Effect of NAFLD on Regulation of Hepatic Transporters and Metabolizing Enzymes Using a High Fat/High Cholesterol Dietary Model in Rat

By

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A thesis submitted in conformity with the requirement for the degree of Master of Sciences

Graduate Department of Pharmaceutical Sciences

University of Toronto

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Master of Sciences, 2012

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University of Toronto

Abstract

Non-alcoholic fatty liver disease is affecting an increasing population worldwide. NAFLD is closely associated with obesity and diabetes. Research has shown that the expression of some important hepatic transporters and enzymes are altered under inflammatory conditions. We examined the effect of NAFLD on the gene expression of several hepatic transporters and enzymes, as well as the impact of exercise in attenuating the effect of NAFLD.

We have demonstrated that the mRNA expression of several hepatic transporters and enzymes, as well as FXR were significantly downregulated in liver of rats treated with a HFHCD. We concluded that HFHCD-induced hepatic steatosis, together with the reduced expression of FXR, contributed to the downregulation of expression of hepatic transporters and enzymes. The mRNA expression of TNF-\(\alpha\) and IL-1\(\beta\) were unaffected. Interestingly, exercise was found to improve the expression levels of some transporters and enzymes.
Acknowledgements

First, I would like to extend my gratitude to my supervisor, Dr. Michileline Piquette-Miller who gave the opportunity and guidance for my graduate studies. For a student being out of school for more than ten years, it would not have been possible to complete my master education in the department of Pharmaceutical Sciences without her support and assistance. I would like to thank Dr. Lavoie and his research team who did tremendous animal work and provided valuable information for this project. I am also thankful to have Dr. Scott Walker, Dr. Shinya Ito and Dr. Peter O’Brien to participate in my advisory committee.

Secondly, I would like to express my thanks and appreciation to those I had been working with over the last two years. You have made my graduate experiences memorable and enriched. In particular, my great thanks go to my lab fellows Greg Anger, Raquel De Souza1, Vanja Petrovic and Ji Zhang, for their technical assistance, continuous support, encouragement and friendship.

Last but not least, my heartfelt thanks go to my dearest husband, who stood by me even when I felt lost and did not believe in myself. Without him, I would have given up during the course of my studies. His love, encouragement and being positive are my strength to go through all the up-and-down moments during my journey of graduate studies. Thank you.
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<tr>
<td>ABC transporter</td>
<td>ATP-binding cassette transporter</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>Angiotensin-converting enzyme inhibitor</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>APP</td>
<td>Acute phase protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BSEP/Bsep</td>
<td>Bile salt export pump</td>
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<td>BCRP/Bcrp</td>
<td>Breast cancer resistance protein</td>
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<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate regulatory element-binding protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive protein</td>
</tr>
<tr>
<td>CYP/Cyp</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HEP G2</td>
<td>Hepatocellular carcinoma G2</td>
</tr>
<tr>
<td>HF</td>
<td>High fat</td>
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<tr>
<td>HFHCD</td>
<td>High fat/high cholesterol diet</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HMG-CoA reductase</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>HNF 4α</td>
<td>Hepatic nuclear factor 4α</td>
</tr>
<tr>
<td>HNF 1α</td>
<td>Hepatic nuclear factor 1α</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic stellate cell</td>
</tr>
<tr>
<td>HVB</td>
<td>Hepatitis virus B</td>
</tr>
<tr>
<td>HVC</td>
<td>Hepatitis virus C</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Inter-cellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-Y</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>JNK-1</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MCD</td>
<td>Methionine-choline deficient</td>
</tr>
<tr>
<td>MDR/Mdr</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRP/Mrp</td>
<td>Multidrug resistance-associated 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>NF-kappaB</td>
<td>Nuclear factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NTCP/Ntcp</td>
<td>Na+–taurocholate cotransporting polypeptide</td>
</tr>
<tr>
<td>OATP/Oatp</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>Ob/ob mice</td>
<td>Obese mutant mice</td>
</tr>
<tr>
<td>PCN</td>
<td>Pregnenolone-16 alpha-carbonitrile</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfloride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHP</td>
<td>Short herterodimer partner</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered-saline with Tween</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>Vd</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
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1. Introduction

1.1. Non-alcoholic Fatty Liver Disease

The increasing population of obesity worldwide has become a health concern, as obesity is associated with a cluster of co-morbidities such as hepatic steatosis, type II diabetes, and cardiovascular disease. According to the World Health Organization (WHO), the prevalence rate of obesity (BMI > 30) has been doubled since 1980 and nearly 500 million adults were obese worldwide in 2008. Non-alcoholic fatty liver disease (NAFLD) is closely related with obesity. It accounted for 46.8% of chronic liver disease cases in 1988 and increased to 75.1% in 2008 along with the rapidly increasing prevalence of obesity in the United States (Younossi et al., 2011).

NAFLD refers to any fatty liver disease that is not caused by excessive alcohol use. NAFLD probably results from abnormal hepatic lipid metabolism and insulin resistance. It is a component of metabolic syndrome, which features visceral obesity, dyslipidemia, insulin resistance and atherosclerosis (Kong, Luyendyk, Tawfik, & Guo, 2009). NAFLD encompasses a spectrum of symptoms ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH), which can ultimately lead to cirrhosis and hepatocellular carcinoma (HCC).

Hepatic steatosis, also known as fatty liver, refers to the accumulation of triglyceride fat and subsequent formation of lipid droplets in the cytoplasm of hepatocytes, resulting in liver enlargement (Anderson & Borlak, 2008). Steatosis alone is considered a relatively benign and reversible condition. The transition from steatosis toward NASH represents a key step in pathogenesis, because it will cause liver damage including fibrosis, cirrhosis and HCC (Wouters et al., 2008). At the beginning, it was thought that the fat accumulation alone in the liver could lead to the development of NASH, because the large quantity of fat in the liver is the source of
peroxidation. However, a growing body of work in animal models suggests a two-hit hypothesis, whereby the first hit is steatosis and the second hit is the oxidative stress resulting from lipid peroxidation and inflammation (Day & James, 1998; Day, 2002; Leclercq et al., 2000; Tilg & Diehl, 2000).

NASH represents an extreme stage of NAFLD, characterized by inflammation, cell damage with concurrent fat infiltration in the liver. Patients with NASH have no or few symptoms. Currently a biopsy of the liver is the only test that is widely accepted to distinguish NASH from other chronic liver diseases and can be used to assess the severity of the inflammation and resultant fibrosis (Adams & Angulo, 2006). The general histopathologic criteria for NASH diagnosis are macrosteatosis, lobular inflammation, and hepatocyte ballooning. It is estimated that NASH and NAFLD affect 5.7 to 17% and 17 to 33% of American adults, respectively (Lickteig et al., 2007). This indicates that more than one third of the American patients with NAFLD are at the NASH stage. Therefore, understanding the progression of the disease and seeking potential therapeutic agents that inhibit the progression are of emerging importance.

Although the pathogenesis of NAFLD is not fully understood, it is believed that NAFLD is closely linked to obesity and insulin resistance. The prevalence of steatosis in patients with obesity is about 75% and nearly 35% develop NASH (Adams et al., 2005; Anderson & Borlak, 2008; Anderson & Borlak, 2008). This indicates that obesity is one key risk factor in development of NAFLD and NASH. Insulin resistance promotes lipolysis of peripheral adipose tissue and de novo triglyceride synthesis with resultant increased fat accumulation in the liver (Adams & Angulo, 2006). Studies have indicated a tight inverse correlation between the intrahepatic triglyceride and insulin sensitivity measured by whole body glucose disposal (Hwang et al., 2007; Korenblat, Fabbrini, Mohammed, & Klein, 2008). Obesity and diabetes also
exacerbate liver damage in patients with hepatitis. One study found that the risk of HCC increased by more than 100 fold in hepatitis virus B (HVB) and hepatitis virus C (HVC) carriers with obesity and diabetes, indicating the promoting effect of metabolic syndrome on HCC in patients with hepatitis (Chen et al., 2008).

The modern lifestyle including high calorie intake and relatively low activity promotes obesity and other metabolic syndromes such as fatty liver. In Asian and Western countries, over-nutrition is the most common risk factor causing NAFLD, with an estimated incidence of 15% to 20% of the population (Anderson & Borlak, 2008; Bedogni et al., 2005; Zhou et al., 2007). Thereby healthy eating and moderate exercise could be beneficial for patients with NAFLD.
1.2. Influence of Obesity and other Metabolic Diseases on Drug Pharmacokinetics

It has been reported from studies that the disposition of some drugs can be changed under the influence of metabolic disorders such as obesity and fatty liver disease. For example, a study of the disposition process of acetaminophen in rats with NASH induced by methionine-choline deficient (MCD) diet resulted in a decreased biliary excretion of sulphate, glucuronide and glutathion metabolites. (Lickteig et al., 2007). Jang et al. showed that the hepatic uptake of metformin was significantly higher in obese mice fed a high fat diet compared with lean mice (Jang, Kim, Park, & Kang, 2010). Alteration of drug disposition was also found in obese humans. For example, the clearance of chlorzoxazone was elevated approximately three folds in morbidly obese patients compared with healthy volunteers (Emery et al., 2003). The altered drug responses in patients with metabolic diseases raise a drug safety concern as it can lead to either toxicity or decreased therapeutic efficacy.

Influence by Physiological Changes

The fate of a drug in human body relies on four pharmacokinetic properties: absorption, distribution, metabolism and elimination. Obesity and other metabolic disorders dramatically affect the body’s physiologies and consequently influence the pharmacokinetics of many drugs.

Obesity results in increased adipose tissue mass, organ mass and blood volume, which can influence the volume of distribution (Vd) of lipophilic and hydrophilic drugs, leading to sub- or supra-therapeutic concentration. In fact, dosing adjustment in obesity has been established in several commonly used antibiotic drugs such as gentamicin and amikacin to account for the larger Vd in obese patients. (Corcoran, Salazar, & Schentag, 1988; Leader, Tsubaki, & Chandler, 1994).
not only is affected by the amount of adipose tissue, but also by the drug plasma protein binding and cardiac output. Hyperlipidemia is frequently found in obese and diabetic patients with altered concentration of alpha-1-acid glycoprotein and lipoproteins (Benedek et al., 1983; Hanley, Abernethy, & Greenblatt, 2010). Recently a study by our group found decreased plasma protein binding of glyburide and saquinavir in diabetic pregnant rats resulting from hyperlipidemia (G. J. Anger & Piquette-Miller, 2010). Amiodarone, a lipophilic antiarrhythmic agent, displayed significant pharmacokinetic alteration in hyperlipidemic rats than in normal rats after feeding a high fat meal. The plasma AUC increased by 1.83 fold and the clearance decreased by 1.5 fold in the hyperlipidemic rats compared with the normal rats. The author proposed that the increased level of lipoprotein in plasma, resulting from hyperlipidemia, contributed to the altered pharmacokinetics of Amiodarone (Shayeganpour, Jun, & Brocks, 2005).

The excessive fluid in adipose tissue might have repercussion in obese individuals with heart failure if this extra volume is re-distributed into the circulation. (Poirier et al., 2006). However, although in general the $V_d$ of drugs is increased in obese individuals, adipose tissue blood flow is reduced. These physiological changes might have potential impact on the distribution of drugs and complicate dosing adjustment in the obese patients.

