed as a percentage of the chow-fed control group level.

by approximately 40% compared to the chow-fed group (p=0.006 and 0.017). This decrease was also significant when compared with the CLXR group (p=0.02 and 0.029, respectively). After 14
days, a similar result is observed; the addition of either T0901317 or cholesterol to the FF diet caused a decrease in ChREBP protein level in the liver (Figure 3.4.8).

Figure 3.4.8. Changes in protein expression after 14 days of treatment.

Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide). Proteins were then transferred to a PVDF membrane and immunoblotted with the respective antibodies. Data (mean + SD) are expressed as a percentage of the FF-fed control group level.
PPAR-alpha, a third transcription factor examined, is known to respond to intracellular fat. This factor may, therefore, be a key player in the interaction between dietary lipids and LXR activation. Similar to the effects seen with ChREBP, PPAR-alpha protein levels did not change significantly after FF diet feeding alone or LXR activation on its own, but did show a significant decrease of slightly more than 50% after either the LXR agonist or cholesterol were administered in concert with the FF diet (p=0.01 and 0.02, respectively). The downregulation of PPAR-alpha in the FFC and FFLXR animals may explain, in part, the increase in plasma TG seen with these diets, since PPAR-alpha is known to ameliorate hypertriglyceridemia (Fruchart 2007).

In order to determine the effect of the different diet treatments on VLDL assembly, the protein mass of hepatic MTP was measured. There was no significant difference between the different groups with respect to this protein. There was a trend towards an increase when comparing the FFC group against the chow-fed group (30% increase, p=0.067). These results are consistent with previous experiments in our laboratory, which showed no change in MTP with either the LXR agonist or the FFC diet (unpublished data).

The lipogenic enzymes SCD and FAS were also examined to determine the effect of FF diet feeding and LXR activation on hepatic lipogenesis. Hepatic levels of SCD increased with both diets containing the LXR agonist. After 16 days, the largest increase was seen with the FFLXR diet, which caused a 60% rise in the protein compared to the chow-fed level, although this was not significant (p=0.10). The addition of T0901317 to the FF diet did significantly increase SCD expression when compared to the FF diet alone in both the 16 and 14 day cohorts (p=0.049, 0.046 respectively). The observed increase in SCD may explain the increases in TG synthesis observed in the LXR-activated groups. The hepatic level of FAS, on the other hand, did not increase in response to any of the stimuli at either time point. The FFC diet did, however, trigger a 43% decrease in FAS expression relative to the chow-fed animals (p=0.006) in the 16-day group.

In order to try to explain the observed decrease in glucose tolerance in the FFLXR and FFC animals, several negative regulators of insulin signaling were also examined. PTP-1B and PTEN are both phosphatases that inhibit the transduction of the insulin signal downstream of the insulin receptor. Increases in either of these enzymes would give a potential explanation for decreased insulin signaling. Instead of an increase, however, a slight but significant decrease was seen in
the FF and FFLXR diet treatments compared to chow (p=0.005 and 0.002, respectively). The hepatic level of PTEN decreased by 30% in response to the FFC diet (p<0.02 compared to all other groups). Since expression of these phosphatases did not increase in any of the diet treatments, the explanation for decreased glucose tolerance must lie elsewhere; either in a different part of the insulin signaling cascade, or in a different facet of glucose uptake.

![Figure 3.4.9. FFC diet feeding decreases AKT mass level.](image)

* p<0.05 compared to all other groups, n = 6

**Figure 3.4.9. FFC diet feeding decreases AKT mass level.**

Fifty micrograms of protein from hepatic tissue homogenates were separated by size using SDS-PAGE (5-10% acrylamide) and then transferred to a PVDF membrane. Membranes were first blotted for phospho-AKT using an antibody specific for phosphorylation at Serine 473. Membranes were then stripped and re-blotted to measure total AKT mass. Data (mean ± SD) are expressed as a percentage of the chow-fed control group level.

Another component of the insulin signaling pathway is the protein AKT, which is phosphorylated upon stimulation of the insulin signaling pathway. Both the protein mass and level of phosphorylation was measured by Western blot to determine the effect of the different treatments. The results are presented in Figure 3.4.9. AKT mass remained constant after
stimulation by any combination of the FF diet and/or T0901317, but the FFC diet triggered a substantial and significant 80% decrease in the protein mass (p<0.004 for all comparisons). This was accompanied by a parallel, but not greater, decrease in AKT phosphorylation. When the ratio of phosphorylated protein to total protein mass level was calculated, there were no significant differences among any of the groups. The FFC group even appeared to have an increased phosphorylation ratio, although this was not significantly different from the other treatment groups. The observed decrease in AKT mass may, in part, explain the decrease in glucose tolerance with the addition of cholesterol to the FF diet, but still does not explain the similar effect observed with LXR activation. LXR activation may have an effect at another point in the insulin signaling pathway, or may directly affect glucose uptake itself.
4 :: Discussion

4.1 Acute LXR Activation and Hepatic Lipid Metabolism

Acute activation of LXR was previously shown in our laboratory to induce metabolic
dyslipidemia in the hamster model. Several studies were repeated using 50 mg/kg T0901317 for
four days in order to confirm previous results, either because the first results were unreliable, or
because a new method had since been developed. The four-day experiments were then repeated
using a lower dose of 10 mg/kg to determine if the lower dose presented similar effects. The
higher agonist dose was not found to affect steady-state lipid synthesis, although it did cause an
increase in total plasma TG levels. The lower agonist dose (10 mg/kg) caused increases in
VLDL-TG and in plasma apoB levels, but not in total plasma TG levels.

Based on these new studies, there was a lack of effect of T0901317 administration (50 mg/kg) on
steady-state lipid synthesis, which is surprising, considering the strong effect of LXR activation
on plasma lipids. LXR has also been shown to interfere with PPAR-alpha activation (Ide et al.
2003), however, which would have the net result of preventing the activation of lipid beta-
oxidation. This mechanism may increase plasma lipids in the short-term, with increases in
lipogenic genes increasing lipid synthesis after a longer term of LXR activation.

The absence of a difference in hepatic lipid deposition (lipid mass) after LXR activation was also
very surprising. This may indicate that the livers of these hamsters may be preferentially
exporting the lipids rather than storing them, contributing to the increase in plasma TG. This
contradicts one previous study, however, which showed massive hepatic lipid accumulation in
mice given 10 mg/kg of T0901317 for four days (Grefhorst et al. 2002). These differences may
be attributable to species differences, as hepatic lipid mobilization appears to differ among

Acute studies at a lower dose of the LXR agonist, T0901317 (10 mg/kg/day) showed a similar
effect in the ability to increase plasma apoB levels, and to increase plasma VLDL-TG. The lower
agonist dose did not, however, induce a comparable increase in plasma total TG, which had been
observed at the higher dose of 50 mg/kg/day. Previously published results in mice have used
doses ranging from 1 mg/kg to 100 mg/kg (Bobard et al. 2005, Cao et al. 2003, Grefhorst et al.
2002, Schultz et al. 2000), and in hamsters ranging from 3 mg/kg to 50 mg/kg (Schultz et al.
2000), with different results. Doses as low as 1 mg/kg have been shown to activate LXR in mice with increases in LXR’s transcriptional targets (Schultz et al. 2000), but the physiological effects vary among animal models and at different doses. In hamsters, doses as low as 10 mg/kg, but not 3 mg/kg, have been shown to increase VLDL-TG in the hamster (Schultz et al. 2000). Another study found that doses of at least 30 mg/kg are necessary for substantial increases in total plasma TG in rats and mice (Cao et al. 2003). This study also found that a dose of at least 30 mg/kg T0901317 is necessary for LXR-mediated decreases in gluconeogenic enzyme mRNA levels. Activated LXR may have preferential targets that are activated at lower agonist doses, with other targets only transcribed at higher doses. Species differences also cannot be overlooked. Few studies have previously used the hamster model with T0901317, making it difficult to compare historical observations. There may be differences among different rodent models with regards to the drug’s metabolism. Subtle changes in the sequence of the LXR promoter among species may also affect the power of the drug to activate it.

