The Role of Intestinal Scavenger Receptor Class B Type I in Chylomicron Production in Normal and Insulin Resistant States

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

In recent years, studies have revealed a central role for the intestine in regulation of lipid homeostasis and development of insulin resistance and type-2 diabetes. The function of intestinal Scavenger Receptor Class-B type-I remains unknown, however it is believed to play a role in dietary lipid uptake. Recently, our laboratory demonstrated a correlation between intestinal SR-BI expression and chylomicron secretion. We hypothesized that intestinal SR-BI is involved in chylomicron secretion and contributes to chylomicron oversecretion in insulin resistance. I first characterized chylomicron production in healthy and insulin resistant Syrian golden hamsters. Inhibition of SR-BI resulted in reduced postprandial chylomicron accumulation in plasma, and resistance to diet-induced hyperlipidemia and weight-gain. Lower postprandial triglyceride levels were also observed in SR-BI/- mice. In summary, these data demonstrate a key role for intestinal SR-BI in chylomicron secretion and control of lipid homeostasis, implicating intestinal SR-BI in chylomicron overproduction in insulin resistant states.
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<tr>
<td>ABCG5</td>
<td>ATP-binding cassette G5</td>
</tr>
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<td>ABCG8</td>
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<td>acLDL</td>
<td>acetylated LDL</td>
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<tr>
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<td>alanine aminotransferase</td>
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<td>cyclic-AMP</td>
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<td>chylomicron</td>
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</tr>
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<td>GLP-2</td>
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<tr>
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<td>GLP-2 receptor</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>IR</td>
<td>insulin resistance</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LLD</td>
<td>luminal lipid droplets</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacyl glycerol</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MG</td>
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<td>monoglycerol acyltransferase</td>
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<tr>
<td>MTP</td>
<td>microsomal transfer protein</td>
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<tr>
<td>NPC1L1</td>
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<tr>
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<td>PDZ domain containing 1</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PKA</td>
<td>protein kinase-A</td>
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<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>PPM</td>
<td>postprandial micelle</td>
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<tr>
<td>PS</td>
<td>Phosphoserine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
</tr>
<tr>
<td>recHDL</td>
<td>reconstituted HDL</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y Gastric Bypass</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>SF-1</td>
<td>splicing factor-1</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelins</td>
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<tr>
<td>SR-BI</td>
<td>scavenger receptor class-B type-I</td>
</tr>
<tr>
<td>SRE</td>
<td>sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>SRE-binding protein</td>
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<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
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<tr>
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<td>tris buffered saline with Tween-20</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF-A</td>
<td>tumour necrosis factor-α</td>
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<tr>
<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VLDL</td>
<td>very-low density lipoprotein</td>
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1 Introduction

1.1 Overview of lipoprotein metabolism

Lipoprotein metabolism is a complex process involving multiple organs such as the liver, intestine, adipose tissue, and even the brain. Lipoproteins are essential for the efficient transport and delivery of dietary or synthesized lipids to and from tissues in the body, since lipids are insoluble in plasma (Shepherd, 1994). Lipoproteins are made up of an amphipathic exterior with a hydrophobic core containing varying amounts of triglycerides (TG), cholesterol (CHOL), and cholesteryl-ester (CE), along with an array of exchangeable and non-exchangeable proteins (Shepherd, 1994). Lipoproteins are divided by density into chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) in order of descending density and size.

CMs are the largest lipoproteins and are slightly larger than VLDL. However, while VLDL is produced in the liver, CMs are produced exclusively in the intestine (Xiao et al., 2011). IDL and LDL are derived from VLDL as it undergoes lipolysis while in circulation, becoming progressively smaller (Shepherd, 1994). HDL, on the other hand, is produced by all tissues as they eliminate excess CHOL in HDL particles. HDL can then either deliver CHOL to other tissues in need or be taken up by the liver for excretion of excess CHOL in bile. This process is known as the reverse cholesterol transport pathway (RCT) (Lewis & Rader, 2005; Tall, 1998; Velde, 2010). The protein responsible for binding HDL in the liver, and involved in the process of RCT, is known as scavenger receptor class B type I (SR-BI) and its function and potential role in intestinal CM production will be discussed in greater detail in section 1.4.

Lipoprotein metabolism can be simplified by dividing all the processes into two states: the fasting state and the postprandial (post-meal) state. During the fasting state, the liver is the main regulator of lipoprotein metabolism as it produces VLDL, carrying primarily TG and some CHOL and CE to meet the body’s energy demands (Xiao et al., 2011). Upon secretion into the plasma, VLDL undergoes hydrolysis by lipoprotein lipase (LPL) which removes TG from
VLDL, turning it into IDL and subsequently to LDL which then contains primarily CE (Xiao et al., 2011).