Altered drug clearance is observed in obese and diabetic patients. The major organs for drug clearance are the liver and the kidney. Obesity is associated with fatty liver, in which the fat accumulation can alter hepatic blood flow rate and might impact the hepatic drug clearance (Hanley et al., 2010). A study of acetaminophen disposition in obese men and women showed that the clearance of the drug increased with body weight and therefore is much greater in obese patients and in men (Abernethy, Divoll, Greenblatt, & Ameer, 1982). Similarly, the clearance of ciprofloxacin is significantly increased by 21% in obese individuals, and the renal clearance
was 29% higher than that of the controls. The higher clearance of ciprofloxacin in the absence of change of half time ($t_{1/2}$) indicated that the larger $V_{ss}$ ($V_d$ at steady state) of the drug in obese patients was the main determinant for the changed ciprofloxacin clearance (Cheymol, 2000).

**Influence by Altered Activity of Metabolic Enzymes and Hepatic Transporters**

The liver is the critical organ for drug metabolism and metabolic enzymes, especially cytochrome p450 enzymes, which are most abundant in this organ. The cytochrome p450 enzyme family is one large and diverse superfamily of metabolic enzymes, which function to facilitate the biotransformation of drugs by addition of functional groups suitable for conjugation and ultimate elimination from the human body (Danielson, 2002).

The biotransformation of drugs may be altered in obesity or other metabolic diseases as a result of chronic liver diseases such as fatty infiltration, cirrhosis etc. Obese individuals with NASH exhibited higher CYP2E1 activity than those without NASH, as evidenced by increased clearance of chlorzoxazone, an in vivo probe for CYP2E1 (Emery et al., 2003). Fisher et al. further demonstrated the altered functional activity of some P450 enzymes toward their specific substrates along with the progressive stages of NAFLD in humans. With the progression of NAFLD, CYP1A2 and CYP2D6 displayed significant decrease in their enzymatic activity. In contrast, the enzymatic activity of CYP2A6 and CYP2C9 is increased with NAFLD severity (Fisher, Lickteig, Augustine, Ranger-Moore et al., 2009).

Drug transporters are a group of transporting proteins that bind to drugs to facilitate the uptake and excretion of drugs and metabolites between the organs and the circulating blood (See section 1.3). Changes in the activity of transporters under metabolic disease states may also affect the pharmacokinetics of drugs. (Q. Cheng et al., 2008; Fisher, Lickteig, Augustine, Oude Elferink et
al., 2009; Lickteig et al., 2007). For example, the dietary-induced NAFLD in rats elicited a striking increase in the activities of several MRP efflux transporters (i.e. Mrp3 and Mrp4), resulting in a corresponding increase of acetaminophen metabolites in the plasma and a shift in the elimination route of acetaminophen metabolites from bile to urine (Lickteig et al., 2007). A study of gestational diabetes in rats indicates that the increased Mdr1 expression partially contributes to the lower hepatic lopinavir exposure (G. Anger & Piquette-Miller, 2011).

However, to date, information regarding the influences of metabolic diseases on the expression and activity of drug transporters and its consequent impact on drug pharmacokinetics is limited.

Since patients with metabolic diseases are often coping with co-existing conditions that require administration of multiple prescription drugs, the understanding of the disease complication and how does it affect the activities of drug transporters and metabolic enzymes are of particular importance. The majority of drugs is metabolized and eliminated through the liver and NAFLD is one common metabolic syndrome occurred in obese or diabetic patients. Therefore, we have focused our study on the influence of NAFLD on the expression and activities of some hepatic transporters and metabolic enzymes, which play pivotal roles in the pharmacokinetics of drugs.
1.3. **Overview of Hepatic Transporters**

The liver is a crucial organ for the metabolism and excretion of many important drugs and their metabolites. Hepatocytes, the predominant cell type in the liver, are polarized cells with two distinct membrane domains: basolateral and apical. Hepatic transporters are a group of membrane proteins that are located in the basolateral or apical domains of hepatocytes, facilitating the uptake and excretion of xenobiotics and endogenous substances between the basolateral membrane and the sinusoidal blood (basolateral), or pumping the xenobiotics and metabolites from the liver into the bile canaliculus for elimination (apical) (See Figure 1).

![Figure 1: Schematic illustration of Hepatic Transporter Localization Domains: Apical and Basolateral](image-url)
**Hepatic Efflux Transporters**

The excretion of xenobiotics from the liver may occur across either the canalicular membrane into the bile, or the basolateral membrane into the blood. Hepatic efflux transporters belong to the ATP-binding cassette (ABC) transporter family. The ABC transporters utilize the energy of ATP hydrolysis to carry various substances across the cellular membranes.


**P-glycoprotein** (MDR1, \textit{ABCB1}) is one of the most widely studied ABC transporters with a 170kda molecular size, encoded by the multidrug resistance 1 (MDR1) in human and Mdr1a/Mdr1b in rodents. It was first identified in multidrug resistance tumor cells (Juliano & Ling, 1976). The protein comprises two membrane bound domains, each made up of six transmembrane helices and a nucleotide binding domain which binds and hydrolyzes ATP (Sharom, 1997). The unusual flexibility of the binding domains of P-gp has made it a transporting protein for an extensively diverse array of substrates including anticancer agents (e.g. paclitaxel, doxorubicin), steroids, cardiac glycoside (e.g. digoxin), HIV protease inhibitors (e.g. saquinavir, indinavir), lipids (Orlowski, Martin, & Escargueil, 2006; Oza, 2002; Park & Sinko, 2005). The protein is found at the apical surface of epithelial cells of tissues with excretory roles such as the bile duct. In the liver, P-gp is the key transporter responsible for the biliary excretion of bulky hydrophobic and cationic substrates in order to protect the liver from
xenobiotic toxicity. Besides the protective role, over-expression of P-gp on tumor cell surface is one cause of multidrug resistance (Oza, 2002). Much evidence indicate that P-gp (MDR1/Mdr1a/Mdr1a) is regulated mainly by PXR in human and rodents (Owen, Chandler, Back, & Khoo, 2004; Teng & Piquette-Miller, 2005a). Recent studies demonstrate that CAR is also involved in the regulation of P-gp. For example, MDR1 expression in human hepatocyte carcinoma HepG2 cells was induced via activation of CAR by valproic acid (Cerveny et al., 2007).

**MRP2/Mrp2** (*ABCC2*) is a xenobiotic efflux pump belonging to the multidrug resistance associated protein (MRP) family. The 190kda protein locates exclusively at the apical membrane domains of polarized cells, such as hepatocytes. MRP2 plays an important role for the excretion and detoxification of endogenous and xenobiotic organic anions, particularly in the efflux of substances conjugated with glutathione, glucuronate, or sulphate. Mrp2 plays a role in the detoxification of bilirubin glucuronosides and some steroid sulphates (Nies & Keppler, 2007). MRP2 is a key transporter in the biliary excretion of numerous drugs including sulfopyrazone, indomethacin, penicillin, and methotrexate (You & Morris, 2007). Mutations in the MRP2 gene has been observed in patients with Dubin-Johnson Syndrome, an inherited liver disorder characterized by conjugated hyperbilirubinemia (Kanda et al., 2009). Reduced Mrp2 expression was found in rats with intrahepatic and obstructive cholestasis (Trauner et al., 1997). For example, endotoxin-induced cholestasis by LPS down-regulates Mrp2 mRNA in rats (Kubitz, Wettstein, Warskulat, & Haussinger, 1999). Mrp2 expression in rats can be regulated by PXR, CAR and FXR via induction by their respective ligands occurred on a common binding site in the proximal promoter of Mrp2 gene (Kast et al., 2002).
**BSEP/Bsep** (ABCB11) represents the major bile salt efflux transporter that is exclusively expressed on the canalicular membrane of hepatocytes. The human BSEP protein is highly similar with its rat and mouse orthologs with similar transporting properties (Noe, Stieger, & Meier, 2002). BSEP has high a affinity for conjugated bile acids and mainly mediates the excretion of monovalent conjugated bile acids from liver into bile canaliculi. The rank order of BSEP affinity to conjugated bile acids is taurochenodeoxycholate (TCDCA) > taurocholate (TCA) > taurodeoxycholate (TDCA) > glycocholate (Kosters & Karpen, 2008; Noe et al., 2002). Defective expression or function of BSEP is associated with cholestasis. Mutations in the BSEP gene (ABCB11) results in progressive familial intrahepatic cholestasis type 2 (PFIC2), a chronic cholestatic disorder that begins in infancy (Jansen et al., 1999; Strautnieks et al., 1998). BSEP induction is mediated via interaction with FXR/RXR heterodimer, in response to bile acids (Zollner, Marschall, Wagner, & Trauner, 2006). FXR null mice exhibit a much lower level of Bsep compared with wild type mice when feeding with a control diet. Cholic acid feeding substantially increases Bsep mRNA level in the livers of wild type mice but the Bsep mRNA level remains unchanged in the livers of the FXR null mice, implicating that FXR is a crucial activator of Bsep (Sinal et al., 2000). Recent studies implicate that PXR can be a potential regulator of BSEP. Spironolactone, a ligand of PXR, has been shown to induce Bsep expression, resulting in an increase of bile flow. Bsep expression levels were lower in PXR-/- mice compared to wild-type mice and spironolactone induction of Bsep expression was absent, indicating the involvement of PXR (X. Cheng, Buckley, & Klaassen, 2007; Kosters & Karpen, 2008).

**MDR3** (ABCB4) is an ABC transporter that involves in the biliary secretion of phospholipids and choliephilic compounds in the liver. Phospholipids are essential for the formation of micelles.
that carry cholesterol and solubilize bile salts in the bile ducts. Excessive cholesterol crystal deposits lead to intrahepatic cholestasis and bile duct inflammation and often leading to gallstone formation. Mutation of MDR3 gene (*ABCB4*) causes low phospholipid-associated cholelithiasis (LPAC), which is characterized by symptomatic and recurring gallstone (Rosmorduc & Poupon, 2007). The human MDR3 and MDR1 gene sequences are highly homologous, suggesting that MDR3 may share similar drug substrate specificity to Pgp as well (van der Bliek, Kooiman, Schneider, & Borst, 1988).

**MRP3/Mrp3** (*ABCC3*) is another important ABC transporter belonging to the MRP family. MRP3 is localized in the basolateral membrane of hepatocytes. Rat Mrp3 shares substantial structural similarity with Mrp2 and Mrp1 (Ortiz et al., 1999). Similar to MRP2, it mainly exports endogenous conjugated substances, particularly glucuronide conjugates, from the liver to the circulating blood. MRP3 and MRP2 have overlapping substrate specificities. In fact, hepatic MRP3 expression is often induced as an adaptive change when the biliary excretory function of MRP2 is impaired in order to protect the liver from over-toxicity. For instance, hepatic expression of Mrp3 is upregulated in obstructive cholestasis in rats with impaired Mrp2 canalicular function (Donner & Keppler, 2001).

**Hepatic Uptake Transporters**

The hepatic uptake of many drugs relies on the function of transporters and sometimes it could be the rate-determining step for the drug elimination process. The transport proteins of the solute carrier family (SLC) are the predominant transporters responsible for the uptake of numerous xenobiotics including drugs from the sinusoidal blood to the liver. Important uptake transporters
are the Na\(^+\)-taurocholate cotransporting polypeptide (NTCP, \textit{SLC10A1}) and the organic anion transporting polypeptides (OATPs, \textit{SLCO}).