4.2 Chronic LXR Activation and the Interaction with Dietary Background

Many previous studies on LXR activation have been conducted in the short term (4-10 days). A series of experiments were also conducted to determine the chronic effect of activating LXR in hamsters consuming a lipogenic diet high in fructose and fat. This is an important question as many studies have previously reported interactions among dietary components (Enmark and Gustafsson 2002, Tobin et al. 2000). LXR has been shown to cause metabolic dyslipidemia, a major component of the metabolic syndrome, when administered with the chow diet. This occurs through a variety of mechanisms. LXR activates transcription of several genes involved in lipogenesis, including Fas, Scd, and Acc (Joseph et al. 2002, Schultz et al. 2000, Talukdar and Hillgartner 2006). In addition to directly activating these genes, LXR also increases transcription of the transcription factor Srebp-1c, a known master regulator of lipogenesis (Repa et al. 2000).

In the present study, feeding a combination of a high fat and high fructose (FF) diet induced increases in both plasma TG and cholesterol. Interestingly, these effects were enhanced even further by the addition of the LXR agonist to the FF diet, demonstrating that LXR activation exacerbates the lipogenic effects of these two macronutrients. The effect on plasma TG was stronger, which is expected because of LXR’s known effects on Scd and Fas expression, two
known lipogenic enzymes. This was mirrored by the increase in plasma apoB, the protein that associates with TG and cholesterol to form VLDL and LDL particles. The relative similarity in both plasma ALT and AST levels between the two dietary treatments suggests that the LXR agonist did not induce any observable hepatic injury. Although there were slight increases in plasma ALT and AST levels on the FF diet alone, these did not reach statistical significance (when compared to their respective baseline levels) suggesting no appreciable hepatic injury with this dietary treatment over the course of the study.

Although LXR is known to activate lipogenesis, it has also generated interest because of its ability to stimulate reverse cholesterol transport (Brunham et al. 2006, Naik et al. 2006). When LXR was administered to hamsters along with the FF diet, there was not only no increase in HDL cholesterol, but there was a significant shift toward VLDL cholesterol. Since all previous studies examining the effect of LXR activation on cholesterol distribution were conducted in mice, it is possible that differences in LXR action between species may account for differences in observed effects. Hamsters express the gene cholesterol ester transfer protein (Cetp), an LXR target gene (Luo and Tall 2000, Xu et al. 2003), while mice and rats do not. It is known that CETP inhibition in hamsters increases reverse cholesterol transport (Tchoua et al. 2008), leading to the idea that the presence of Cetp may worsen existing lipid problems. Indeed, LXR agonists have been found to have no effect on plasma HDL, and to increase LDL in Cetp-expressing species including hamsters and cynomolgus monkeys (Groot et al. 2005). This perhaps accounts for some of the differences observed between current hamster studies and previously published data in mice and rats.

While hepatic TG and cholesterol accumulation did not increase at any of the time points examined following LXR activation, the physical mass of the liver did increase with LXR activation, in proportion to the hamsters’ total body mass. Since this weight increase cannot be attributed to increased lipid storage, there must be another reason for the added mass. This may be due to an inflammatory response and accumulation of macrophages in the liver.

LXR, when activated, has previously been shown to specifically target genes involved in lipogenesis, including Fas and Scd (Schultz et al. 2000). The level of SCD mRNA was found to increase dramatically at all time points following the addition of the LXR agonist to the FF diet. While this is expected, it is also a likely explanation for the observed increase in plasma TG
levels. FAS mRNA levels also increased significantly after four and eight days of LXR agonist treatment, as expected, but showed no difference from the FF diet-fed group after 16 days. This pattern of Fas expression was mirrored by the hepatic FAS protein levels at each time point. There was an initial increase in FAS protein levels that was tempered after 16 days of treatment and feeding. This may be the result of a feedback mechanism; the build-up of lipids in the plasma may somehow reduce the initially increased FAS mRNA level. The regulation must be at the transcription level, since that is the first level at which the change is seen. FAS mRNA is known to be upregulated by increased AKT phosphorylation (Furuta et al. 2008, Wang et al. 2005), an effect seen in many cancers. Since AKT phosphorylation decreased over the course of the experiment, this may play a role in the expression of FAS protein as well. The level of hepatic SCD protein was also substantially increased after four and eight days, but this level of increase is reduced after 16 days, even though the level of mRNA continued to be sharply increased. This may indicate negative regulation at the translational or post-translational level.

PPAR-alpha, which is activated by lipids, is known to activate fatty acid beta-oxidation (Reddy et al. 1986). Since LXR has been shown to interfere with PPAR-alpha dimerization with RXR (Ide et al. 2003), LXR activation may negate any of the beneficial effects that may occur because of dietary lipid activation of PPAR-alpha (Ide et al. 2003). Results from this set of experiments showed that LXR activation in fact reduced PPAR-alpha protein level after 16 days of feeding, perhaps indicating that LXR activation may also interfere with PPAR-alpha expression. PPAR-alpha has also been shown to increase expression of LXR itself (Li and Glass 2004), further perpetuating the cycle. After fat feeding, LXR-alpha protein and mRNA levels have been shown to be increased in a PPAR-alpha dependent manner (Tobin et al. 2000).

The level of hepatic MTP mRNA was measured to determine if the LXR-induced increase in plasma VLDL was due to more efficient lipid packaging. Mtp gene transcription is known to be regulated in several ways: its transcription level is increased after exposure to oleic acid (Qiu et al. 2005) and by increased PPAR-alpha activity (Ameen et al. 2005), and is repressed by hepatocyte nuclear factor 4 (HNF-4) expression (Sheena et al. 2005) and in response to sterol depletion (Sato et al. 1999). Each of these regulatory pressures is consistent with one another, and is consistent with the effect seen in our study at four days of FF diet feeding following LXR activation. The lipids provided by the diet and produced after LXR activation may promote Mtp gene transcription, and the presence of cholesterol would eliminate the inhibition associated with
sterol depletion. After eight days of feeding, the level of MTP mRNA was still increased with LXR activation, but after 16 days, its level decreased by almost 50%. It therefore appears that increased MTP is not a sufficient explanation for the observed increase in plasma VLDL at the later time point. Although increased MTP may contribute to VLDL assembly and secretion, it is possible that VLDL packaging can further increase under these experimental conditions due to increased lipid availability and decreased apoB degradation (leading to increased apoB availability for VLDL assembly).