Postprandial lipid metabolism is quite distinct from the fasting state and is characterized by a decrease in hepatic VLDL production and a rapid increase in CM production as dietary lipids are taken up by the small intestine (Mansbach & Siddiqi, 2010; Nakajima et al., 2011; Xiao et al., 2011; Xiao & Lewis, 2012). Following the uptake of dietary lipids, CM production and secretion is rapidly induced. A lot of work has been done by our laboratory and others in elucidating the mechanisms driving CM assembly, which will be discussed in greater detail in section 1.3.

Finally, once CMs enter the circulation, they circulate through adipose tissue and other tissues where they undergo lipolysis by LPL, depositing FA and glycerol which are then reassembled into TG in those tissues (Dalla-Riva et al., 2010; Parthasarathy, 2010). The CMs are now smaller in size with less TG content and are known as CM remnants, which are taken up by the liver and macrophages and broken down (Bravo & Napolitano, 2007; Dalla-Riva et al., 2010; Luft, 2004; Redgrave, 2004). The present thesis will focus on the early stages of CM metabolism, namely dietary fat uptake followed by CM production and secretion into the plasma and the potential role of intestinal SR-BI on postprandial CM production.

1.2 Chylomicrons in disease: Insulin resistance, type-2 diabetes and cardiovascular disease

Lipoprotein metabolism has been shown to be significantly altered in states of insulin resistance and type-2 diabetes (T2D). In insulin resistance and T2D, dyslipidemia occurs and is characterized by elevations in fasting and postprandial TG and CHOL levels, an increase in VLDL and CM production, and reduced clearance of VLDL and CM from plasma (Adeli & Lewis, 2008; Curtin et al., 1994; Curtin et al., 1996; Schaefer et al., 2002; Xiao & Lewis, 2012).

VLDL and CM are hydrolyzed by LPL, which binds to its anchor membrane protein GPIHBP1 expressed on vascular endothelial cells. Here LPL catalyzes the hydrolysis of TG from VLDL and CM, resulting in a reduction in VLDL and CM TG levels (Redgrave, 2004; Williams, 1998; Willnow, 1997). Aside from the CM overproduction observed in T2D and insulin
resistance, it has been shown that LPL activity is also reduced, leading to reduced CM clearance and further contributing to the elevated CM levels associated with insulin resistance and T2D (Hogue et al., 2007; Kobayashi et al., 2007; Patsch, 1998; Xiao & Lewis, 2012).

Our laboratory has extensively studied the mechanisms behind intestinal CM overproduction in insulin resistant states. In the fructose-fed hamster model of insulin resistance, we have shown a reduction in insulin signaling and responsiveness, increased apoB48 secretion, increased de novo lipogenesis and elevated MTP mass and activity (Basciano et al., 2005; Federico et al., 2006; Haidari et al., 2002; Leung et al., 2004). Tumor necrosis factor-alpha (TNF-α) was also shown to stimulate CM secretion by impairing insulin signaling and increasing MTP mass, suggesting that CM overproduction may be driven by inflammation (Qin et al., 2007).

Furthermore, the insulin-sensitizer rosiglitazone has been shown to improve insulin resistance while also resulting in reductions in CM overproduction in our fructose-fed hamster model of insulin resistance (Leung et al., 2004). Rosiglitazone is a PPAR-γ agonist that has been shown to improve whole-body insulin sensitivity, but it is unclear whether it acts directly on the intestine or through some other mechanism. The authors stipulated that a decrease in FFA flux from adipose to other tissues including the intestine may account for the observed reductions in intestinally-derived postprandial lipoprotein secretion. There have also been several studies by other laboratories linking insulin resistance and T2D to increased intestinal lipoprotein production (Chan et al., 2002; Cohn et al., 1999; John et al., 2001; Phillips et al., 2000; Xiao & Lewis, 2012). Therefore, there exists a substantial amount of evidence suggesting that increased CM production is associated with insulin resistance.

Further, postprandial dyslipidemia and elevated CM levels in plasma have also been linked to cardiovascular disease (CVD). Early studies helped to establish a relationship between coronary heart disease (CHD) and postprandial hyperlipidemia (Brown et al., 1965; Zilversmit, 1979), while more recently CM and CM remnants have also been shown to be associated with CVD (Patsch et al., 1992; Weintraub et al., 1996). In fact, apoB48 itself has been found in atherosclerotic lesions (Karpe et al., 1994; Proctor & Mamo, 2003; Tomkin & Owens, 2012). These findings suggest that CM production is not just up-regulated in disease, but can also contribute to the development of diseases such as CVD.
In summary, intestinally derived lipoproteins are essential in the delivery of lipids and nutrients to tissues, but dysregulation of intestinal CM production leads to and is associated with insulin resistance, T2D and cardiovascular disease. It is important to study how CM production and intestinal lipid uptake are regulated. Improving our understanding of intestinal lipid uptake and CM production can be beneficial in our understanding of the pathophysiology of metabolic dyslipidemia commonly observed in insulin resistance, T2D, and CVD, while paving the way for the development of novel treatments.