**NTCP/Ntcp (\textit{SLC10A1})** is the principal bile salt uptake transporter expressed exclusively in liver. The NTCP-mediated bile salt uptake is sodium-dependent and it accounts for more than 80% of the hepatic conjugated taurocholate uptake (Trauner & Boyer, 2003). During cholestasis, NTCP expression is downregulated in order to prevent the accumulation of bile acid in the liver. In rodent, the repression of Ntcp is believed to be regulated through the FXR-SHP pathway (Zollner et al., 2005). FXR, activated by bile acid, induces the expression of SHP, which in turn represses the Ntcp expression by inhibiting transactivation of heterodimer complex formed by retinoid X receptor \(\alpha\) (RXR\(\alpha\):RXR\(\alpha\)).

**OATPs/Oatps (\textit{SLC21})** Transporters of the OATP family play an essential role in the hepatic uptake of both drugs and other endogenous substances. OATPs have a very broad substrate specificity ranging from organic anions, bulky cations and neutral steroids. Unlike NTCP, OATPs mediate the uptake of substances across the sinusoidal membrane through a sodium independent manner. Out of the 11 OATP transporters in human, OATP1B1, OATP1B3 and OATP2B1 are expressed on the sinusoidal membrane of hepatocytes and can facilitate the liver uptake of their substrate (Kalliokoski & Niemi, 2009). In human OATP1B1 (\textit{SLCO1A4}) is clearly the most important uptake transporter mediating the absorption of a variety of drugs. OATP1B1 and Oatp1a4 are regulated by PXR and binding sites have been identified in the promoters of OATP1B1 and Oatp1a4 genes of human and rats (Frank et al., 2005) Activation of PXR by pregnenolone-16alpha-carbonitrile (PCN) significantly induces the expression of Oatp1a4 in rat (Guo, Staudinger, Ogura, & Klaassen, 2002). Drug substrates of OATP1B1 include the HMG-CoA reductase inhibitors such as atorvastatin and pravastatin, angiotensin-
converting enzyme (ACE) inhibitors such as enalapril and angiotensin II receptor antagonists such as olmesartan. Besides clinical drugs, OATP1B1 also transports endogenous substances such as bile acids, conjugated steroids, and thyroid hormones (Kalliokoski & Niemi, 2009). OATP1B1 expression is repressed in patients with cholestatic liver diseases probably through a bile acid-mediated pathway involving FXR, SHP, HNF4α and HNF1α (Zollner et al., 2006). On the other hand, treatment of chenodeoxycholic acid (CDCA) increases OATP1B3 gene expression in human hepatocyte cells via FXR regulation, suggesting a physiologic role of OATP1B3 to maintain hepatic uptake of xenobiotics and endogenous substances for metabolic elimination even during cholestasis (Jung et al., 2002; Zollner et al., 2006).
**1.4. Chronic Inflammation and NASH**

Inflammation is the body’s defence response that occurs in reaction of injuries such as tissue damage or infection, with the attempt to remove the harmful injurious stimuli and initiate the healing process. Inflammation can be classified as acute inflammation or chronic inflammation based on its onset, persistence and histopathology (Ho & Piquette-Miller, 2006; Teng & Piquette-Miller, 2008). Acute inflammation is the short-term response of the body to harmful stimuli, resulting in the healing process under normal circumstances. However, if the cause of the inflammation persists or if the healing process fails, the inflammation becomes chronic. Under chronic inflammatory condition, the balance between pro-inflammatory and anti-inflammatory mediators is compromised. Cells of the immune system liberate reactive oxygen species, chemokines, cytokines, and other small molecules that maintain and intensify the inflammatory response, resulting in consistent tissue degradation and regeneration (Vassileva & Piquette-Miller, 2010).

As mentioned in section 1.1, NAFLD is associated with chronic hepatic inflammation (NASH), which exacerbates the liver injury and affects the normal physiological function of the organ. Studies of patients with NASH show significant elevation of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) compared with healthy volunteers (Bahcecioglu et al., 2005; Kugelmas, Hill, Vivian, Marsano, & McClain, 2003). Similar findings have been reported from experimental models of NAFLD in rodents (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Hebbard & George, 2011; Yu et al., 2006).
Recent studies in both human and rodent models strongly implicate that NF-kappaB seems to be the major determinant for hepatic inflammation in NASH and its progression to fibrosis. NF-kappaB is a family of dimeric transcription factors that regulate inflammation, innate and adaptive immunity, wound healing responses, and cell fate and function (Elsharkawy & Mann, 2007). Feeding of MCD in mice caused hepatic NF-kappaB activation and development of equivalent steatohepatitis in wild type, TNF -/- and TNF receptor (R)-1/- mice, suggesting that steatohepatitis is independent of TNF expression. (Dela Pena et al., 2005). Hepatocyte apoptosis, degree of hepatic inflammation and fibrosis are markedly increased in patients with NASH and correlate with active NF-kappaB expression (Ribeiro et al., 2004). Activation of NF-kappaB can recruit and activate other liver cells such as endothelial and Kupffer cells, which in turn trigger intercellular cascades to induce and maintain inflammation. Activated Kupffer cells and hepatic stellate cells (HSCs) contribute to additional cytokine production and oxidative stress (TNF-α, IL-6 and ROS) (Anderson & Borlak, 2008; Marra, 2008).

Besides the NF-kappaB activation, studies on NASH pathogenesis indicate other molecular pathways in promoting hepatic steatosis to inflammation. For example, cytokine-induced c-Jun N-terminal kinase 1 (JNK1) promotes the development of steatohepatitis in MCD diet fed mice as JNK1 null mice had significantly reduced levels of hepatic triglyceride accumulation, inflammation, lipid peroxidation, liver injury, and apoptosis compared with wild-type mice (Schattenberg et al., 2006). Overall, inflammation is a critical step in NASH development and cytokines are involved in the recruitment and activation of macrophages and transcription factors that regulate steatosis, inflammation and fibrosis in liver.

To date, a number of literatures have reported that acute inflammation is linked to alteration in drug transporter and metabolic enzyme expression. Morgan E.T. has demonstrated in the 90s
that infection or inflammation in patients caused variation in drug metabolism due to the suppression of P450 enzyme expression and activity resulted from inflammation (Morgan, 1997). Many of our previous studies, as well as others, showed the substantial downregulation of the expression and activity of ABC transporters mediated by acute inflammation or cytokines in human and rodents (Fardel & Le Vee, 2009; Lee & Piquette-Miller, 2001; Petrovic, Wang, & Piquette-Miller, 2008; Sukhai, Yong, Pak, & Piquette-Miller, 2001). For example, turpentine-induced acute inflammation in rats causes 50-70% decrease in the hepatic protein expression of P-gp with corresponding 45-65% decrease in the efflux of rhodamine 123 (Piquette-Miller, Pak, Kim, Anari, & Shahzamani, 1998). Significant decrease of P-gp protein expression is found in rat hepatocytes treated with IL-6 and IL-1β, suggesting the downregulation of P-gp expression is mediated by these pro-inflammatory cytokines (Sukhai et al., 2001). Administration of endotoxin to rodents imposed a significant downregulation of hepatic transporters. Most recently, studies in our group demonstrate that viral-induced inflammation caused downregulation of mRNA expression of several key hepatic transporters (Mrp2, Bcrp, Oatp1a4 and Ntcp) in polyinosinic/polycytidylic acid-treated rats (Petrovic & Piquette-Miller, 2010).

However, knowledge regarding the regulation of transporters and enzymes under the influence of low grade, chronic inflammation is limited and is of emerging importance as many prevalent metabolic diseases are associated with a state of chronic inflammation. Since NASH is a stage of severe hepatic inflammation and is associated with elevated levels of several proinflammatory cytokines (TNF-α, IL-6, and IL-8) in human patients, we hypothesized that NAFLD might elicit similar regulatory pattern on hepatic transporters and metabolic enzymes as that of acute inflammation.
Important cytokines induced by liver inflammation include TNF-α, IL-1 and IL-6. In endotoxin-induced liver injury in mice, TNF-α, as well as IL-1 are markedly induced and are the main mediators responsible for the upregulation of mRNA inter-cellular adhesion molecule 1 (ICAM-1), which stimulated inflammatory infiltration and fibrogenesis (Essani et al., 1995).
1.5. Overview of Nuclear Receptors: PXR and FXR

Nuclear hormone receptors are an abundant class of transcription factors found within cells of animal, in which the receptor regulate functions as diverse as reproduction, differentiation, development, metabolism, metamorphosis or homeostasis (Duarte, Perriere, Laudet, & Robinson-Rechavi, 2002). Nuclear receptors can be activated by their specific ligands and bind directly to DNA response element to regulate adjacent genes, resulting in upregulation or downregulation of gene expression.

Nuclear receptors control the gene transcription of transporters and enzymes either directly through ligand-activation or indirectly through a molecular pathway involving other transcription factors as well. Below is a brief introduction of several important nuclear receptors that have roles in the regulation of transporters and enzymes of our study.

**Pregnane X Receptor (PXR)**

PXR is an important member of the nuclear hormone receptor family. PXR is highly expressed in liver and intestine in both rodent and human, with primary function as transcription regulator to sense foreign toxic substances and in response upregulate expression of target genes for the detoxification and elimination process. PXR can be activated by a large number of endogenous substances or xenobiotics including steroids, bile acids, antibiotics, the herbal antidepressant St. John’s wort and many other drugs (Kliewer, Goodwin, & Willson, 2002).

Like other hormone nuclear receptors, PXR when activated, forms a heterodimer with the retinoid X receptor (RXR) and binds to the response elements on DNA to regulate target gene expression. Much evidence indicates that PXR is the key transcription regulator to induce
CYP3A4, the predominant oxidative enzyme that is responsible for metabolism of many drugs (Lehmann et al., 1998; Li & Chiang, 2006; Pascussi, Drocourt, Fabre, Maurel, & Vilarem, 2000). PXR is activated by compounds that regulate CYP3A4 in human and rats (e.g. rifampicin, PCN) and thus causes significant drug interaction (Lehmann et al., 1998). However, the effect is interspecies different.

PXR is shown to stimulate the expression of hepatic transporters that are involved in the detoxification and elimination of endogenous substances and xenobiotics from the body. For example, a previous study showed that PCN-activated PXR induced expression and activity of Mrp3 in mice treated with cholic acid, which was found to protect the liver from hepatic cholestatic injury (Teng & Piquette-Miller, 2007). A recent study in rats with gestational diabetes demonstrated that PXR-mediated upregulation of hepatic and placental Mdr1 activity partially contributed to the reduced accumulation of lopinvir (a HIV protease inhibitor) in the liver and fetus (G. Anger & Piquette-Miller, 2011).

PXR is also involved in the downregulation of several hepatic transporters during inflammation. IL-6 imposed significant decrease in the expression of Bsep, Mrp2 and Cyp3a11 in PXR+/+ mice but not in PXR−/− mice, signifying the involvement of PXR. Of note, PXR expression is suppressed by IL-6 administration although the mechanism is not known yet (Teng & Piquette-Miller, 2005b).

**Farnesoid X receptor (FXR)**

FXR, also known as the bile acid regulator (BAR), is a member of the nuclear receptor superfamily of ligand-activated transcription factors. FXR, encoded NR1H4, is highly expressed in liver and intestine. Similar to PXR, when activated, FXR translocates to cell nucleus, forms a
heterodimer with RXR and binds to hormone response elements on DNA, which up- or down-regulates the expression of target genes.