The Chrebp gene is known to be under the control of LXR (Cha and Repa 2007), and should therefore be upregulated upon LXR activation. ChREBP mRNA level was upregulated after eight days of treatment, confirming this expectation. There was, however, no change in ChREBP protein level observed after either four or eight days, and there was in fact a decrease after 16 days. This may indicate a feedback mechanism in place at the cellular level, preventing excessive ChREBP increases. A recent study shows that ChREBP undergoes post-translational regulation; the transcription factor can undergo phosphorylation under high glucose conditions, reducing its ability to translocate into the nucleus (Tsatsos et al. 2008). This or other post-translational changes may alter ChREBP function at different points during the treatment course.

The mRNA level of the cholesterol-sensitive transcription factor, SREBP-2 increased significantly after four and eight days with LXR activation, but subsequently decreased significantly after 16 days. The initial increase in the mRNA level of this enzyme may help explain the increase in plasma cholesterol seen in these animals, since SREBP-2 is responsible for upregulating HMG-coA reductase, the rate-limiting enzyme in cholesterol synthesis (Brown and Goldstein 1997). SREBP-2 is also known to upregulate itself, providing a feedback mechanism for its upregulation (Sato et al. 1996). The decrease in SREBP-2 mRNA after extended feeding is less expected, however. While the effect of LXR on Srebp-2 gene regulation is unclear, it is known that SREBP-2 mRNA levels increase in LXR alpha -/- mice (Peet et al. 1998, Wojcicka et al. 2007). This perhaps means that in the long term LXR may exert negative control over the Srebp-2 gene, as seen at the longer time point in this study.

Although insulin sensitivity and glucose tolerance were not measured during this phase of the study, the hepatic protein levels of two negative regulators of insulin signaling, PTEN and PTP-1B were measured. These two enzymes negatively regulate insulin signaling by
dephosphorylating components of the insulin signaling pathway, tempering its downstream actions. Higher levels of these two proteins would normally indicate an impairment of insulin signaling under the experimental conditions studied. In the present study, there was no observable change in the protein levels of either PTP-1B or PTEN. This means that either the insulin signaling pathway was intact, or that if it was impaired, it was due to dysregulation at another point in the pathway. Another possibility is that the FF diet alters insulin signaling, but that this effect is not changed by the addition of the LXR agonist (as the effects were not compared to a normal chow diet in this first phase of the study; this was done subsequently in other experiments).

In conclusion, LXR activation in the hamster model appears to worsen the lipogenic effects of the FF diet, suggesting additive or synergistic effects. These effects included the accumulation of TG in the plasma and the activation of several lipogenic genes, including Fas and Scd. LXR activation likely contributes to a greater enhancement of hepatic lipogenesis over that stimulated by the FF diet alone. The consequence of this interaction is a substantial increase in production of hepatic VLDL and increased circulating VLDL-TG. These observations suggest that factors that can stimulate LXR chronically (such as high dietary cholesterol feeding) can exacerbate the metabolic effects of a lipogenic diet and promote progression to metabolic dyslipidemia.

4.3 Long-term LXR Activation in the Hamster Model on Chow and FF Diets

Following the preliminary observations discussed above, further experiments were conducted over 30 days. Hamsters were fed either the chow diet or the FF diet, and each was supplemented with either vehicle or 10 mg/kg T0901317. Both the FF diet and LXR activation caused some measure of dyslipidemia after long-term administration. Both groups consuming the FF diet showed an increase in plasma cholesterol, while the FF and CLXR groups showed increases in plasma TG. The FF diet triggered a significant increase in hepatic triglyceride accumulation. Administration of the LXR agonist caused a significant shift in plasma cholesterol to the VLDL density range, regardless of the accompanying diet. Consumption of the FF diet caused a trend toward decreased glucose tolerance, while the combination of the FF diet with LXR activation caused a significant increase in AKT phosphorylation. The effect of the LXR agonist was not
very strong in any of the measures, perhaps because of the lower dose of agonist used (10 mg/kg).

In these studies, the effect of the LXR agonist appeared to be weaker after 30 days of treatment than after 16 days. This may likely be due to the lower LXR agonist dose used for this experiment; after four days of treatment, the 10 mg/kg dose was not seen to have substantial effects on plasma TG levels. Importantly, the FF diet appeared to significantly interact with chronic LXR agonist treatment, as the more severe effects of the T0901317, particularly in plasma and liver lipid perturbations, occurred uniquely in hamsters consuming the FF diet.

The LXR-mediated increase in plasma TG after 30 days with or without the FF diet was consistent with previous studies. When LXR was activated on the chow diet alone, it caused a gradual increase in plasma TG over time. The animals receiving both the FF diet and LXR agonist showed a sharper increase initially followed by a gradual decline to the level of the chow-LXR activated (CLXR) group. This effect may be due to the inclusion of fat and fructose in the diet, which would help to generate the spike in plasma TG. The FFLXR group showed the largest increase in plasma apoB100, indicating the highest increase in circulating VLDL particles.

None of the diet treatments resulted in large changes in either of the enzymes ALT or AST. Increases in the levels of these enzymes would indicate liver damage, and thus none of the diets employed appeared to severely affect liver function at the time points examined. Previous work in our laboratory has shown that LXR activation in chow-fed animals causes a significant rise in both ALT and AST after just four days (Basciano et al, 2008, in submission). Although this experiment lasted up to 30 days, a comparable increase was not seen. Since the four-day study used 50 mg/kg of T0901317, the absence of liver injury in this study is likely due to a smaller dose of the agonist.

Each of the diet combinations caused a shift of plasma cholesterol towards the VLDL fraction when compared with the chow group after 30 days of treatment. The magnitude of this effect appears to be similar whether the stimulus is the LXR agonist, the FF diet, or the combination of the two. The addition of the LXR agonist to the FF diet did not therefore exhibit any additive effect to the level of VLDL cholesterol. There was no significant change in the distribution of VLDL-TG among the different lipoprotein fractions. This is surprising, as the combination of the
FF diet and T0901317 in another group of hamster studies prompted a significant shift in TG toward the VLDL fraction after 16 days of feeding (see Figure 3.2.2). The reason for this lack of effect on VLDL-TG in the 30 day treatment studies may be due to the lower dose of the agonist employed.

The FF diet triggered a significant increase in hepatic TG mass. This is expected because of the additional fat being introduced through the diet. Of note, however, is the lack of TG accumulation in the livers of those hamsters fed both the FF diet and the LXR agonist. The level of TG in these livers was comparable to that in the chow and CLXR groups. No significant changes in hepatic cholesterol accumulation were brought about by LXR activation or by FF diet feeding. Interestingly, the changes in liver mass did not reflect the differences in lipid accumulation. LXR activation and FF diet feeding, in any combination, increased the liver mass in proportion to the body weight by approximately 20% compared with the chow-fed group. Since the increase in liver mass cannot be ascribed to increases in lipid accumulation, there must be another explanation for this increased hepatic bulk. Adipose tissue mass did not change with any of the dietary manipulations. This however, does not guarantee that there was no change in overall lipid storage patterns, since only the gonadal fat pads were weighed. There may have been changes in the amounts of subcutaneous or visceral fat that were not assessed this study.