1.3 Intestinal lipid uptake and chylomicron production

Food ingestion initiates the postprandial state, where the body focuses its energy on absorbing, processing, and storing dietary sources of energy, including lipids. During the postprandial state, CM secretion is induced. CMs are the largest lipoproteins (75-450 nm in diameter) and are produced exclusively in enterocytes in response to dietary lipid uptake during the postprandial state (Beisiegel & Heeren, 1999; Cartwright et al., 2000; Frazer, 1954; Kindel et al., 2010; Hussain, 2000; Mansbach & Siddiqi, 2010; Redgrave, 2004; Williams et al., 2004; Xiao & Lewis, 2012).

1.3.1 Dietary lipid uptake in enterocytes

The uptake of dietary lipid by the intestine is one of the most crucial functions in the body. It is a very efficient process that is now generally accepted to be protein-mediated, although the proteins involved and their roles in lipid uptake are still under investigation. Dietary lipid consists of TG, PL, CHOL, and many other lipids and fat-soluble vitamins (Carey et al., 1983; Cianflone et al., 2008; Iqbal & Hussain, 2009).

The process of lipid digestion begins upon ingestion of lipid into the oral cavity. Here, lingual lipases initiate TG digestion, which continues in the stomach by gastric enzymes (Iqbal & Hussain, 2009). The emulsification of lipids begins in the stomach aided by its peristaltic motion. This process continues as the lipids enter the duodenum, where bile salts and pancreatic lipase
work together to form lipid micelles containing hydrolyzed TG products (Iqbal & Hussain, 2009; Mu & Høy, 2004; Xiao et al., 2011).

In the duodenum, pancreatic lipase acts on the sn-1 and sn-3 position of the TG molecule, generating 2-monoacylglycerol (2-MAG) and free fatty acids (FFA) (Iqbal & Hussain, 2009; Mattson & Beck, 1956; Mattson & Volpenhein, 1968). Dietary fat is now ready for uptake by enterocytes, which occurs in the jejunum.

The jejunum is the major site of dietary lipid uptake and it is now thought that the process of lipid uptake is mainly protein-mediated as opposed to passive. There are many candidates of lipid uptake. Proteins like CD36 appear to be involved in FA uptake, FATP4 is thought to be involved in FA transport within the enterocyte, while proteins like SR-BI and NPC1L1 have been proposed to play a role in CHOL uptake (Haikal et al., 2008; Hsieh et al., 2009; Levy et al., 2007; Levy et al., 2010; Tran et al., 2011; Wang & Lee, 2008).

It remains unclear whether either NPC1L1, SR-BI, or both are involved in intestinal CHOL uptake, although research points to NPC1L1 being the key transporter involved in intestinal CHOL uptake (Altmann et al., 2004; Knöpfel et al., 2007). More research is needed in understanding intestinal lipid uptake, particularly to improve our understanding of the role of intestinal SR-BI. The fact that SR-BI/- mice show no impairment of intestinal CHOL uptake suggests that SR-BI is not essential in CHOL uptake, raising questions about its main function in the intestine (Mardones et al., 2001). The absence of a reduction in intestinal CHOL uptake in SR-BI/- mice suggests that there are other proteins (such as NPC1L1) that either act alone or in collaboration with SR-BI to facilitate CHOL uptake.

Once hydrolyzed dietary lipid makes its way to the jejunum, it enters the enterocyte, where FA and MG must travel to the ER in order to be re-synthesized into TG molecules. This transport is believed to occur by fatty-acid binding proteins (FABPs) found in enterocytes and the liver (Agellon et al., 2002; Besnard et al., 2002). TGs are re-assembled in a two-step process involving, first, the addition of a fattyacyl-CoA molecule to MG by monoacylglycerol acyltransferases (MGATs), followed by addition of the second and third fatty acyl-CoA molecules forming diacylglycerol (DG) and triacylglycerol (TG) by diacylglycerol acyltransferases (DGATs) [reviewed in: (Yen et al., 2008)].
The next step is the assembly of CM, which will be discussed in greater detail in section 1.3.2. It is important to note that dietary lipid is not the only source of TG to be used in CM production, as pools of TG exist in the enterocyte that may also be derived from de novo lipogenesis. These lipid pools are organized and trafficked around the cell and can be made available for CM assembly. The mechanisms behind the organization and trafficking of intestinal lipid pools remains poorly understood and more research is warranted.

1.3.2 Chylomicron assembly

VLDL and CM lipoproteins are similar in terms of their function and size but there are some key differences. They both consist of a single apoB molecule, which is crucial in maintaining the structural integrity of the particle. Similarly, both CM and VLDL are TG-carriers, containing mostly TG as opposed to CHOL. The key difference between the two lipoproteins lies in the site of synthesis and the nature of the apoB molecule. VLDL are synthesized in the liver and contain apoB100, whereas CM are exclusively synthesized in the intestine and contain apoB48—a truncated form of apoB100 (Hussain, 2000; Xiao et al., 2011; Xiao & Lewis, 2012).