FXR is the central nuclear receptor in maintaining the bile acid homestasis in the enterohepatic circulating system. FXR plays a crucial role in all aspects of bile acid regulation. 1) Control of hepatic bile acid synthesis. Bile acids are the natural ligands for FXR. One important role of FXR is the suppression of bile acid synthesis in liver when the bile acid level is too high. Activated by bile acid, FXR controls the expression of CYP7A1, the rate-limiting enzyme in the conversion of cholesterol to bile acids, through a negative feedback pathway. FXR induces expression of SHP, which then inhibits the transcription of CYP7A1 gene, resulting in the reduction in CYP7A1 expression and bile acid production (Zollner et al., 2006). 2) Bile acid export and re-absorption. The uptake and excretion of bile acids in the liver is accomplished by the function of several hepatic transporters and metabolic enzymes that is directly or indirectly mediated by FXR. The biliary excretion of bile acid is essentially dependent on the expression of BSEP, an efflux transporter that is directly regulated by FXR (Zollner et al., 2003). ABC transporter MRP2 and MDR3, responsible for biliary excretion of divalent bile acid conjugates and phospholipids, are also upregulated by FXR (Huang et al., 2003; Kast et al., 2002). On the other hand, activated-FXR downregulates the expression of sinusoidal uptake transporter NTCP, through which 80% of the bile acids uptake occurs (Lefebvre, Cariou, Lien, Kuipers, & Staels, 2009). Like the suppression of CYP7A1, SHP may be involved in this regulation (Zollner et al., 2003).

FXR is also able to control triglyceride and cholesterol metabolism. Hence FXR has been identified as a molecular link between bile acids and lipid metabolism (Lefebvre et al., 2009). FXR activated by bile acid or synthetic agonist (GW4064) inhibits hepatic de novo lipogenesis
and VLDL production in mice through suppression of SREBP-1c via SHP induction (Sinal et al., 2000; Watanabe, Houten, Wang, Moschetta, Mangelsdorf, Heyman, Moore, & Auwerx, 2004b). This suggests a potential role of FXR activity in lowering hepatic triglyceride and VLDL production. On the other hand, FXR deficiency in mice results in fatty liver formation following a high cholesterol diet (Sinal et al., 2000). Moreover, the FXR-deficiency renders the mice more susceptible to NASH in a high-fat dietary model (Kong et al., 2009). Therefore, FXR is a crucial factor in lipid metabolism and disruption of the nuclear receptor can lead to the development of obesity, NALFD and other metabolic syndrome.
1.6. **Hepatic Cholestasis**

Cholestasis refers to the impairment of bile flow from the liver to the duodenum. Cholestasis can be classified into two types: intrahepatic cholestasis and extrahepatic cholestasis. Intrahepatic cholestasis is resulted from a functional defect at the level of the hepatocyte, whereas extrahepatic cholestasis is mainly due to obstruction at the bile duct level (Claudel, Zollner, Wagner, & Trauner, 2011).

Cholestasis is associated with chronic liver diseases such as inflammation and cirrhosis. During cholestasis, toxic bile acid is accumulated in liver causing hepatobiliary damage. In the liver, bile acid homeostasis is maintained by the function of bile acid transporters such as the Bsep, Mrp2 and Ntcp. It has been shown that the expression of several hepatic transporters is downregulated during cholestasis induced by inflammation. For example, the Mrp2 expression and activity have been dramatically reduced in rat models of cholestasis induced by endotoxin (Trauner et al., 1997).

FXR is the central regulator of bile homeostasis and lipid metabolism in the liver and intestine. FXR deficiency has been implicated in the development of cholestasis, NAFLD and NASH in rodent and human (Zhu, Li, & Guo, 2011). Cholestasis is common in patients with chronic liver disease such as NASH and can exacerbate liver injury.
1.7. **Lifestyle Factors and NAFLD**

NAFLD is a chronic metabolic disorder in liver that affects 30% of adults and the majority of obese individuals in the urban population of the United States and its prevalence is at an increasing trend (Browning et al., 2004). Due to its close association with obesity and type II diabetes, NAFLD has been considered the hepatic manifestation of the metabolic syndrome, together with hyperlipidemia, hypertension and insulin resistance. Unhealthy lifestyle such as excessive calorie intake and lack of physical activity are two common risk factors for NAFLD development. NASH patients showed high BMI, fat mass and visceral fat accumulation coupled with remarkably high energy intake and low resting metabolic rate (Capristo et al., 2005). Hence, weight loss strategies including exercise have been proposed in treatment of NAFLD.

Exercise is known to exert control of the progression of obesity and fatty liver diseases resulting from excessive calorie intake. Data from studies indicate that poor physical fitness and inactivity is associated with an increased prevalence of NAFLD (Centis, Marzocchi, Di Domizio, Ciaravella, & Marchesini, 2010; Church et al., 2006). There is an inverse association between physical fitness categories (eg, cardio-respiratory fitness) and NAFLD prevalence.

NAFLD is characterized by hepatic steatosis with increased insulin resistance (Kawaguchi et al., 2011). Physical activity has been shown to reduce intrahepatic fat content and improves liver function and insulin sensitivity (Perseghin et al., 2007; Thomas et al., 2006). Exercise stimulates lipid oxidation and suppression of free fatty acid uptake and lipid synthesis in liver (Hannukainen et al., 2007; Lavoie & Gauthier, 2006). Exercise is also beneficial to diabetic patients as it enhances insulin sensitivity through increase of muscle glucose uptake by upregulation of glucose receptor GLUT4 (Goodyear & Kahn, 1998).
Since exercise was beneficial in reversing or preventing some features of NAFLD (e.g., intrahepatic fat accumulation), we hypothesized that exercise might have an impact on the regulation of hepatic transporter and metabolic enzymes in NAFLD.
1.8. Experimental Models of NAFLD

To understand the mechanisms by which NAFLD and NASH develop, numerous clinical studies have been undertaken in patients. However, NAFLD can take decades to progress and the ethical concern around obtaining human liver tissue has been a challenge for the study in humans. In response, researchers have developed various animal models of NAFLD to mimic the pathology and metabolic dysfunction of NAFLD in human. However, so far none of the experimental models can perfectly replicate the pathology of NAFLD in human. Below is a brief description of some frequently used models for NAFLD.

As mentioned in 1.1, it has been suggested that the pathogenesis of NAFLD may require ‘two hits’ (See Figure 2). The ‘first hit’ is accumulation of intrahepatic lipid. Anstee et al. summarized that the intrahepatic lipid accumulation can occur through the following mechanisms: 1) Increased delivery and uptake into hepatocytes of long chain fatty acids (LCFAs) due to excess dietary intake or release of from adipose tissue stores. 2) Increased de novo hepatic LCFAs and TG synthesis through activation of SREBP-1c or ChREBP. 3) Failure of VLDL and TG export from liver. 4) Failure of elimination of excessive fatty acid caused by impaired hepatic mitochondrial β-oxidation (Anstee & Goldin, 2006). Most of the experimental models of NAFLD utilize some of these mechanisms to develop steatosis, through either nutritional manipulation or genetic modification.

The ‘second hit’ involves oxidative stress and inflammation in the liver. The cause of hepatic inflammation in NAFLD is not quite clear and could be multi-factorial. For example, the adipocytes monitor the energy storage levels and release proinflammatory cytokines to report over-nutrition to the rest of the body. These proinflammatory cytokines promote macrophages
localization in adipose tissue and trigger the inflammatory response (Baker, Hayden, & Ghosh, 2011). Unhealthy diet is a risk factor to elicit hepatic inflammation. Diets with high cholesterol have been shown to evoke hepatic inflammatory response, as well as atherosclerotic disease. Kleemann et al. demonstrated that high cholesterol induced inflammation in liver in mice through four key pathways (IFNγ, TNF-α, IL-1 and PDGF), which are also critical in the lesion development of atherosclerosis (Kleemann et al., 2007).

**Dietary-based Models**

The dietary-based animal models are among the most popular types of models for the study of NAFLD because over-nutrition or unhealthy eating style is the primary driving force for the disease in humans. The dietary-based models provide a source for fat accumulation and damage in liver that mimics the pathology of NAFLD. The most common types of diet in these models are the methionine and choline deficient (MCD) diet, high fat (HF) diet and the cholesterol and cholate enriched diet (Hebbard et al., 2011).

**Methionine-choline Deficient (MCD) Diet**

The MCD diet model is frequently used to produce more progressive liver pathology, resulting in the development of macrovesicular hepatic steatosis, lobular inflammation and fibrosis (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Leclercq et al., 2000). The MCD diet is usually high in fat and sucrose but lacks methionine and choline, which are essential for hepatic β-oxidation and formation of very low density lipoprotein (VLDL). The MCD model results in impaired VLDL synthesis and increase of intrahepatic fat accumulation due to impaired synthesis of phosphatidylcholine, leading to the development of steatosis and inflammation (Anstee & Goldin, 2006). The MCD-fed mice develop prominent steatosis and subsequent
necro-inflammation and fibrosis, which resembles those seen in human with NASH. MCD diet promotes inflammation and liver damage by ways of activated macrophages infiltration in the liver, NF-kappaB activation and concomitant increase of proinflammatory cytokines (IL-6, ICAM-1) and serum alanine aminotransferase (ALT) level (Dela Pena et al., 2005; Leclercq, Farrell, Sempoux, dela Pena, & Horsmans, 2004).

Although the MCD model induces some hepatic features that correlate with the pathology observed in humans with NAFLD, its replication of phenotype and mechanism of metabolic syndrome in human is in doubt. In contrast to human, mice with MCD-induced NASH exhibit weight loss, low plasma triglyceride and cholesterol level and lack of insulin resistance (Anstee & Goldin, 2006; Hebbard & George, 2011).

**High Fat Diet**

High fat (HF) dietary model is used in an attempt to imitate the Western style diets, which is the primary causative factor for development of obesity and NAFLD in North America.

In high fat diet (HFD), the majority of calorie intake is from fat or variations such as fructose or trans-fat. Animals fed a HFD develop features of NAFLD such as steatosis inflammation and oxidative stress. In contrast to MCD dietary model, in a rat model of HF diet (71% energy from fat, 11% energy from carbohydrate and 18% energy from protein), increased insulin levels and insulin resistance were found in rat fed with HFD (Lieber et al., 2004). However, the severity of hepatic steatosis and liver injury induced by HFD is relatively mild compared with that formed by a MCD diet (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Hebbard & George, 2011). HFD-induced livers of rats showed primarily microvesicular steatosis with mild lobular inflammation, while MCD livers showed marked diffuse macrovesicular hepatic steatosis with
mild lobular inflammation as well as early signs of bridging fibrosis. Therefore, it may take a longer time to develop NALFD, especially NASH in animals using this type of dietary model compared with the MCD dietary model.

**Cholesterol and Cholate Enriched Diet**

Cholesterol and cholate enriched diets are widely used to induce atherosclerosis in animals. Recent studies found that the diets produced not only atherogenic lipoprotein profile and vascular fatty streak lesion, but also induced NASH characteristics such as liver steatosis, inflammation, macrophages infiltration and fibrosis (Jeong et al., 2005). In mice, increased cholesterol levels are a critical factor for the pathogenesis of NASH as it sensitizes the liver to TNF and Fas-mediated steatohepatitis through mGSH depletion (Mari et al., 2006).