In order to determine whether the FF diet feeding or LXR agonist had effects on the hamsters’ ability to respond to insulin, glucose and insulin tolerance tests were performed on select animals from each group. There were no significant differences between any of the groups examined. The LXR agonist did not appear to alter glucose tolerance beyond the effect of the FF diet, indicating that dietary fat and fructose may have played a stronger role. Dietary fructose has previously been shown to cause insulin resistance in the hamster model (Taghibiglou et al. 2000), identifying the importance of this dietary factor in insulin signaling. Although the FF diet did not cause a significant impairment in glucose tolerance, there was a trend toward glucose intolerance that was consistent with both animal groups consuming the FF diet. This may not have reached significance simply due to the small sample size; since both insulin and glucose tolerance tests were conducted, the six animals in each group were divided equally between the two tests, leaving three animals per group, per test.
To determine whether the insulin signaling pathway was disturbed at the molecular level with either LXR activation or FF feeding, the mass and phosphorylation state of AKT were measured. All diet combinations resulted in approximately equal AKT mass levels, but hamsters given both the LXR agonist and the FF diet were shown to have increased levels of AKT phosphorylation. AKT, a component in the insulin signaling pathway, is activated by phosphorylation. Phosphorylated AKT then propagates the insulin signaling pathway by phosphorylating downstream factors. One of the main results of this pathway is increased glucose uptake from plasma, as AKT is known to increase translocation of GLUT4 to the surface of muscle and adipose tissue cells (Cong et al. 1997, Wang et al. 1999b). It is therefore surprising that the FF diet increased AKT phosphorylation, since both dietary fat and fructose are known to cause glucose intolerance (Sumiyoshi et al. 2006). LXR may have effects on insulin resistance in tissues other than liver. In future, the integrity of the insulin signaling pathway in tissues other than liver, including muscle and adipose, could be measured to determine that effect.

The increase in SCD expression in the CLXR group is expected, since Scd is a known target of LXR (Schultz et al. 2000). The lack of increase in hepatic SCD protein level after FF diet feeding, with or without the LXR agonist, and the lack of change in FAS protein level with any treatment, is surprising considering the increase in plasma and hepatic TG seen with FF feeding. The hepatic protein levels of FAS and SCD decreased over time in FFLXR animals, with only slight increases after 16 days of feeding. This may be explained by a potential feedback mechanism caused by excess lipid accumulation. There is so far no evidence that increased lipid availability can directly downregulate FAS expression; however, increased AKT phosphorylation has been shown to negatively regulate FAS expression (Furuta et al. 2008, Wang et al. 2005). The increase in AKT phosphorylation observed in the FFLXR group may thus provide an explanation for the lack of change in FAS expression after 30 days of activation.

In conclusion, long-term FF diet feeding together with LXR activation causes gradual increases in plasma TG and cholesterol. The initially strong effects of LXR activation on VLDL levels seen after four to 16 days of feeding seem to be tempered after 30 days. The effects of the FF diet were manifested by increases in plasma lipids and hepatic triglyceride accumulation. The FF diet also prompted a trend toward an increase in glucose intolerance, indicating weaker insulin sensitivity. The increase in AKT phosphorylation shown by the FFLXR group, however, indicated that regulation of this pathway might in fact be more complex. The weaker effects
observed in the two LXR agonist groups may be attributed to the lower agonist dose employed, since the dose of 10 mg/kg didn’t elicit an identical response to the 50 mg/kg dose.

4.4 Comparison of the Metabolic Effects of Chronic LXR Activation with Cholesterol Feeding

Dietary cholesterol, when fed along with the FF diet, has previously been found to induce severe metabolic dyslipidemia and insulin resistance. Cholesterol supplementation appears to play a major role in inducing these metabolic effects, as increases in the concentration of dietary cholesterol from 0.05% to 0.25% severely exacerbated the effects of the FF diet (Basciano et al 2008, in submission). The dyslipidemic effects seen with this diet were intriguingly similar to the effects observed with chronic LXR activation. Since oxysterols, which are metabolic derivatives of cholesterol, are natural ligands for LXR, it is possible that cholesterol exerts at least some of its effects via LXR activation. To test this hypothesis, the metabolic effects of administering the LXR agonist in hamsters consuming the FF diet were directly compared with the effects of cholesterol supplementation of the same diet. It is reasonable to expect that oxysterols derived from cholesterol may be acting via LXR activation. A study by Chen et al showed LXR inactivation in mice with overactive oxysterol-metabolizing enzymes, and thus with unnaturally low oxysterol levels. In addition, those mice without oxysterol-producing enzymes also have low LXR activity (Chen et al. 2007). In both cases, LXR itself was not impaired, as T0901317 continued to activate LXR to the same level as in the wild-type animals (Chen et al. 2007). This study supports the idea that dietary cholesterol plays a direct role in activating LXR in rodents.

In support of this concept is the similarity between cholesterol feeding and LXR activation in the hamster model on the background of the FF diet. Both LXR activation and cholesterol feeding caused increases in both plasma TG and cholesterol beyond the effect of the FF diet alone. Dietary cholesterol feeding had a stronger impact on plasma cholesterol, as expected. The LXR agonist, on the other hand, had a stronger impact on plasma TG, especially in the short term. Added cholesterol began to approach the effect of LXR activation with respect to plasma TG after 16 days of feeding. This may be due to a delay in cholesterol processing; whereas T0901317 activates LXR immediately, cholesterol must first be converted to oxysterols, which must reach an appreciable level in the liver before they are able to activate LXR to the same extent. One of the most abundant oxysterols in the body, 24-OH cholesterol, is mainly
manufactured in the central nervous system, and must be transported into general circulation (Olkkonen 2004) before reaching the liver. Dietary cholesterol levels of 3-5% have, however, shown to cause significant increases in hepatic oxysterols within four days of feeding (Schroepfer 2000). Such oxysterols can function as natural ligands to stimulate LXR gene activity.

The fact that cholesterol feeding with the FF diet triggered increases in gene levels of several known LXR targets also lends support to the idea that dietary cholesterol acts partly through LXR activation. Since LXR is a transcription factor, mRNA levels were measured to determine the effect on the transcription of target genes. The well-known LXR target Scd (Schultz et al. 2000) was most strongly activated by the CLXR and FFLXR diets, as would be expected. Scd transcription was also increased after feeding with the FF and FFC diets. The effect of the FFC diet was not stronger than that of the FF diet alone, indicating that the cholesterol feeding had no additional effect on this gene. All four treatments significantly augmented the mRNA level of the LXR target ABCA1, with the FFC and FFLXR groups showing almost identical effects. One surprising result was the FF-diet group’s ability to cause the strongest level of activation in Abca1.