This tissue specific expression of apoB can be explained by the fact that enterocytes express apobec-1, a mRNA editing protein responsible for the post-transcriptional editing of APOB at codon 2153 from CAA (glutamine) to UAA (stop codon). This causes the machinery responsible for protein translation to halt, resulting in the synthesis of a truncated apoB48 protein (Hussain, 2000; Xiao & Lewis, 2012). This editing is not essential to fat uptake, as apoB100 would also be effective but less efficient. Studies in apobec-1 knockout mice reveal that apoB-editing leads to more efficient fat uptake and can be tought of as an evolutionary advantage under conditions of food shortage (Kendrick et al., 2001).

Aside from apoB48, CMs also contain other apolipoproteins like apoA-I, apoA-IV, and apoCs which, in contrast with apoB48, are exchangeable (Hussain, 2000). CMs are composed of an outer layer consisting of PL, free cholesterol (FC) and proteins, and a lipid core containing mostly TG (85-92%), and some CE (1-3%) (Hussain, 2000). More than 80% of the surface area
of CMs is composed of PL (mainly phosphatidylcholine), while the rest of the surface is composed of protein and small amounts of TG and FC (Miller & Small, 1983; Hussain, 2000).

CM assembly is a complicated and highly regulated process. It takes place exclusively in enterocytes and it begins with the transcription, modification, and translation of apoB48, followed by its lipidation by MTP in the ER. This leads to the formation of primordial CM particles that are then further lipidated and enlarged as they make their way through the golgi and are eventually secreted as CM into the lymph (Hussain, 2000; Xiao & Lewis, 2012).

The rate limiting step of CM production is the lipidation of apoB48 by MTP (Hussain, 2000). This can be seen in patients with abetalipoproteinemia, which is characterized by severe fat malabsorption and a virtual absence of apoB48 and apoB100 from plasma as a result of a mutation in the gene coding for MTP (Gregg & Wetterau, 1994; Sharp et al., 1993; Wetterau et al., 1992). Furthermore, co-expression of apoB and MTP in non-intestinal and non-hepatic cells leads to the assembly and secretion of apoB-containing lipoproteins (Gordon et al., 1994; Leiper et al., 1994; Wang et al., 1996).

Following lipidation of apoB48 by MTP, the next step in CM assembly is “core expansion,” which is proposed to occur by the fusion of primordial CM particles with lipid droplets (Hussain, 2000). Lipid droplets are found in the cytosol and in the ER lumen. Therefore, there are two kinds of lipid droplets: cytosolic lipid droplets (CLD) and luminal lipid droplets (LLD), which have been shown by electron microscopy to increase in size in response to fat uptake into the enterocyte (Béaslas et al., 2009; Hansen et al., 2003a; Hussain, 2000; Xiao & Lewis, 2012).

1.3.3 Regulation of chylomicron assembly

As is the case with all metabolic processes, chylomicron secretion is subject to regulation by a multitude of factors. Those factors can be broken down into two categories: (1) hormonal and (2) nutritional. Some of the major nutritional factors in the regulation of CM production include fructose and glucose and FFA (Basciano et al., 2005; Federico et al., 2006; Miller & Adeli, 2008; Xiao & Lewis, 2012). Some of the hormonal factors in the regulation of CM production include insulin, glucagon, GLP-1 and GLP-2 (Adeli & Lewis, 2008; Dekker et al.,
Fructose has been shown to increase intestinal TRL production and chronic fructose consumption has been shown to increase de novo lipogenesis, and to induce insulin resistance, hypertriglyceridemia, and visceral adiposity in animals as well as in humans (Basciano et al., 2005; Dekker et al., 2010; Federico et al., 2006; Haidari et al., 2002; Miller & Adeli, 2008; Rutledge & Adeli, 2007; Xiao & Lewis, 2012). Similarly, glucose has been shown to result in increased plasma TG and CM and apoB48 secretion (Robertson et al., 2003; Xiao & Lewis, 2012).

Insulin also acts to regulate CM production and, in healthy individuals, insulin suppresses CM production (Harbis et al., 2001; Levy et al., 1996; Pavlic et al., 2010). As stated previously, there is an increase in CM production in insulin resistance. Even though insulin resistance is characterized by elevated insulin levels, insulin fails to suppress CM production due to attenuated insulin signaling in enterocytes (Adeli & Lewis, 2008; Avramoglu et al., 2006; Haidari et al., 2002; Hsieh et al., 2008).

Other modulators of CM production include glucagon, GLP-1, and GLP-2 and have been reviewed elsewhere (Drucker, 2006; Hsieh et al., 2008; Hsieh et al., 2009; Xiao & Lewis, 2012). GLP-2 is known to increase CM secretion and was used to assess intestinal SR-BI in CM secretion in the present thesis. The regulation of CM production by GLP-2, a positive regulator of intestinal CM production, will be covered in greater detail in section 1.5.