Matsuzawa et al. demonstrated that with atherogenic diet alone (1.25% cholesterol and 0.50% cholate %w/w), the mice developed features of NASH, including cellular ballooning, a critical histological feature defining human NASH, on a time dependent manner from 6 to 24 weeks (Matsuzawa et al., 2007). This is the first report that cellular ballooning is induced in livers of mice fed with atherogenic diets. Furthermore addition of a high fat component (60.0% fat %w/w) to the atherogenic diet accelerated the progression of cellular ballooning and caused hepatic insulin resistance and oxidative stress by the activation of HSCs. (Mari et al., 2006). The author concluded that overall the high fat atherogenic diet-induced steatohepatitis is a better experimental model of human NASH for the following reasons: 1) The model is more physiological dietary model of NASH as it does not require artificial manipulation in the diet such as depletion of methionine and choline (MCD diet). 2) The pathology involved hepatic
ballooning, a necessary feature defining human NASH; 3) The high fat component exacerbates the liver damage and causes hepatic insulin resistance.

**Genetic Models**

Besides the dietary models, researchers also developed genetic models to study pathogenesis and effects of NAFLD and NASH.

The ob/ob mouse is genetically deficient in leptin, an 16kDa adipokine produced by white adipose tissue to mediate the innate adaptive neuroendocrine response to starvation. Due to the lack of leptin, ob/ob mice exist in a state of perceived starvation, resulting in excessive food intake and obesity (Faggioni, Feingold, & Grunfeld, 2001). The development of hepatic steatosis by ob/ob mouse is a complex obesity-related process involving lipid and carbohydrate metabolism disorder. For example, ob/ob mice exhibit increase of fatty acid synthesis by activation and accumulation of SREBP-1c in ob/ob hepatocytes. However, unlike human NAFLD population, ob/ob mice do not spontaneously progress from steatosis to steatohepatitis. Thus the ob/ob mice need a ‘second hit’ such as administration of endotoxin (LPS) (S. Q. Yang, Lin, Lane, Clemens, & Diehl, 1997). Similar to the ob/ob mouse model, the db/db mouse model is another obesity model, wherein the leptin receptor activity is deficient. Researchers have also used other genetic models to study the pathogenesis of NAFLD in order to understand the roles of specific genes in fatty liver formation.
Figure 2: The two hit hypothesis for the pathogenesis of NAFLD: First hit – fat accumulation and Second hit – hepatic inflammation.
2. **Rationale and Experimental Justification**

2.1. **Background**

As mentioned in the introduction, numerous studies obtained from our group or other groups demonstrated a decreased expression and activity of several important hepatic transporters and metabolic enzymes in rodent or human under acute inflammatory conditions. We further demonstrated that this alteration in transporters and enzymes expression and activity is mediated by proinflammatory cytokines (Fardel & Le Vee, 2009; Lee & Piquette-Miller, 2001; Petrovic & Piquette-Miller, 2010; Piquette-Miller et al., 1998; Sukhai et al., 2001).

NAFLD is characterized by intrahepatic fat accumulation, hepatic inflammation and fibrosis. A ‘two hit’ hypothesis has been proposed to delineate the progression of NAFLFD from simple steatosis to steatohepatitis (NASH) (Day & James, 1998). NASH represents a severe stage of NAFLD, leading to liver cell damage, inflammation, progressive fibrosis and ultimately cirrhosis and hepatocellular carcinoma ((Reynaert, Geerts, & Henrion, 2005). NASH closely associated with obesity and chronic hepatic disease. Studies of patients with NASH show significant elevation of proinflammatory cytokines such as TNF-α, IL-6 and IL-8 compared with health volunteers (Bahcecioglu et al., 2005; Kugelmas et al., 2003). With our experiences of acute inflammation, we predicted that chronic inflammation such as NASH also elicited alteration in the expression and activities of hepatic transporters and metabolic enzymes. Indeed, a recent study of NASH induced by MCD dietary model in rats has indicated decreased hepatic uptake transporter expression (NTCP and OATPs) and significant retention of plasma bromosulfophthalien (BSP). On the other hand, the proinflammatory cytokine IL-1β is strongly expressed, suggesting a plausible downregulation mechanism of hepatic uptake transporters mediated by the cytokine (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009).
However, as discussed in the introduction, the MCD dietary model might not be an appropriate model for NAFLD as the lipid metabolism of MCD diet differs from that of human patients and the model does not replicate some features that are observed in NAFLD human patients such as weight gain and insulin resistance.

For the present study, a high fat atherogenic dietary model (high fat and high cholesterol) was developed and used to induce NAFLD in rats by the research group of Dr Lavoie. As mentioned earlier the atherogenic diet is more physiological and can generate features that match NASH pathology in human patients (eg, dyslipidemia, oxidative stress and cellular ballooning). The addition of a high fat component induces insulin resistance and downregulates genes of antioxidant enzymes and thus further aggravates NASH (Matsuzawa et al., 2007).

Exercise has been used as an alternative approach to treat NAFLD and related metabolic diseases other than medical therapies. Poor physical fitness or inactivity are associated with increased NAFLD prevalence in human population (Centis et al., 2010). Studies show that exercise exhibits beneficial effects on NAFLD such as reduction of intrahepatic fat accumulation, increase of insulin sensitivity and improved liver function (Hannukainen et al., 2007; Lavoie & Gauthier, 2006). We postulated that exercise might improve the dysregulation of hepatic transporters and metabolic enzymes caused by NAFLD in the current study.

Studies of the effect of NAFLD on the regulation of drug transporters and metabolic enzymes allows us to establish guideline in predicting potential alteration in drug pharmacokinetics due to the changed expression and activities of these transporters and enzymes caused by this epidemic disease.
2.2. Hypotheses

NAFLD can decrease in the expression of hepatic transporters and metabolic enzymes as a result of hepatic inflammation.

By exercise, the effect of NAFLD on transporters and enzymes will be diminished due to the reduction in hepatic fat accumulation and improved liver function.

2.3. Objectives

1) To evaluate the effect of NAFLD on the regulation of hepatic transporters and enzymes in liver using a high fat/high cholesterol dietary (HFHCD) model in rats.

2) To understand the molecular mechanisms involved in the regulation of hepatic transporters of NAFLD.

3) To see if exercise could be a factor in improving the dysregulation of the drug transporters and enzymes resulting from NAFLD.
3. Materials and Methods

3.1 Animal Treatment

*Note: Animal Work was conducted by the research group of Dr. Lavoie from the University of Montreal.*

Sprague-Dawley female rats (Charles River, St-Constant, PQ, Canada) were treated in a temperature controlled environment on a 12:12 dark night cycle with free access to water and food upon arrival. All animal experiments described in this report were conducted according to the directives of the Canadian Council on Animal Care after institutional approval.

For this study, the rats were fed with either a normal standard diet or a high fat, high cholesterol diet (HFHCD) for 7 weeks and stayed sedentary during the treatment (n=8 each group). The HFHCD contains 21% fat, 1.2% cholesterol and 0.5% cholic acid (% w/w). During the dietary treatment, a third group of rats (n=7) receiving HFHCD was submitted to an exercise training program, which consisted of continuous running on a rodent treadmill (Quinton Instruments, Seattle, WA) for 60 minutes, 5 times per week for the duration of the experiment.

After 7 weeks, all animals were sacrificed and liver tissues were harvested and stored at -80 degree until use.
3.2. **Determination of Hepatic Triglyceride and Cholesterol**

For extraction of hepatic triglyceride and cholesterol, approximately 100mg of frozen liver tissue (stored at -80°C) were weighed and thawed on ice. 4ml of chloroform/methanol (2:1 v/v) was added to each sample in a glass tube which was homogenized at 15000 rpm for 30 seconds. After adding 1ml of 50mM of NaCl, the homogenate was vortexed for 15 seconds following by centrifuging at 1500 g at 4°C for 30 minutes. The organic layer was removed to a new glass tube and washed with 1ml of 0.36M of CaCl2/Methanol (1:1v/v) by repeating the vortexing and centrifuging process. The final organic extract was placed in a 5ml volumetric flask and diluted to volume with chloroform (Carlson & Goldfarb, 1977).

For assay of triglyceride and cholesterol, 50µl or 25 µl of each sample (50µl for triglyceride and 25µl for cholesterol) was transferred to a new glass tube, which contained 10µl of 50% Triton-X 100 solution in chloroform. The samples were vortexed and dried under nitrogen. Standard solutions of triglyceride and cholesterol were prepared in the same way as samples. The samples and standards were re-constituted in the enzymatic assay reagents (Infinity Liquid Stable Reagent, Thermo Fisher Scientific). The triglyceride and cholesterol assays were performed following the instruction of an enzymatic assay kit (Thermo Fisher Scientific, VA, USA). In brief, triglyceride and cholesterol in samples were converted to their respective derivatives by reactions with enzymatic reagents and then measured by spectroscopy at 500nm against their standards.

**Reagents Used**

Chloroform, Methanol: Caledon, Canada; NaCl: Bioshop, Canada; CaCl2, Triton X: Sigma, USA
### 3.3. Total RNA Isolation and PCR Analysis

Total crude RNA was extracted from thawed liver tissue using the TRIZOL method (Invitrogen, Burlington, ON, Canada). Single-stranded complementary DNA (cDNA) was synthesized from 2µg of RNA according to the Reverse Transcription Kit (Applied Biosystems, Fosterity, CA). The mRNA levels of the targeted genes were determined by quantitative real time polymerase chain reaction (qRT-PCR). The amplification of the cDNA product (20ng or 40ng) was detected via reaction with SYBR Green fluorescence reagent (Roche Diagnostics, IN, USA) by the Roche Light Cycler (Roche, Laval, QC, Canada). The relative quantitation of the target genes was determined by the efficiency corrected ∆CT method (Bookout, Cummins, Mangelsdorf, Pesola, & Kramer, 2006). The mRNA level of the target gene was normalized to that of reference gene - 18S. The PCR primers were obtained from the DNA Synthesis Centre (Sick Kid Hospital, Toronto). The sequences of the primers are listed in Table 1.