The gene Bsep is a target of another nuclear receptor, FXR. Since T0901317 has been shown to dually activate both LXR and FXR (Houck et al. 2004), the mRNA level of this target gene was measured to determine to what extent this was happening. None of the diet groups had a very substantial effect on Bsep expression, indicating no major activation of FXR with either T0901317 or dietary cholesterol feeding.

The FFLXR and FFC groups also showed similar changes in SREBP-2 mRNA levels. Since the LXR agonist alone did not cause parallel effects, the combination of the FF diet with either LXR activation or cholesterol feeding was likely necessary to bring about this change.

The Srebp-1 promoter is known to contain an LXRE. It is therefore not surprising that both the LXR agonist and cholesterol feeding caused similar increases in SREBP-1c mRNA. The surprising fact is that the FF diet alone had the strongest effect on Srebp-1 expression. Since Srebp-1 is also activated by insulin (Eberle 2001), the decrease in insulin sensitivity that was seen in both the FFC and FFLXR treatment groups may have affected their ability to transcriptionally activate Srebp-1.
Interestingly, in all treatment groups examined, the expression pattern of Chrebp was not mirrored by a corresponding change in protein level. All four treatment groups, particularly CLXR, showed increased ChREBP mRNA levels above the level of chow-fed controls. LXR is known to activate Chrebp gene transcription, and this should translate into both increased mRNA and protein levels. When the protein level was measured, however, both the FFLXR and FFC groups showed a significant downregulation of ChREBP protein level. The similarity of expression in these two groups shows that there may be a common pathway mediating their effects, namely LXR activation. The increase in ChREBP mRNA expression is expected since this is a known LXR target. The post-translational decrease is more unexpected, however. A recent study showed that ChREBP activity is negatively regulated under high glucose conditions by phosphorylation at the N-terminus (Tsatsos et al. 2008). This post-translational modification may also affect the total mass of the protein. In future, the phosphorylation state of ChREBP could be measured to determine whether this is affected by the different experimental conditions.

Interestingly, there were differences among the treatments when examining hepatic lipid accumulation. After 14 days, the LXR agonist prompted more TG accumulation in the liver, and after 16 days all groups consuming the FF diet demonstrated higher hepatic TG levels. Cholesterol feeding had no additional effect with respect to TG levels. Cholesterol feeding did, however, affect hepatic cholesterol levels, as would be expected. After both 14 and 16 days, dietary cholesterol triggered significantly greater cholesterol accumulation in the liver than any of the other groups. These differing observations suggest that the effects of cholesterol in terms of hepatic lipid accumulation may not be completely mediated by LXR activation, and that any effects of LXR activity may not be wholly mediated by oxysterols, but instead may be partially mediated by other dietary factors.

Dietary cholesterol and LXR activation produced very similar shifts in plasma lipoprotein profile. After 16 days, both conditions produced very similar shifts in TG toward the VLDL fraction, regardless of the inclusion of fat and fructose in the diet. This is in slight contradiction with the effects seen in mice (Brunham et al. 2006, Naik et al. 2006, Zanotti et al. 2008). While it is well known that LXR activation increases lipogenic gene expression, it is also known to increase reverse cholesterol transport through increased Abca1 expression (Naik et al. 2006). Increased ABCA1 in macrophages increases the transport of cholesterol to the HDL fraction in plasma (Levin et al. 2005). The observed disagreement in lipid effects is likely due to species
differences, as discussed in section 4.2. Hamsters express CETP, an enzyme also expressed in humans that inhibits reverse cholesterol transport, and that is not expressed in mice or in rats (Luo and Tall 2000, Xu et al. 2003). The increase in TG synthesis in the hamster livers, promoted by LXR activation through increased Scd and Fas expression, likely drives increased VLDL production and secretion.

LXR activation and dietary cholesterol diet supplementation also showed similar effects on glucose tolerance. While FF diet feeding appeared to decrease the animals’ ability to dispose of plasma glucose, this effect did not reach statistical significance until either the LXR agonist or cholesterol was added to the diet. LXR has been previously shown to ameliorate increases in plasma glucose (Laffitte et al. 2003). The previously observed decrease in plasma glucose has been attributed to LXR-mediated inhibition of gluconeogenic enzymes (Cao et al. 2003, Chisholm et al. 2003, Laffitte et al. 2003). LXR activation has also been shown to increase glucose tolerance in the rat model, as measured by a hyperinsulinemic-euglycemic clamp (Commerford et al. 2007). They also noted an increase in the glucose transporter GLUT4 at the surface of adipocytes, which is expected since LXR has been shown to directly upregulate Glut4 expression (Dalen et al. 2003). The rats in the study conducted by Commerford et al. were fed 60% fat and were given the LXR agonist GW3965 for 17-19 days. The main difference between this study and the current one is the presence of fructose in the diet. The addition of fructose alone to hamster and rat diets has been shown to cause insulin resistance (Taghibiglou et al. 2000, Tobey et al. 1982, Zavaroni et al. 1980), demonstrating that this simple sugar may have a significant influence on insulin signaling in the hamster model. LXR activation and cholesterol feeding, however, significantly worsened glucose tolerance beyond the effect of the FF diet. This indicates that these dietary additives are working in a concerted fashion with the FF diet to worsen glucose intolerance. To underline the potential effects of carbohydrates in the diet, a recent study has also found that glucose can directly activate LXR (Mitro et al. 2007). Glucose is thought to both compete with oxysterol binding in the same pocket, and to bind additional pockets as well. This glucose-induced activation leads to the next question: does fructose have a similar effect? This question remains unanswered at this point but deserves further investigation.

Finally, the FFC diet significantly decreased AKT mass, while the addition of the LXR agonist did not show a substantial effect. Therefore, the mechanisms underlying the insulin resistance induced by LXR activation and cholesterol supplementation may differ. An examination of other
components of the insulin signaling pathway and factors involved in glucose uptake (including the glucose transporter GLUT4) would shed some light on the mechanisms involved.

The evidence presented here supports the idea that cholesterol exerts many of its dyslipidemic effects through LXR activation. Additional support for this hypothesis comes from a study conducted by Maxwell et al, who measured gene expression by microarray in the livers of mice fed either 0.5% cholesterol or 10 mg/kg T0901317 for one week. They found that of the 32 genes upregulated by cholesterol feeding, 13 of them were also upregulated after LXR activation (Maxwell et al. 2003). The overlap in gene expression seen between LXR activation and cholesterol feeding indicates that at least parts of cholesterol’s effects are mediated by LXR activation.

It is possible, however, that some of the effects seen in cholesterol-fed animals are not due to direct LXR stimulation by oxysterols. The results from the FFC and FFLXR groups were not exactly identical, indicating that cholesterol may act through alternate pathways. FFC diet-feeding decreased AKT mass while LXR activation did not. The two stimuli also had different effects on hepatic lipid mass accumulation. There are other mechanisms by which cholesterol may act to exert its dyslipidemia-inducing effects. A recent study showed that cholesterol accumulation in hepatic mitochondria sensitizes the liver cells to TNF-alpha and Fas-mediated steatohepatitis. The Fas mentioned here should not be confused with the previously mentioned lipogenic enzyme FAS. Fas is a receptor involved in the mediation of apoptosis when activated by the Fas ligand, or FasL. The accumulation of cholesterol in the mitochondria and the subsequent inflammatory sensitivity is proposed to be the switch from the simple accumulation of lipids in the liver (steatosis) to the more inflammatory and more dangerous steatohepatitis (Mari et al. 2006). This is supported by further studies showing that mitochondrial dysfunction is firmly linked to development of steatohepatitis (Begriche et al. 2006, Froment et al. 2004). The most recent study by Mira et al. is particularly interesting because they show that the accumulation of fat or carbohydrates on their own isn’t enough to cause the complications seen with cholesterol feeding. Cholesterol is proposed to cause the observed effects because of mitochondrial glutathione (mGSH) depletion.