1.4 Scavenger receptor class B type I (SR-BI)

Scavenger receptor class B type I (SR-BI) is a cell-surface glycoprotein expressed in tissues with different functions. Being a scavenger receptor, it binds to a range of ligands that share certain structural characteristics that make binding to SR-BI possible. Its expression has been characterized in rodents and humans and it has been shown to play an important role in cholesterol metabolism and lipid transport in the body. Intestinal SR-BI expression has also been detected and well characterized. However, the function of intestinal SR-BI has yet to be
demonstrated. The focus of the present study is to shed some light onto the role of SR-BI in the intestine and to determine whether SR-BI plays a role in CM production and lipid uptake in the intestine. This section will cover in detail the present knowledge about the expression, regulation, and functions of SR-BI in different tissues.

1.4.1 SR-BI Structure

SR-BI is a cell-surface glycoprotein consisting of 509 amino acids and with an apparent molecular weight of 57kDa (Rhainds et al., 2004). That differs from the observed molecular weight of 82-85kDa largely due to multiple glycosylations (Rhainds et al., 2004). Human SR-BI is very similar to hamster SR-BI, with 9 glycosylation sites compared to 11 sites on murine SR-BI (Rhainds et al., 2004). SR-BI has a characteristic “horseshoe” shape, anchored into the membrane by its C and N-termini, with a large extracellular domain as shown in the diagram below, adapted from a review on SR-BI (Rhainds et al., 2004).

![Figure 1. Diagram of SR-BI protein structure (adapted from Rhainds et al., 2004).](image-url)

This is a diagram of the putative structure of SR-BI showing its N and C-termini as well as PDZK1, an adaptor protein that binds to the C-terminus of SR-BI and is important to its function and expression in specific tissues.
Other interesting features of SR-BI include phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) suggesting its function may be modulated by phosphorylation on its cytoplasmic tails (Rhainds et al., 2004). PDZK1 is also depicted in Figure 1, as it is an important adaptor protein. Upon deletion of PDZK1, there is a 90% down-regulation in hepatic SR-BI and 50% down-regulation in intestinal SR-BI (Kocher et al., 2003). Furthermore, a recent review summarizes some of SR-BI’s signaling pathways that are mediated by PDZK1 (Al-Jarallah & Trigatti, 2010).

1.4.2 Expression, localization and regulation of SR-BI

SR-BI expression has been detected in many tissues, including the liver and the intestine—two major players in lipid metabolism (Acton et al., 1996; Altmann et al., 2002; Cai et al., 2001; Cao et al., 1997; Cao et al., 1997; Lobo et al., 2001; Xiao et al., 2011; Xiao & Lewis, 2012). Expression of SR-BI has also been detected in other tissues like vascular smooth muscle cells (Yeh et al., 2002), endothelial cells (Goti et al., 2001; Uittenbogaard et al., 2000), pneumocytes (Buechler et al., 1999; Kolleck et al., 1999), macrophages (Buechler et al., 1999), and kidney cells (Johnson et al., 2003). SR-BI has a multitude of functions that can be different depending on the particular tissue. The function of SR-BI may thus be modulated both by regulation of its expression as well as through other means, many of which remain poorly understood. The regulation of SR-BI has been best studied in the liver and in various cell lines.

Due to key elements in its promoter region, regulation of SR-BI expression occurs primarily via cellular cholesterol levels and hormones (Rhainds & Brissette, 2004). The SR-BI promoter region has been shown to contain 2 E-box consensus sequences (CANNTG) that can be bound by basic helix-loop-helix transcription factors (Cao et al., 1997; Rhainds & Brissette, 2004). The SR-BI promoter also contains a sterol regulatory element (SRE) motif (ATCACCCCCAC), which is bound by SRE-binding-proteins (SREBPs) (Kim et al., 1995; Rhainds & Brissette, 2004).

Expression of SR-BI has been shown to be increased by liver receptor homolog-1 (LRH-1) found in the pancreas, steroidogenic tissues, and liver (Sirianni et al., 2002). The LRH-1 induction of SR-BI expression is independent of liver X receptor (LXR)/ retinoid X receptor
(RXR) expression, which has also been shown to induce SR-BI expression. Interestingly, SREBP-1a has been shown to reduce LXR activation, thereby suggesting that SREBP-1a is acting as a negative modulator of LXR-mediated activation of SR-BI.

In contrast, in rats and in various cell lines, SREBP-1a has been shown to activate transcription of SR-BI (Lopez & McLean, 1999; Rhainds & Brissette, 2004). SREBPs mainly function by controlling fatty acid and cholesterol synthesis in tissues. They regulate the expression and functions of genes and proteins involved in the respective synthesis pathways and control cholesterol homeostasis via the LDL-receptor pathway (Horton et al., 2002; Rhainds & Brissette, 2004). It is therefore reasonable that SREBP-1a would regulate SR-BI expression, further implicating SR-BI in lipid metabolism.

SR-BI expression has also been shown to be regulated by cyclic AMP (cAMP). When steroidogenic cells were treated with cAMP, SR-BI mRNA expression was increased. This was due to protein kinase A (PKA) phosphorylation of steroidogenic factor-1 (SF-1), which binds to the SF-1 binding site found on the SR-BI promoter (Cao et al., 1997; Lopez et al., 1999). It has also been shown that gonadotropin induces SR-BI promoter activation and it may do so in a cell specific manner (Rhainds & Brissette, 2004; Shea-Eaton et al., 2001).