**Table 1: Oligonucleotide primers used for qRT-PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcb1a(Mdr1a)</td>
<td>5’-GACGGAATTGATAATGTGGACA-3’</td>
<td>5’-AAGGATCAGGAACAAATAA-3’</td>
</tr>
<tr>
<td>Abcc2 (Mrp2)</td>
<td>5’-GTCACCGCTTCTTCTG-3’</td>
<td>5’-AACCCCAAACCTGCTA-3’</td>
</tr>
<tr>
<td>Abcb11 (Bsep)</td>
<td>5’-GCCATTGTGCAGATCCTAAA-3’</td>
<td>5’-TGCAAGGCTCCACCTCTCT-3’</td>
</tr>
<tr>
<td>Slco1a4(Oatp1a4)</td>
<td>5’-TTGCTTGTTGGATGTGAGTT-3’</td>
<td>5’-GCCAATGTCATTCTGTTT-3’</td>
</tr>
<tr>
<td>Slc10a1(Ntcp)</td>
<td>5’-CGTGGCCGGAATGTTT GTCT-3’</td>
<td>5’-TGCCCTTCTGTCTCAGTTCATG-3’</td>
</tr>
<tr>
<td>Cyp3a2</td>
<td>5’-GATTCTAAGCATAAGCACCAGATGT-3’</td>
<td>5’-ACAGGGCTTTTATGACACTTCTCTTT-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-CACCTCTCAACAGCGACAGA-3’</td>
<td>5’-GGTTCCATGTTGAAGCT-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-AAATGGGCTCCCTCTCAGTTGC-3’</td>
<td>5’-TCTGCTTGGTGGTCTACGAC-3’</td>
</tr>
<tr>
<td>PXR</td>
<td>5’-GCTCTCTGCGAGCAGGCTA-3’</td>
<td>5’-GTACCCAGCGAGGGCAGCT-3’</td>
</tr>
<tr>
<td>FXR</td>
<td>5’-CCGCACTCGAGGCCATGTTCC-3’</td>
<td>5’-TCATCGGAGATGCGCCACTTTG-3’</td>
</tr>
<tr>
<td>18S</td>
<td>5’-GTCTGTTGATGCCCTTAGAT-3’</td>
<td>5’-AGCTTATGACCCGACTTAC-3’</td>
</tr>
</tbody>
</table>
3.4. Western Blot Analysis

**Protein Extraction** - Total crude membrane protein was isolated from 300 mg of liver tissue in 4ml of lysis buffer (1X RIPA buffer [Cell Signalling Technology], P8340 protease inhibitors [4µl/ml, Sigma-Aldrich] and PMSF [25 ul/ml of 200 mM stock]). After homogenization at 15000rpm for 2 minutes, the homogenate was centrifuged at 2000g for 20 minutes at 4°C. The procedure was repeated twice and the resulting supernatant was collected and centrifuged at 10,000g for 60 minutes at 4°C using an ultra-centrifuge (Optima™ L-80 XP, Beckham). The resulting pellet containing the membrane protein was suspended in 0.5X lysis buffer and the suspension was stored at -80°C until use. Prior to western blot test, the total protein in each sample was measured by spectrophotometer using Bradford reagent (Sigma).

**Western Blot** – 30µg of each protein sample was separated on a 10% SDS-polyacrylamide gel and transferred to an Immun-Blot™ PVDF membrane (Bio-Rad, CA, USA). After blocking with TBST with 5% milk, the membrane was incubated with primary antibodies of P-glycoprotein (Pgp) and Mrp2 (Pgp antibody C219 & MRP2 antibody [M2 III-6], Abcam) in TBST with 2% milk at 4°C overnight. After being washed a few times with TBST, the membrane was incubated with secondary antibodies (Peroxidase conjugated goat anti-mouse IgG, Jackson Immuno Research) and the antibody of the housekeeping protein β actin (Sigma) in TBST with 2% milk. Bound antibody was detected by use of a Western blotting enhanced chemiluminescence detection kit (GE Health, UK). The level of target protein was normalized to that of the β actin.
3.5 Correlation between mRNA Expression of Hepatic Transporters and FXR

To establish the potential involvement of FXR in the regulation of hepatic transporters during NAFLD, we examined the relationship between changes in the expression of hepatic transporters and FXR. Pearson’s correlation coefficient was used to assess the linear relationship between mRNA expression values of FXR and transporter target genes obtained from individual liver samples from the control and HFHCD groups. The Pearson’s correlation coefficient was calculated using Microsoft Office Excel (2007) and significance was set to $p \leq 0.05$, which corresponds to a $R = 0.574$ for $n = 10$.

3.6 Data Analysis and Statistics

Values are expressed as mean ± S.E.M (n = 6-8). Statistical analyses were performed using either a two-tailed student T-test or a one-way ANOVA analysis. The statistical significance was set to $p \leq 0.05$. 
4. Results

4.1. Indication of Fatty Liver: Hepatic Triglyceride and Cholesterol

The rats treated with HFHCD developed massive fatty liver whereas the liver of the normal diet treated rats remained normal. As depicted in Figure 3, the hepatic triglyceride and cholesterol content of the HFHCD treated rats were profoundly higher than that of the normal rats. The mean triglyceride and cholesterol levels in the liver of the HFHCD rats were more than 3 fold and 5 fold higher than those in the liver of the controls (HFHCD vs. Control: triglyceride: 21.6mg/g vs. 6.4mg/g; cholesterol: 23.3mg/g vs. 4.3mg/g).

4.2. Effect of HFHCD on mRNA levels of hepatic transporters and enzymes

After 7 weeks HFHCD treatment, the rats exhibited a significant reduction in the hepatic mRNA levels of efflux transporters Mdr1a, Mrp2 and Bsep, as well as the uptake transporters Oatp1a4 and Ntcp, compared with those of the controls (Figure 4 and Figure 5). In HFHCD treated rats, Mdr1a, Mrp2 and Bsep mRNA expression was decreased by 72%, 48% and 75%, respectively. Similarly, the mRNA expression levels of Oatp1a4 and Ntcp were decreased by 64% and 71%. The mRNA expression of metabolic enzyme Cyp3a2 was significantly decreased by 59% in the HFHCD treated rats.

4.3. Effect of HFHCD on protein levels of Pgp (Mdr1a/1b) and Mrp2

Consistent with mRNA expression, the hepatic protein level of Mrp2 of the HFHCD treated rats was significantly decreased by 25% compared with that of the controls. The protein level of Pgp, which is encoded by mdr1a and mdr1b in rodent, remained unchanged between the two groups of rats. (Figure 10)
4.4. **Effect of Exercise on mRNA Expression of hepatic transporters and metabolic enzyme**

To determine the effect of exercise, the mRNA levels of the above transporters and metabolic enzyme were examined in the trained rats as well. As depicted in Figure 6 and Figure 7, the exercise training did not exert significant effect on the hepatic mRNA expression of Mdr1a, Mrp2 and Oatp1a4 in the HFHCD treated rats. Similar to the sedentary HFHCD treated rats, hepatic mRNA reduction of these transporters was found in the trained rats as well (% downregulation compared with controls: Mdr1a: 70%, Mrp2: 70% and Oatp1a4: 46%). However, the mRNA expression levels of the bile acid transporters Bsep and Ntcp, together with the metabolic enzyme Cyp3a2, were significantly improved by exercise training and their levels were comparable with those of controls.

4.5 **Levels Cytokines and Nuclear Receptors in HFHCD Treated Liver**

The levels of pro-inflammatory cytokines were measured by qRT-PCR to determine if inflammation was induced in the fatty livers of the HFHCD treated rats. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are two important pro-inflammatory cytokines involved in hepatocellular inflammation (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Krohn et al., 2009). However, the mRNA levels of IL-1β and TNF-α in the liver of the HFHCD treated rats did not differ significantly from those of the control rats (Figure 10).

The mRNA levels of nuclear receptors, which regulate the expression of the above hepatic transporters and enzymes in the HFHCD and control rats, were also determined. The nuclear receptors were the pregnane X receptor (PXR), the liver X receptor (LXR) and the farnsoid X receptor (FXR). While no difference was found in the expression of PXR and LXR between the
two groups, the expression of FXR was profoundly reduced by more than 3 fold (HFHCD vs. Control: 30.05 ± 4.78% vs. 100.00 ± 16.14%, Figure 9).

4.6 Correlation of mRNA Expression between FXR and Mrp2, Bsep and Cyp3a2

The mRNA expression of Mrp2, Bsep and Cyp3a2 shows significant linear correlation with the mRNA expression of FXR ($p \leq 0.05$) The Pearson correlation coefficient values for the linear relationship between FXR and MRP2, Bsep and Cyp3A2 were 0.663, 0.870 and 0.658, respectively. Refer to Figure 11.
Figure 3: Levels of Triglyceride (A) and Cholesterol (B) in liver tissue of HFHCD treated and control rats. The values represent the mean ± S.E.M (n = 7) of each group. Statistical significance is set to $p < 0.05$: *, $p < 0.01$: ** and $p < 0.001$: ***.
Figure 4: Effect of HFHCD on the mRNA expression of biliary efflux transporters - Mdr1a, Mrp2, and Bsep. The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M (n = 6 or 8) as percentage of the control values. Statistical significance is set to $p<0.05$: * and $p<0.01$: **.
Figure 5: Effect of HFHCD on the mRNA expression of hepatic uptake transporters and metabolic enzyme - Oatp1a4, Ntcp and Cyp3a2. The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M (n = 6 or 8) as percentage of the control values. Statistical significance is set to $p<0.05$: * and $p<0.01$: **.
Figure 6: Effect of exercise on the mRNA expression of biliary efflux transporters - Mdr1a, Mrp2 and Bsep. The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M. (n=7 or 8). Statistical significance is set to $p<0.05$: *, † and $p<0.01$: **, ‡‡ (*: HFHCD or HFHCD + exercise vs. control, †: exercise +HFHCD vs. HFHCD)
Figure 7: Effect of exercise on the mRNA expression of hepatic uptake transporters and metabolic enzyme - Oatp1a4, Ntcp and Cyp3a2. The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M. (n = 7 or 8). Statistical significance is set to p< 0.05: *, † and p< 0.01: **, †† (*: HFHCD or HFHCD + exercise vs. control, †: HFHCD + exercise vs. HFHCD).
Figure 8: Effect of HFHCD on the mRNA expression of pro-inflammatory cytokines - interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M. (n = 6 or 8) as percentage of the control values. Statistical significance is set to $p<0.05$. 
Figure 9: Effect of HFHCD on the mRNA expression of nuclear receptors - liver X receptor (LXR), pregnane X receptor (PXR) and farnesoid X receptor (FXR). The mRNA levels were determined by qRT-PCR and normalized to 18S. The values represent the mean ± S.E.M. (n = 6 or 8) as percentage of the control values. Statistical significance is set to $p < 0.05$: * and $p < 0.01$: **.
Figure 10: (A) Effect of HFHCD on hepatic protein levels of Pgp and Mrp2. The protein levels were determined by Western blot and results were normalized to β actin. The values represent the mean ± S.E.M. (n = 5 or 8). Statistical significance is set to $p < 0.05$: * and $p < 0.01$: **. (B) Representative Western Blot
Figure 11: Correlation of mRNA expression between FXR and Mrp2(A), Bsep(B) and Cyp3a2 (C). Expression of FXR and transporter genes were determined from individual liver samples obtained from rats receiving HFHCD or control diet. Significance of Pearson correlation is set to $p \leq 0.05$, with corresponding $R = 0.574$ for $n = 10$. 
5. Discussion

5.1 Experimental Models of NAFLD and Regulation of Transporters and Enzymes

NAFLD is closely associated with obesity and diabetes. Due to the increasing population of obesity worldwide, information pertaining to the liver function and its impact of drug disposition under the pathological condition of NAFLD is of particular importance.

Studies suggest that the hepatic transporters and metabolic enzymes are altered in rodents with NAFLD. However, the level of alteration is dependent on the severity level of the disease and the animal models being used (Q. Cheng et al., 2008; Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Lickteig et al., 2007; More & Slitt, 2011; Vinaixa et al., 2010). Fisher et al. compared the effect of NASH and simple hepatic steatosis on the mRNA and protein levels of some hepatic uptake transporters (NTCP, OATP1a1, 1a4, 1b2 and 2b1) in the liver of male rats using methionine-choline deficient (MCD) diet or high fat (HF) diet respectively. It was found that both groups of rats exhibited downregulation of these transporters, but the effects in rats with NASH were more prominent (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009). In a similar experimental setting, Lickteig et al. found that the mRNA and protein expression of hepatic efflux transporters (Mrp3 and Mrp4) were strikingly increased in rats fed with MCD diet but not in rats fed with high fat diet. However, the mRNA levels of Bsep and Mrp2 were decreased in rats fed with both MCD and HF diets. In contrast with the mRNA expression, the protein expression of hepatic Mpr2 was increased in rats fed with MCD diet (Lickteig et al., 2007). Nevertheless, the mechanism governing the alteration of transporters and metabolic enzymes of NALFD is still not clear.