Conversely, the effects seen with LXR activation are likely not wholly due to activation by diet-derived oxysterols. As mentioned in section 4.4, glucose has the ability to directly activate LXR
Mitro et al. 2007). LXR can also be activated by components of the cholesterol synthesis pathway (Yang et al. 2006), including the unsaturated cholesterol precursors desmosterol and zymosterol. Although these are similar ligands to oxysterols, they demonstrate other non-dietary routes of LXR activation.

In conclusion, dietary cholesterol supplementation and LXR activation show similar effects in several measures of hamster metabolism after administration for at least two weeks. When given to hamsters consuming the FF diet, the LXR agonist and dietary cholesterol showed similar changes in plasma triglycerides, similar plasma lipoprotein profiles and similar changes in glucose tolerance. Many of the effects are exacerbated by the concurrent FF diet feeding, and became more severe with chronic administration (at least 14 days). Cholesterol feeding and LXR activation were not parallel in terms of Akt mass or phosphorylation, or hepatic lipid accumulation, indicating that although cholesterol may exert many of its effects through LXR activation, the effects of these two stimulations may also have divergent effects.

4.5 Conclusions

The LXR agonist is well known for its lipogenic effects. The object of this study was to determine whether LXR activation could exacerbate the effects of a high fat/high fructose diet, to determine the long-term effects of LXR activation on the background of this diet, and to compare LXR activation with cholesterol feeding.

Previous studies examining LXR activation had used a dose of 50 mg/kg. Concerns that this dose was super-physiological were addressed with studies employing a dose of 10 mg/kg. Findings from these experiments indicated that the LXR agonist T0901317 can be administered in a dose of 10 mg/kg to hamsters and have certain metabolic effects comparable to those observed at 50 mg/kg, including increased hepatic apoB synthesis and secretion. While there was not a significant effect on plasma TG, neither was there a measurable change in ALT, an indicator of hepatic damage. Perhaps an intermediate dose of T0901317 would be most appropriate in future studies in order to maximize the dyslipidemic effects while minimizing hepatic injury.

The experiments described here present evidence that the dyslipidemic effects of cholesterol may be mediated via LXR activation. Activation of LXR in hamsters consuming the FF diet was
remarkably similar to results of previous experiments showing that cholesterol dose-dependently induces metabolic dyslipidemia. Direct comparison of cholesterol supplementation with LXR activation on the FF diet background showed that both treatments caused decreased glucose tolerance, increased plasma lipids and dysregulated lipoprotein profiles. The similarity in the effects of cholesterol and the LXR agonist on the expression of SREBP-1c, ABCA1, ChREBP and PPAR-alpha provides some explanation for their similar manifestations. Since cholesterol molecules are naturally converted to oxysterols after consumption, and since oxysterols are natural LXR activators, it is very likely that the parallel results observed in the FFLXR and FFC treatment groups are due to parallel mechanisms.

Time was found to augment the dyslipidemic effects of the examined treatments. The dyslipidemia seen with LXR activation and cholesterol supplementation were more pronounced after 14-30 days, compared with acute four-day treatments. The mechanisms of these time-dependent increases aren’t completely clear, however, as many of the initial increases in lipogenic proteins were subsequently reduced. Feedback mechanisms may be in place to mitigate initial increases in factors such as ChREBP, FAS, SCD, SREBP-1c and MTP.

One final but important consideration is the exaggerated effect of cholesterol feeding or LXR administration when administered to FF diet-fed hamsters. Many of the noted effects, especially decreased glucose tolerance and increased lipogenesis, were particularly augmented in hamsters consuming the FF diet compared to those consuming regular chow. This indicates that the diet components of fat, fructose and cholesterol somehow act in collaboration when administered concurrently. The precise mechanism of this overlap must be further examined.

The main limitation of this study was its observational nature. Since this study simply compared the effects of different diet groups, there can be no firm conclusions on the mechanism by which cholesterol exerts its metabolic effects.

The similarity of the effects of chronic cholesterol supplementation and LXR activation in the FF diet-fed hamster model strongly supports the hypothesis that dietary cholesterol acts at least in part through LXR activation. Further studies, which will be proposed in the following section, will provide clarification on the extent and mechanism of LXR’s cholesterol-mediating effects.
4.6 Future Directions

Future experiments should further examine the interaction between dietary cholesterol and LXR activation to more clearly determine the effects that this relationship has. Future studies should also investigate alternate pathways and mechanisms by which cholesterol may exert its metabolic effects.

The effect of dietary cholesterol, fat and fructose on mitochondrial function can be assessed by several methods. Several studies have shown that hepatic lipid accumulation leads to disruptions in the respiratory chain, leading to increased release of lipid peroxidation products, which in turn contributes to the progression of Non-alcoholic steatohepatitis (NASH). A recent paper by Koves et al demonstrates how mitochondrial dysfunction may decrease insulin sensitivity through incomplete lipid oxidation (Koves et al. 2008). Malondialdehyde and 4-hydroxynonenal are two by-products of lipid peroxidation that have been shown to interfere with the respiratory chain function in mitochondria (Wei et al. 2008). Lipid peroxidation products can be measured easily by measuring the formation of thiobarbituric acid reactants (Berson et al. 1998, Fau et al. 1992). Accumulation of lipid peroxidation products has also been implicated in increased mitochondrial size, or megamitochondria (Day and James 1998). Mitochondrial size has been shown to increase with progressive NASH (Caldwell et al. 1999, Wei et al. 2008). Electron microscopy has shown to be very useful for measuring mitochondrial size, and for identifying lipid droplets that may accumulate in the organelles (Sobaniec-Lotowska and Lebensztejn 2003). This technique could be employed in future studies to determine mitochondrial size and the extent of mitochondrial lipid accumulation after LXR activation and cholesterol feeding.

The increased lipid peroxidation products produced by dysfunctional mitochondria may promote fat-laden liver to a steatohepatotic state by providing a second ‘hit’. In 1998, it was recognized that hepatic fat accumulation was the first step towards progression to NASH, but a second insult was required (Day and James 1998). Factors that were identified as being capable of triggering this promotion were the production of lipid peroxidation products due to reactive oxygen species, and the presence of cytokines (Day 2002, Day and James 1998). Cytokine infiltration could be easily measured by immunohistochemistry with preserved liver sections to determine the level of inflammation. Cytokines may be involved in more than simply liver disease; increased hepatic
TNF-alpha has also been shown to modulate insulin signalling through serine/threonine phosphorylation of IRS-1 and IRS-2 (Day 2002).