Adding to the complexity of SR-BI regulation, it has been shown that SR-BI is not only regulated at the mRNA level but also at the post-translational level by its adaptor protein PDZK1 (Fenske et al., 2008; Kocher et al., 2003; Komori et al., 2006; Nakamura et al., 2005; Yesilaltay et al., 2006). This was first discovered in PDZK1-/- mice, which showed tissue specific reduction of SR-BI protein mass with a 90% reduction in the liver and 50% reduction in the intestine of PDZK1-/- mice (Kocher et al., 2003). Interestingly, it was later shown that the lack of PDZK1 did not alter SR-BI localization or function (Yesilaltay et al., 2005). The interaction between SR-BI and PDZK1 is currently under investigation and considerable work is underway to determine how PDZK1 affects SR-BI expression and function.

In summary, it is clear that SR-BI is a complex, heavily regulated protein with a multitude of tissue-specific functions. Its ubiquitous expression further complicates its study since its role in the body is often difficult to delineate. However, it is possible to focus on the function of SR-BI in specific tissues like the intestine and in specific states including its potential role in intestinal lipid uptake and chylomicron production.
1.4.3 SR-BI ligands and its role in lipid uptake

As is the case with most scavenger receptors, SR-BI has a multitude of ligands. It has been shown that SR-BI can bind with high affinity to HDL (Acton et al., 1996), LDL (Al-Jarallah & Trigatti, 2010; Krieger, 2003; Lewis & Rader, 2005; Acton et al., 1994; Shetty et al., 2006), oxidized LDL (oxLDL) (S et al., 1994), acetylated LDL (acLDL) (Acton et al., 1994), and VLDL (Calvo et al., 1997). Other SR-BI ligands include all classes of lipoproteins as well as PL (Rhaiands & Brissette, 2004). There are some key residues on SR-BI that determine its binding efficiency. For example, when glutamines 402 and 418 are replaced by arginine, binding to HDL is markedly reduced, as is the selective uptake of CE (Gu et al., 2000; Rhainds & Brissette, 2004). Interestingly, the binding of apoE disks is also reduced (Li et al., 2002), while LDL binding is not affected in these mutants (Rhainds & Brissette, 2004).

Replacement of arginine 158 for methionine prevents binding of both HDL and LDL but not acLDL and leads to the abolishment of CE selective uptake (Gu et al., 2000). Interestingly, mutations at residues 108 and 173 (both are arginines that are glycosylated) lead to abolishment of CE selective uptake without affecting binding of HDL, proving that it is possible to dissociate the binding of SR-BI to its ligand from its selective uptake (Vinals et al., 2003). This effect has also been seen in the interaction of SR-BI with its inhibitor BLT-1.

BLT-1 is a semithiocarbazone that acts as a small chemical inhibitor of SR-BI function. It has been shown that although BLT-1 binds to SR-BI with high affinity, significantly reducing CE selective uptake, this also leads to an increase in the binding affinity of SR-BI for HDL (Nieland et al., 2008; Penman et al., 2002; Yu et al., 2011).

It is apparent that SR-BI has multiple binding sites and is flexible in terms of its ability to bind different ligands under various conditions. It has been suggested that one general requirement for SR-BI binding may be the distribution of negatively charged residues in alpha-amphipathic helices present in proteins as well as apolipoproteins (Rhaiands & Brissette, 2004). This net negative charge can arise from glycation and acetylation of proteins (Rhaiands & Brissette, 2004). SR-BI has also been shown to bind apoA-I, apoA-II, and apoC-III present in HDL particles (Xu et al., 1997), as well as apoE—although with much lower affinity than HDL apolipoproteins (Li et al., 2002).
Another important set of ligands for SR-BI are the PLs such as PC and phosphatidylserine (PS), which have been shown to bind to SR-BI and compete for LDL and HDL binding to SR-BI (Fluiter & van Berkel, 1997; Murao et al., 1997; Rigotti et al., 1995). There are detailed and extensive reviews covering the variety of SR-BI ligands and SR-BI binding sites that depend on the conformation of its extracellular domain and may vary across different tissues (Connelly et al., 2001; Vinals et al., 2003; Rhainds & Brissette, 2004).

One property of SR-BI that makes it truly unique is its ability to mediate selective uptake. Although it is mostly known for its selective uptake of CE from HDL, it is actually capable of selectively uptaking other lipids including polar lipids such as PC and sphingomyelin (SM) (Ji et al., 1997; Urban et al., 2000), as well as TG (Greene et al., 2001). Using reconstituted HDL (recHDL) particles, one group was able to measure the efficiency of selective uptake by SR-BI of its different ligands (Thuahnai et al., 2001). It was determined that the preferred lipid substrates for SR-BI-mediated selective uptake are: FC > CE > TG >> PL >> SM.