In the current study, the high fat, high cholesterol dietary (HDHCD) model was used to induce NAFLD. The HFHCD contains a high fat content (21% w/w) with additional cholesterol.
(1.2% w/w) and cholic acid (0.5% w/w). Researchers have indicated that cholesterol is a causal factor in the development of both steatosis and hepatic inflammation, which are the key components of NASH (Wouters et al., 2008). Dietary cholesterol enhances the accumulation of hepatic cholesterol, triglyceride and the peripheral adipose macrophages that stimulate cytokine production for inflammation in liver (Subramanian & Chait, 2009; Vinaixa et al., 2010). In the current study, cholic acid was added to the HFHCD to enhance cholesterol absorption as the plasma cholesterol level in rat is naturally low (Kovar, Tonar, Heczkova, & Poledne, 2009). In fact, Vergners et al. found that cholesterol and cholate induced distinctive sets of genes involved in different aspects of inflammatory response, with cholesterol being required for the acute inflammatory response, whereas cholate was responsible for activating genes associated with hepatic fibrosis (Vergnes, Phan, Strauss, Tafuri, & Reue, 2003). In an attempt to mimic the pathological characteristics of NASH, the high fat, high cholesterol dietary model was employed for this study.

As expected, HFHCD dietary treatment induced steatosis in rats of our study, with more than a 3 fold and a 5 fold increase in triglyceride and cholesterol in the liver of the HFHCD rats compared the controls. As mentioned earlier, hepatic lipid accumulation is the ‘first hit’ of NAFLD and can elicit further liver damage such as NASH and fibrosis. This indicates that the high fat, high cholesterol dietary model was able to elicit steatotic condition of NAFLD.

Our results demonstrated that the mRNA levels of several important hepatic transporters (Mdr1a, Mrp2, Bsep, Oatp1a4, and Ntcp) and metabolizing enzyme Cyp3a2 were significantly decreased in liver of the HFHCD treated rats compared with that of the controls. Our findings are consistent with those found from other experimental animal models (e.g. MCD dietary model). For example, levels of hepatic uptake transporters such as Ntcp and Oatp1a4 are downregulated
in rats with NAFLD (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009; Geier et al., 2005; Lickteig et al., 2007; Z. X. Yang, Shen, & Sun, 2010).
5.2 Inflammation and Proinflammatory Cytokines

We postulated that the downregulation of transporter and enzyme expression might be mediated by inflammation caused by NAFLD as similar downregulation of transporters and enzymes has been frequently observed under acute inflammatory conditions induced by endotoxin, viral mimics or cytokines. (Refer to 1.4).

Expression of a few pro-inflammatory cytokines were examined in the liver tissue of both the HFHCD treated rats and the controls. Interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) are two pro-inflammatory cytokines that have been linked to hepatic inflammation. Although significant differences in the mRNA levels of these two cytokines were not detected between the HFHCD treated and the control groups (Figure 4), we cannot rule out the possibility of inflammation induced by NAFLD as the disease is linked to a number of other inflammatory mediators (Wouters et al., 2008).

For example, IL-6 and IFNγ are also key proinflammatory cytokines that can be induced by hepatic inflammation (Baker et al., 2011). However, we were unable to measure the hepatic levels of these cytokines by PCR in this present study. This is likely due to a low expression as the production of these cytokines in the liver is minimal under normal conditions. With pathological stimulation, cytokine production is increased by cells of immune system to mediate liver inflammation, apoptosis and fibrosis (Tilg & Diehl, 2000). Unfortunately, mRNA levels of cytokine IL-6 may have still been too low to be detected by qRT-PCR.

Another way to confirm hepatic inflammation in our HFHCD treated rats is to measure the systemic levels of acute phase proteins (APPs). In response to local injury or inflammation, the liver produces large amount of APPs in the blood circulating system. Thus a significant increase
of APP concentration in plasma is an indication of inflammation. For example, C-reactive protein (CRP) can be as an indicator for liver inflammation and failure after resection (Ananian, Hardwigsen, Bernard, & Le Treut, 2005). In the future, the levels of CRP should be measured in both groups of rats to determine whether systemic inflammation is a factor.
5.3 Role of FXR under NAFLD

To understand the molecular mechanism involved in (Y. Zhang, Chan, & Cummins, 2009)(Y. Zhang, Chan, & Cummins, 2009) the alteration of transporters and enzyme, we measured the mRNA levels of several nuclear receptors that have roles either in the regulation of hepatic transporters and enzymes or in the pathologic development of fatty liver disease. Refer to section 1.5.

Previous studies in our laboratory suggest partial involvement of PXR in suppression of hepatic gene expression under acute inflammation condition and PXR expression is reduced in mice treated with endotoxin or IL-6 (Teng & Piquette-Miller, 2005a). As NAFLD is associated with chronic inflammation and downregulation of mRNA levels of several hepatic transporters was observed in the HFHCD treated rats, it was thought that PXR might be involved in mediating the downregulation of these transporters. FXR and LXR are the principal regulators for hepatic lipid metabolism and bile acid homeostasis. As NAFLD is associated with a state of metabolic disorder and cholestasis (Z. X. Yang et al., 2010; Y. Zhang et al., 2009), we examined the effect of NAFLD on these nuclear receptors, which might affect the regulation of transporters and enzymes in the rats that developed NAFLD.

Interestingly, the hepatic mRNA levels of PXR and LXR were unchanged between the HFHCD treated and the control groups. However, hepatic FXR mRNA level was profoundly decreased by more than 3 fold in the HFHCD treated group relative to the controls. Moreover, we observed a positive correlation between the mRNA expression levels of Mrp2, Bsep and Cyp3a2 with FXR(Figure 11), suggesting that downregulation of the transporters and enzyme could be mediated by FXR as many of these transporters and enzyme have roles in bile acid homeostasis.
FXR is a ligand-activated nuclear receptor, which functions as the central regulator for bile acid homeostasis in the enterohepatic circulation system (Kalaany & Mangelsdorf, 2006; Sinal et al., 2000). Bile acids are endogenous ligands for FXR. Upon activation by bile acid, FXR together with other nuclear receptors (PXR, CAR and vitamin D) collectively maintain bile acid homeostasis by regulating bile acid synthesis, transport and metabolism in the liver (Zhu et al., 2011). One consequence of impaired function of FXR is hepatic cholestasis, a condition that exacerbates liver injury by excessive accumulation of toxic bile acids (Pizarro et al., 2004). As we observed a profound decrease of FXR expression in the liver of the HFHCD treated rats, it is likely that hepatic cholestasis occurred concurrently with fatty liver. In addition, the cholesterol and cholic acid in the HFHCD could also serve as a source of bile acids, which could either elicit or further aggravate hepatic cholestasis. Cholestasis, resulting from reduced bile formation or flow, is associated with an accumulation of bile within hepatocytes causing cell injury. It is known that during cholestasis the expression and activities of several hepatic transporters such as Mrp2, Mdr1a and Ntcp are decreased (Cherrington, Slitt, Li, & Klaassen, 2004; Pizarro et al., 2004; Trauner et al., 1997). The downregulation of hepatic transporters during cholestasis could be related to inflammation, a common condition occurred with chronic liver disease. It has been shown that LPS-induced hepatic cholestasis in rats is associated with downregulation of hepatic Mrp2, Bsep and Ntcp expression mediated by proinflammatory cytokines such as TNF-α (Elferink et al., 2004). Therefore, it is highly plausible that hepatic cholestasis is induced by NAFLD in the HFHCD treated rats.

FXR plays a pivotal role not only in regulating bile acid homeostasis but also in lipid metabolism in liver. FXR downregulates LXR expression via SHP activation and subsequently the LXR target genes SREBP-1C and FAS, resulting in inhibition of triglyceride synthesis and decreased
accumulation in liver (Watanabe, Houten, Wang, Moschetta, Mangelsdorf, Heyman, Moore, & Auwerx, 2004a). Hence decrease in FXR expression promotes hepatic steatosis. Histology analysis reveals that FXR/LDLr double knockout mice fed high fat diet exhibited striking macrosteatosis, lobular inflammation in liver, whereas no inflammatory cell infiltration is detected in the livers of LDLr knock-out mice fed the same high fat diet, suggesting that FXR plays a protective role in NASH development (Kong et al., 2009). Consistently, a recent study of human patients with NAFLD show significantly reduced level of hepatic FXR expression, whereas the expression levels of LXR, SREBP-1C and FAS are increased in liver relative to healthy individuals (Z. X. Yang et al., 2010). Therefore, decreased activity of FXR is an important factor in the pathogenesis of NAFLD.

What causes the downregulation of FXR expression in our NAFLD model remains to be determined. One possible explanation is that downregulation of hepatic FXR expression could be mediated by conditions of altered glucose homeostasis in liver, as NAFLD is related to insulin resistance and diabetes. A study of the influence of diabetes on FXR expression in diabetic Zucker rats shows that FXR expression is dependent on D-glucose concentration and its expression is decreased in liver due to the reduced glucose uptake by liver because of insulin resistance (Duran-Sandoval et al., 2004). This suggests a possible route of FXR downregulation in our NAFLD model.

Alternatively, hepatic inflammation could cause downregulation of FXR. It has been reported that the levels of nuclear receptors involved in lipid metabolism such as FXR, PXR and LXR are downregulated in rats with endotoxin-induced (LPS) inflammation (Fang et al., 2004). Hence, the downregulation of FXR expression observed in our study could be a result of the HFHCD-
induced steatosis and the subsequent conditions associated with conditions such as inflammation and insulin resistance.

On the other hand, FXR deficiency could exacerbate liver injury induced by NALFD as it promotes hepatic steatosis through increased SREBP-1C expression and impairs bile acid excretion from the liver due to decreased expression of Bsep (Refer to Introduction 1.5).
5.4 Effect of NAFLD on mRNA and Protein Expression of Biliary Efflux Transporters

In the current study, many of the transporters and metabolizing enzyme which were altered by the HFHCD diet are known to play a role in various aspects of bile acid homestasis in liver and they are regulated by FXR either directly or indirectly.

mRNA levels of Bsep, which is primarily responsible for the efflux of monovalent bile acids, were significantly reduced in the HFHCD fed rats. It has been shown that Bsep expression is reduced and Bsep induction by bile acid is absent in FXR/-/- mice (Saini et al., 2004; Zollner et al., 2003). Therefore, FXR might be directly involved in the regulation of hepatic Bsep in HFHCD fed rats.

Mrp2 also exports divalent and conjugated bile salts such as sulfated or glucuronidated conjugates from liver into the bile (Keppler & König, 2000). Expression of both mRNA and protein levels of hepatic Mrp2 were significantly decreased in the HFHCD treated rats as well. This indicates that NAFLD can affect Mrp2 at both transcriptional and post-transcriptional levels in the HFHCD rats. Downregulation of hepatic Mrp2 expression has been found in obese Zucker rat with fatty livers, rendering the fatty livers more vulnerable to toxic bile salts and other xenobiotics (Geier et al., 2005). Moreover, downregulation of hepatic Mrp2 expression has been observed in various rat models of cholestasis induced by endotoxin or bile duct ligation (Trauner et al., 1997). Therefore, the Mrp2 expression downregulation might be mediated by cholestasis, a condition that likely occurred in our HFHCD treated rats.