A third possible experiment that was previously alluded to is to determine whether fructose directly activates LXR. A solution to this question would provide another connection between the FF diet and the observed effects. Fructose-binding to LXR could be tested using a number of simple experiments. As an initial test, the expression of LXR-controlled genes could be measured in fructose-treated primary hepatocytes. To test this effect more directly, a scintillation proximity assay could be employed to determine the physical association between fructose and LXR. This method tests the ability of cold fructose to displace $^3$H-labelled fructose from a bead coated with LXR (Mitro et al. 2007). Fructose-induced activation of LXR would also explain some of the effects seen with the LXR agonist that are not explained by cholesterol feeding.

While LXR activation is known to increase $\text{Glut4}$ transcription (Dalen et al. 2003), the effects of the diet combinations, LXR feeding and cholesterol feeding on GLUT4 translocation to the cell membrane are unknown. GLUT4 is translocated to the cell membrane of insulin-responsive tissue after insulin signalling. If insulin signalling is impaired, the presence of GLUT4 on the cell membrane will also be reduced. Quantification of the amount of GLUT4 on the cell membrane after diet treatment and LXR activation would give another indication of the responsiveness of the tissue to insulin, as well as perhaps providing an explanation for the impaired glucose tolerance after FFC and FFLXR treatment.

To determine more directly the effects of oxysterols on LXR activation, a simple experiment could be performed in cell culture. Primary hamster hepatocytes could be treated with different concentrations of oxysterols to measure the response in terms of increased LXR target transcription. The main drawback of this experiment would be the lack of ability to continue the treatment over the long term, since primary hepatocytes typically survive only about two days in culture. Taking the cells out of the context of the whole animal would also be non-representative of the real-life effect. This experiment would, however, give an indication of the ability of oxysterols to actually activate LXR. Once this activation is confirmed, the experiment could be scaled up to oxysterol feeding in hamster, in combination with the FF diet.
In addition to experiments further investigating the effects of both cholesterol and LXR, the data as presented here will need to be analyzed using more rigorous statistical methods, including ANOVA analysis and post-hoc comparison tests.

Many further experiments can be conducted to determine with more precision the effects of LXR activation in the context of diet feeding, as well as the pathway of cholesterol effects. A more complete understanding of these effects will help to understand the metabolic syndrome and its progression to metabolic dyslipidemia and more severe liver disease.
5 :: Appendix

5.1 Abstract

The nuclear receptor Farnesoid X Receptor (FXR) acts in a similar but complementary way to LXR. Instead of activating bile acid production in response to excess oxysterols, FXR inhibits bile acid production after being activated by bile acids themselves. Since FXR is known to oppose some of LXR’s metabolic effects, we asked whether chemically activating FXR in hamsters would ameliorate dyslipidemia. Four hamsters in each group were given either the synthetic FXR agonist GW4064 or vehicle for five days. No significant differences were observed in plasma lipids or lipoproteins, but hepatic FAS protein level decreased significantly by 30%. Further work should be completed to determine both the effect of FXR activation on a lipogenic diet and the effect of chronic FXR activation.

5.2 Introduction

The Farnesoid X Receptor (FXR) is a nuclear receptor belonging to the same family as LXR. FXR was identified before its ligand was known, and was originally put into the Orphan Nuclear receptor family. Its natural ligands have since been identified to be bile acids, and its gene targets include many factors involved in bile acid metabolism. FXR’s tissue distribution is consistent with its role as a modulator of bile acid homeostasis; it is present primarily in the liver, intestine, kidney and adrenal glands (Kalaany and Mangelsdorf 2006). FXR regulates many of its gene targets directly, by interacting with a specific hormone response element consisting of inverted repeat elements separated by one nucleotide (IR-1) (Kalaany and Mangelsdorf 2006). FXR does, however, have the ability to modulate a subset of its targets indirectly, through an intermediate protein. FXR activates transcription of the small heterodimer partner (SHP), which goes on to activate or repress transcription of more genes. One of the main effects of FXR activation is to reduce the conversion of cholesterol to bile acids by repressing transcription of the gene Cyp7a1, the rate-limiting enzyme in bile acid production (Chen et al. 2001). Since FXR is activated intracellularly by bile acids, it acts as a bile sensor, preventing excessive and toxic bile acid accumulation. FXR also upregulates several genes involved in bile salt efflux, including the bile
salt export protein (*Bsep*) (Ananthanarayanan et al. 2001) and the multidrug resistance gene (*Mdr*) (Huang et al. 2003).

With respect to lipid metabolism, FXR activation is implicated in reducing plasma TG levels and increasing plasma HDL. FXR is known to activate apoCII, which is an obligate cofactor for LPL (Kast et al. 2001). FXR also suppresses apoCIII, which is known to inhibit LPL (Claudel et al. 2003, Ginsberg et al. 1986). Increasing LPL activity would increase TG hydrolysis VLDL, thereby reducing total plasma TG levels. FXR is also known to repress expression of *Srebp-1c* and *Mtp* (Hirokane et al. 2004, Watanabe et al. 2004). In recent studies, FXR activation with the natural ligand chenodeoxycholic acid (CDCA) has been shown to reduce dietary fructose-induced dyslipidemia in hamsters (Bilz et al. 2006). FXR activation was also shown to protect against liver fibrosis in rats (Fiorucci et al. 2004).

FXR’s regulation of the lipogenic enzyme *Fas* is currently under dispute. One study shows that FXR -/- mice have increased *Fas* transcript levels, indicating that FXR would negatively regulate this gene if present (Duran-Sandoval et al. 2005b). Another study, however, demonstrates that FXR positively regulates *Fas* through an IR-1 element in the *Fas* promoter (Matsukuma et al. 2006). The evidence for FXR’s positive regulation of *Fas* is strong, as Matsukuma et al. show not only that the FXRE is present in the *Fas* promoter, but also that FXR activation with both natural and synthetic ligands in both cell and animal models increases *Fas* expression. This evidence is not concordant, however, with FXR’s role in ameliorating dyslipidemia. Indeed, in hamsters, FXR activation reduced fructose-induced increase in hepatic *Fas* levels (Bilz et al. 2006).

### 5.3 Rationale, Objectives and Hypothesis

FXR activation has been highlighted as a potential target for the treatment of the metabolic syndrome and associated dyslipidemia (Cariou and Staels 2007, Duran-Sandoval et al. 2005a). Since FXR has been shown to ameliorate dyslipidemia in hamsters and since it is known to counteract the effects of LXR activation, we decided to measure the effect of FXR activation in the Syrian Golden Hamster using the chemical agonist GW4064. The synthetic FXR agonist GW4064 was administered to hamsters by oral gavage at a dose of 30 mg/kg twice per day for five days.
It was hypothesized that treatment with the FXR agonist will ameliorate the metabolic phenotype developed in the fructose-fed/insulin resistant hamsters.