1.4.4 SR-BI-mediated lipid exchange

One of the key questions asked about the function of SR-BI regards the process by which it mediates lipid exchange. Many models have been proposed. In a recent review, the authors presented three possible models: (1) opening of a hydrophobic channel; (2) proper alignment to an already opened hydrophobic channel; or (3) the formation of a hydrophobic channel between multiple SR-BI proteins (Rhainds & Brissette, 2004). The involvement of a hydrophobic channel is key to the selective uptake of lipids by SR-BI and there is evidence to support the idea that SR-BI itself may form such a hydrophobic channel by oligomerization of SR-BI (Papale et al., 2011; Sahoo et al., 2007; Sahoo et al., 2007).

In fact, SR-BI has been shown to form tetramers (Papale et al., 2011; Sahoo et al., 2007; Sahoo et al., 2007). Interestingly, the ability of SR-BI to form dimmers and tetramers is attributed to key cysteine residues on the extracellular domain of SR-BI (Papale et al., 2011). The importance of these cysteines has also been highlighted in another study examining the ability of BLT-1 to inhibit SR-BI-mediated CE uptake (Yu et al., 2011). It is possible therefore that the ability of SR-BI to form dimers and tetramers is crucial to its function and that small
molecular inhibitors like BLT-1 inhibit SR-BI function not only by blocking ligand-binding sites but also by preventing the formation of these chemical structures.

1.4.5 Intestinal SR-BI

There is a considerable body of research detailing the function of SR-BI in the liver and steroidogenic tissues, as well as its function in RCT and selective lipid uptake from HDL. However, the role of SR-BI in the intestine is poorly understood and has yet to be defined. Its functions in the liver and steroidogenic tissues suggest that intestinal SR-BI may be involved in CHOL uptake.

The expression of SR-BI in the intestine grants significant merit to the hypothesis that intestinal SR-BI may be involved in dietary lipid uptake. Intestinal SR-BI is expressed highly in the proximal small intestine, with expression levels decreasing towards the distal small intestine and increasing levels of SR-BI from crypt-to-villus (Bietrix et al., 2006b; Cai et al., 2001; Cao et al., 1997; Han & Lee, 2002; Hansen et al., 2003; Levy et al., 2004; Lobo et al., 2001; Rigotti et al., 2003).

A major determinant of intestinal SR-BI expression is delivery of biliary lipid to the intestine. It has been shown that when bile delivery to the intestine is reduced by means of bile duct ligation or bile diversion, intestinal SR-BI expression and cholesterol absorption are also reduced (Voshol et al., 2001). This finding, along with other data, has suggested that intestinal SR-BI is involved in dietary lipid uptake (Hauser et al., 1998; Rhainds & Brissette, 2004; Rigotti et al., 2003).

Another attractive hypothesis is that intestinal SR-BI plays a role in lipid trafficking within the enterocyte. Following fat absorption, large lipid droplets form in enterocytes that act as storage sites for dietary lipids (Hansen et al., 2003; Olofsson et al., 2009; Redgrave, 1971; Sage & Jersild, 1971; Salentinig et al., 2011). Those lipid droplets can then be made available to the ER lumen, where apoB48 lipidation (the rate-limiting step in CM formation) occurs (Cartwright et al., 2000; Kindel et al., 2010; Mahley & Hussain, 1991; Hussain, 2000; Mansbach & Siddiqi, 2010; Redgrave, 2004; Xiao et al., 2011; Xiao & Lewis, 2012).
It is possible, therefore, that intestinal SR-BI senses dietary lipids in the intestinal lumen and is internalized into cytosolic lipid droplets (CLD). Here it may play a role in the organization of CLD, aiding in their storage or delivery to the ER depending on the state of the enterocyte and signaling cascades.

1.4.6 SR-BI signaling

Yet another important aspect of SR-BI function is SR-BI-mediated signaling. It has been demonstrated that SR-BI is capable of mediating signaling cascades upon binding to HDL in vascular endothelial cells (Al-Jarallah & Trigatti, 2010; Assanasen et al., 2005; Mineo & Shaul, 2003; Saddar et al., 2010; Sahoo & Drover, 2006; Zhu et al., 2008). In response to binding HDL, SR-BI causes the activation of Src upstream of PI3K, leading to signaling that activates endothelial nitric oxide synthase (eNOS) (Al-Jarallah & Trigatti, 2010). The SR-BI-mediated activation of eNOS by HDL occurs in a PI3K-mediated fashion (Al-Jarallah & Trigatti, 2010; Saddar et al., 2010).

Aside from eNOS activation, it has also been shown that SR-BI signaling leads to cell migration and the formation of lamellipodia independent of eNOS activation (Saddar et al., 2010). Interestingly, it was also shown that the C-terminus of SR-BI, which is required for binding to its adaptor protein PDZK1, is essential to SR-BI signaling (Al-Jarallah & Trigatti, 2010; Saddar et al., 2010; Zhu et al., 2008).