On the other hand, upregulation of hepatic Mrp2 expression has been reported in the MCD dietary rat model (Lickteig et al., 2007). The conflicted results of Mrp2 could be due to the
differences in dietary mechanisms (HFHCD vs. MCD), animal gender and treatment duration between the experimental models.

Although the mRNA levels of Mdr1a were decreased in the fatty liver of the HFHCD treated rats, protein levels of Pgp were unchanged. However, it has been reported that the transcriptional regulation of Mdr1a and Mdr1b (isoforms of Pgp gene in rodent) are different in several pathological conditions. For instance, whereas endotoxin-induced inflammation in rats has been shown to elicit decreased Mdr1a mRNA expression, an increase mRNA expression of Mdr1b is seen (Goralski, Hartmann, Piquette-Miller, & Renton, 2003; Vos et al., 1998). Therefore, the unchanged protein level of hepatic Pgp in our study may be a result of the divergent transcription effect of NAFLD on Mdr1a and Mdr1b, whereby HFHCD treatment imposed an upregulation of Mdr1b mRNA expression. The mRNA level of hepatic Mdr1b in the HFHCD treated rats needs to be examined in the future. Anyway, our results indicated that HFHCD-induced NAFLD caused no impact on the protein level of hepatic Pgp.

Previous studies in our laboratory have demonstrated that proinflammatory cytokines IL-1β, TNF-α and IL-6 are involved in Pgp regulation but through different regulatory pathways. Whereas treatment with IL-6 in rat hepatocytes can downregulate hepatic Pgp at both mRNA and protein levels, treatment of IL-1β regulates Pgp expression at protein level only (Sukhai et al., 2001). In vivo treatment with TNF-α causes an induction of Mdr1b. Consistent with this finding is the fact that hepatic IL-1β expression is not changed in rats treated with HFHCD, indicating that IL-1β is not involved in Pgp mRNA and protein regulation in our study. In contrast, IL-6 has been shown to be a principal mediator for Pgp modulation under inflammatory conditions in rodents and humans. Downregulation of Pgp mRNA and protein expression have been found in
rats treated with IL-6 (Hartmann, Kim, & Piquette-Miller, 2001; Lee & Piquette-Miller, 2001). It is possible that IL-6 was induced under the steatotic condition and participated in the downregulation of hepatic transporters in the HFHCD rats. Further study is needed to determine the role of IL-6 in NALFD.
5.5 Effect of NAFLD on mRNA Expression of Hepatic Uptake Transporters

We observed that HFHCD treatment imposed significant downregulation on the mRNA expression of the uptake transporters Oatp1a4 and Ntcp in addition to effects on biliary efflux transporters. Downregulation of Oatp1a4 and Ntcp expression are consistent with results using the HF and MCD dietary models (Fisher, Lickteig, Augustine, Oude Elferink et al., 2009).

NAFLD, especially NASH, is associated with cholestasis, a condition with impaired bile flow in the liver (Pizarro et al., 2004). It has been reported the mRNA expression of Ntcp, Oatp1, Oatp2 and Oatp4 tend to decrease under cholestatic conditions in mice administered with lipopolysaccharide (LPS) (Lickteig, Slitt, Arkan, Karin, & Cherrington, 2007). Obese Zucker rats with fatty liver also exhibit decreased hepatic Oatp2 mRNA and protein expression, in parallel with the decreased hepatic Mrp2 expression (Geier et al., 2005). Based on our results, it is plausible that cholestasis induced by HFHCD contributes to the downregulation of these hepatic uptake transporters.

It is important to note that a decreased hepatic uptake of bile acid during cholestasis is also considered as an adaptive mechanism to prevent hepatocellular toxicity (Zollner et al., 2006). Bile acid accumulation in liver induces a negative feedback inhibition of their uptake through complex regulatory mechanisms involving multiple nuclear receptors: HNF4α, HNF1α (hepatic nuclear factor 4 alpha, 1 alpha), heterodimer RXRα:RXRα, and SHP (short heterodimer partner). SHP is a known target gene of FXR (Jung & Kullak-Ublick, 2003; Zollner et al., 2006).
5.6 Effect of NAFLD on mRNA Expression of Cyp3a2

We observed a pronounced downregulation of the metabolic enzyme, Cyp3a2 in the livers of the HFHCD treated rats. It has been previous reported that NAFLD affects not only hepatic transporters but also a cluster of metabolic enzymes that have function in lipid metabolism. A recent study of obese Zucker rats revealed that the dysregulation of a large set of metabolic genes accompanied with hepatic steatosis using transcriptomic analysis (Buque et al., 2010). This study demonstrates that many genes related to detoxification, tissue structure maintenance and cell survival were downregulated in the steatotic hepatocytes.

Enzymes of the CYP3A family are one group of phase I oxidative enzymes involved in metabolism of numerous xenobiotics including drugs. CYP3A enzymes are actively involved in the detoxification process of bile acids. In the liver, CYP3As catalyze the hydroxylation of lithocholic acid (LCA), which promotes LCA elimination from the liver (Saini et al., 2004). CYP3As can be regulated by PXR, FXR and CAR (Claudel, Staels, & Kuipers, 2005; Eloranta, Meier, & Kullak-Ublick, 2005; Gnerre, Blattler, Kaufmann, Looser, & Meyer, 2004). In our study, FXR expression was downregulated and correlated with Cyp3a2 expression, while PXR expression was unchanged in the fatty liver of the HFHCD rats, indicating that FXR suppression could be a factor in the downregulation of Cyp3a2. Recent studies have also shown that CAR coordinates with FXR in protecting the liver from bile acid induced toxicity (J. Zhang, Huang, Qatanani, Evans, & Moore, 2004). Hence, it is possible that CAR could also be involved in the dysregulation of Cyp3a2 in our study.
5.7. Effect of Exercise on NAFLD

Another purpose of our study was to determine if exercise could diminish the effects of NAFLD as exercise was shown to decrease visceral adipose tissue and hepatic triglyceride concentration in obese individuals (Johnson et al., 2009).

In our study, the expression of bile acid transporters (Bsep and Ntcp) and metabolizing enzyme Cyp3a2 were significantly improved in the rats receiving exercise and their levels are normalized to the same levels as the controls (Figure 4, 5). Exercise slightly improved the expression level of Oatp1a4 but did not reach significance. Indeed, the expression level of Mrp2 was decreased in the trained rats compared with the sedentary rats. Therefore, the effect of exercise is transporter specific.

As discussed in the Introduction 1.6, exercise has been shown to control the progression of fatty liver by reducing intrahepatic fat, fatty acid uptake and improve insulin sensitivity (Goodyear & Kahn, 1998; Perseghin et al., 2007; Thomas et al., 2006). Thus exercise helps to release the burden such as oxidative stress and inflammation caused by fat accumulation in liver. The increased gene expression of Bsep, Ntcp and Cyp3a2 is most likely results from the beneficial effects of exercise on NAFLD. Besp and Ntcp are the main transporters in controlling the bile acid flow in the liver and Cyp3a2 is playing important in bile acid detoxification. This suggests that exercise might also improve the cholestatic condition found in the fatty liver of the HFHCD treated rats. It will be interesting to investigate the expression level of hepatic FXR in HFHCD treated rats receiving exercise.

However, fatty liver was also observed in the trained rats treated with HFHCD. Therefore the exercise training program used in the current study might not be sufficient to compensate the
negative impact of fatty liver on the regulation of hepatic transporters. A longer training period or higher exercise intensity should be considered.
6. Conclusion

Rats in the HFHCD treatment group developed massive fatty liver and displayed features of NAFLD such as triglyceride and cholesterol accumulation in liver. A significant downregulation in the mRNA expression of metabolic enzyme Cyp3a2, and several transporters (Mrd1a, Mrp2, Bsep, Otp1a4 and Ntcp) were seen in the liver of the HFHCD treated rats, compared with those of the controls.

FXR, a key regulator of bile acid and lipid metabolism, was profoundly downregulated in the liver of HFHCD treated rats as compared to controls. FXR downregulation promotes triglyceride synthesis and impairs bile acid excretion from liver, rendering the liver more susceptible to injury by the fat accumulation and toxic bile acid. Since FXR is an important regulator of several hepatic transporters, thus the hepatic FXR expression downregulation, together with hepatic steatosis, most likely contributed to the downregulation of hepatic transporters and metabolic enzyme in our study.

Exercise training has been used as an alternative approach to treat NAFLD and related diseases. It is interesting that our results show significant improvement in hepatic mRNA expression of Bsep, Ntcp and Cyp3a2 in HFHCD treated rats receiving exercise, compared with their sedentary counterpart.

Overall, the HFHCD treated rats developed characteristics of NAFLD and our results are consistent with those found from other experimental models and human patients with NAFLD. Therefore, the high fat/ high cholesterol dietary (HFHCD) model could be a useful tool to study the pathological effects of NAFLD. However, we failed to find liver inflammation in the rats treated with HFHCD. NAFLD can progress in various stages (i.e., simple steatosis,
steatohepatitis (NASH) and fibrosis), which in turn will have different impacts in the regulation of hepatic transporters and metabolizing enzymes. Therefore, future work should focus on characterization of the HFHCD model conditions and how they represent different stages of NAFLD.

In human, hepatic OTAPs, MRP2 and CYP3As are responsible for the uptake and elimination of many drugs and endogenous substances (Cui, Konig, & Keppler, 2001); (Niemi, 2007); (Lynch & Price, 2007). Our study suggests that there is a potential drug accumulation in plasma or liver due to the reduced expression of these drug transporters and metabolic enzyme. If clinical relevance of the current study is confirmed, patients with NAFLD may require dose adjustment to avoid systemic and hepatic drug toxicity caused by the altered levels of the above transporters and metabolic enzymes.
7. Future Direction

We have demonstrated the effect of NAFLD on several hepatic transporters and metabolic enzymes. Since NAFLD affects a network of genes involved in bile acid homeostasis and lipid metabolism, future work should be continued to determine the effect of HFHCD on other transporters, enzymes and transcription factors that have roles in these aspects. (e.g, Mrp3, Mrp4, CAR, HNF4α, SERBP-1C and Cyp7a1).

Based on our results, FXR appears to be a key determinant for the dysregulation of hepatic transporters and metabolic enzymes. However, studies suggest that under cholestatic or steatotic conditions, transcription factors (PXR, FXR, CAR, PPARs) coordinate each other to protect the liver from hepatotoxicity. Our data most likely represents results from crosstalk among these transcription factors. Therefore, knockout rats should be used in order to determine the specific role of each transcription factor under the effect of NAFLD.

In the future, model conditions such as duration of treatment, gender of animals, and composition of diets (HFHCD) should be characterized in order to study the various stages of NAFLD and other concurrent conditions such as hyperlipidemia, diabetes and hepatic inflammation.

The effect of NAFLD on drug distribution and clearance should be examined as well. Many drugs that are substrates of MRP2, OATP2 and Cyp3a2 are commonly used by patients with metabolic syndromes (e.g. statins). The conditions used for the in vivo drug disposition study should be established to mimic the conditions used in human patients.
8. References


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