5.4 Results

FXR activation has previously been shown to ameliorate some of the effects associated with metabolic dyslipidemia. To determine if FXR activation would decrease plasma lipids in the hamster model, hamsters were given the synthetic FXR agonist GW4064 twice each day for five days. According to the manufacturer, GW4064’s half life is on the order of several hours, making bi-daily dosing necessary for continual FXR activation. Plasma samples were taken both at baseline and at the endpoint to test glucose, lipid and enzyme levels. After five days of treatment the hamsters’ livers were perfused, and primary cells were used in a metabolic pulse-chase experiment to measure apoB synthesis and secretion levels. Primary cells were also homogenized and used for Western blot analysis of hepatic protein levels.

5.4.1 Effects of FXR activation on plasma lipid values

FXR activation in the hamster model showed no significant alterations in plasma glucose, cholesterol, TG, AST or in total body weight (Figure 5.3.1 A). The parameters of the vehicle-fed animals remained consistent over the five-day treatment period, with no value varying more than 10% in either direction from its original level. There was a slight decrease in both plasma TG and cholesterol in the GW4064-fed hamsters, but this change was not significantly lower than either their respective baseline levels or the vehicle-fed (control) animal levels.

5.4.2 FXR activation does not alter plasma lipid distribution

Although there was no change in total plasma lipid values, the distribution of these lipids among different density fractions was measured to determine if FXR activation altered plasma lipoprotein profile. FXR activation induced a slight but insignificant shift in plasma cholesterol toward the HDL fraction (p=0.361) (Figure 5.3.1 B, C). There was no difference between the FXR agonist-treated group and the vehicle-treated group with respect to plasma TG distribution.
5.4.3 Effects of FXR activation on apoB synthesis and secretion

To determine whether the FXR agonist altered the rate of apoB synthesis and secretion from the liver, a pulse-chase experiment was performed on primary hamster hepatocytes. Primary liver cells were starved of methionine for 45 minutes and were then given a 45 minute ‘pulse’ of $[^{35}\text{S}]$-methionine. Selected lots of cells were harvested directly following the pulse, and remaining cells were treated with non-radioactive media for 60 or 120 minutes of ‘chase’. There was

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![Graph showing physiological parameters](image)

**Figure 5.3.1. FXR activation does not alter plasma lipids or lipoprotein profile.**

Plasma samples were obtained at baseline and at endpoints from four hamsters per group (n=4). A) Plasma levels of glucose, TG, cholesterol, ALT and AST were measured using a clinical chemistry analyzer. B, C) Endpoint plasma samples were separated by density using FPLC, and fractions were analyzed for TG and cholesterol content.
Figure 5.3.2. FXR activation does not affect apoB synthesis or secretion, but downregulates FAS protein in liver.

A, B) Hepatocytes were isolated from both vehicle and GW4064-treated, chow-fed hamsters. The cells were incubated in methionine-free medium for 45 minutes, and then subjected to a 60-minute pulse with $[^{35}S]$-labelled methionine. Following the pulse, cells were rinsed and allowed to recover for 0, 60 or 120 minutes. Cells and media were collected and solubilized and apoB was immunoprecipitated from each sample overnight. ApoB was then solubilized in SDS sample buffer and subjected to SDS-PAGE. ApoB100 was quantified by physically cutting each band and measuring radioactivity by liquid scintillation counting. Results were normalized according to total radiolabeled protein as measured by TCA precipitation. Data (mean ± SD) are representative of three experiments (n=3). C) Fifty micrograms of protein from hepatic tissue
homogenates were separated by size using SDS-PAGE (6% acrylamide). Proteins were then transferred to a PVDF membrane and immunoblotted with the anti-FAS antibody. Data (mean + SD) are expressed as a percentage of the chow-fed control group level.

no significant difference between the two groups at any of the time points, when measuring either total apoB produced, or secreted apoB (collected in the medium) (Figure 5.3.2 A, B).

\textbf{5.4.4 FXR activation reduces hepatic FAS protein level}

Although there were no significant changes in plasma lipids or their distribution, there was a significant decrease in hepatic FAS protein mass. As seen in Figure 5.3.2 C, GW4064-mediated FXR activation caused a 30% decrease in the level of FAS protein in the liver, when compared with vehicle-treated controls (p=0.008).

\section*{5.5 Discussion}

The lack of change in both the amount and distribution of plasma lipids in this study was surprising, since FXR activation has been shown to reduce plasma lipids in previous studies (Bilz et al. 2006, Maloney et al. 2000). Bilz et al also showed that FXR activation in hamsters decreased plasma VLDL levels, leading to the expectation that apoB secretion from the liver would decrease upon FXR activation as well. There was, however, no difference between FXR agonist-treated and vehicle-treated hamsters with respect to apoB synthesis or secretion in primary hepatocytes. The main difference between the previously published hamster study and the current one is the choice of agonist. Whereas Bilz et al used the endogenous agonist CDCA, the current study employed the synthetic agonist GW4064. This has not been previously used in hamsters and may not activate the nuclear receptor in the same way. Indeed, there are two specific residue differences in the ligand binding pocket of FXR between mice and humans that affects the affinity of the pocket for bile acids (Cui et al. 2002). These residue changes in fact increase the affinity of FXR for its ligand in humans compared to in mice, but this increased affinity may not extend to synthetic ligands. Hamster FXR shares its sequence with that of humans, particularly in the crucial binding pocket residues. This alteration in protein sequence may affect not only the affinity for the ligand, but may also change the affinity of FXR for its diverse targets once activated (Zhang et al. 2003).
The decreased hepatic FAS mass seen after FXR activation is also not congruent with the previous study showing that FXR activates transcription of Fas. However, Matsukuma et al did not measure corresponding FAS protein levels after FXR activation (Matsukuma et al. 2006). This leaves room for the possibility that post-transcriptional and/or post-translational regulation may play a role in altering hepatic FAS levels.

The effects of FXR activation in the hamster model are therefore not as dramatic as had been expected. It is possible that with future experiments the intricacies of FXR activation can be uncovered.

The synthetic FXR ligand GW4064 is not available commercially, making it difficult to continue large-scale or long-term studies in the hamster. For this reason, as well as the lack of change in lipid metabolism in the pilot experiment, this avenue of experimentation was not pursued further.

5.6 Future Directions

Experiments to examine the effect of FXR activation in the hamster model may be expanded in the future. Firstly, the ability of the synthetic FXR agonist to activate hamster FXR in vivo must be determined with more certainty. This could be ascertained by measuring the hepatic mRNA levels of known FXR targets after GW4064 treatment. To further confirm GW4064’s effects, electrophoretic mobility shift assays (EMSAs) could be performed to determine whether FXR indeed binds to its target promoters upon ligand treatment. If GW4064 proves to be ineffective in the hamster model, the physiological ligand CDCA could instead be used, as that is known to reduce dyslipidemia in hamsters (Bilz et al. 2006).

Once the efficacy of the ligand has been confirmed, further experiments could be designed to test the effect of FXR activation both in the context of fat/fructose diet feeding, and over a longer time period. Results from these experiments would yield insight both into the interaction between FXR activation and dietary stimulation, and any changes in effects over time.
References


Macrophage Accumulation and Atherosclerosis in Obese LDL Receptor-Deficient Mice. *Arterioscler Thromb Vasc Biol.*