PDZK1 was first discovered to regulate steady-state level expression of SR-BI in the liver and intestine, based on the finding that in PDZK1/- mice, there is a 95% reduction in hepatic SR-BI and a 50% reduction in intestinal SR-BI (Kocher et al., 2003). Interestingly, there may be an overlap between SR-BI-mediated signaling and glucagon-like peptide-2 (GLP-2) signaling, as they both appear to induce eNOS activation. The role of GLP-2 on CM production and the possible involvement of SR-BI and PDZK1 will be covered in the following section.
1.5 Block lipid transport-1, an SR-BI inhibitor

Block lipid transport-1 (BLT-1) is a small chemical compound generated as an inhibitor of SR-BI (Nieland et al., 2008). Using high-throughput screening, BLT-1 as well as a range of other compounds were discovered to have inhibitory properties towards SR-BI function. Of those compounds, BLT-1 was the most potent and has been utilized as a tool for the study of SR-BI function (Yu et al., 2011).

![Figure 2. Structure of BLT-1 (adapted from Yu et al., 2011).](image)

The compound consists of a five-carbon ring with an alkyl chain and a thiosemicarbazone moiety. The alkyl chain and the sulfur atom in the thiosemicarbazone moiety of BLT-1 are essential to its inhibitory activity (Nieland et al., 2008).

Structure-activity relationship (SAR) studies demonstrated that the sulfur in the thiosemicarbazone moiety of BLT-1 is essential to its high-affinity binding and inhibition of SR-BI-mediated selective lipid uptake (Nieland et al., 2008). This prompted the authors of the follow-up study to investigate the importance of cysteine residues on SR-BI’s extracellular domain on BLT-1’s inhibitory effects. That led to the discovery that Cys384 present on the extracellular domain of SR-BI is the target residue for BLT-1, as mutation of Cys384 but not other free cysteine residues led to the abolishment of BLT-1’s inhibitory effects on SR-BI (Yu et al., 2011).

BLT-1 has been used in the study of SR-BI function. In one particular study, BLT-1 was used to inhibit SR-BI binding to lipid micelles in Caco-2 cells, which in turn inhibited the translocation of sub-apical apoB48 stores towards the ER following lipid stimulation in Caco-2
cells (Béaslas et al., 2009). This effect of BLT-1 on SR-BI function was not observed in Caco-2 cells lacking in SR-BI, suggesting that BLT-1 is specific to SR-BI (Béaslas et al., 2009).

1.6 Regulation of chylomicron production by GLP-2

As mentioned, CM metabolism is regulated by multiple factors. One of the more potent factors resulting in an overproduction of CM is glucagon-like peptide-2 (GLP-2), and recent work from our laboratory has demonstrated that infusion of hamsters and mice with GLP-2 results in a dramatic increase in postprandial CM production (Hsieh et al., 2008).

GLP-2 is a 33 amino acid peptide secreted by enteroendocrine L-cells in response to lipid ingestion that has been shown to be intestinotropic (Estall & Drucker, 2006). GLP-2 binds to its receptor, GLP-2R, found in enteroendocrine cells (Yusta et al., 2000), subepithelial myofibroblasts (Ørskov et al., 2005) and the central nervous system (Estall & Drucker, 2006; Hsieh et al., 2009; Tang-Christensen et al., 2000).

Interestingly, there is no trace of GLP-2R to be found in absorptive enterocytes, suggesting that the observed effects of GLP-2 on CM production must be indirect (Hsieh et al., 2009). Recent work from our laboratory has proposed that GLP-2 acts by binding the GLP-2R found in subepithelial myofibroblasts (SEM). This results in the activation of VEGF, which in turn binds to the VEGF receptor in absorptive enterocytes, causing an increase in endothelial nitric oxide synthase (eNOS) (Hsieh et al., 2010). The induction of postprandial CM overproduction by GLP-2 has been shown to be mediated by CD36, however, it is important to note that CD-36/- animals are still capable of producing CM, suggesting that other proteins may also be involved in this process (Hsieh et al., 2009).

1.7 Rationale

The intestine is now known to be more than a mere absorptive organ. It plays a key role in the regulation of lipid metabolism and is increasingly viewed as a central player in the development of insulin resistance (IR) and T2D. This highlighted by studies revealing that Roux-
en-Y gastric-bypass (RYGB) surgery in morbidly obese patients with T2D leads to significant weight-loss as well as a dramatic improvement or complete resolution of T2D (Dixon, 2009).

One of the major functions of the intestine is the absorption, digestion, assembly, and secretion of dietary lipids such as TG, CHOL, and CE in the form of intestinally-derived CM (Xiao et al., 2011). Lipid uptake in the intestine is a highly efficient process and in recent decades, it has been suggested that the process is protein-mediated rather than passive (Béaslas et al., 2009; Mu & Høy, 2004). The role of hepatic SR-BI in cholesterol homeostasis and reverse cholesterol transport (RCT) has been well established. However, the role of SR-BI in the small intestine remains unknown. Recent studies have pointed towards an involvement of SR-BI in intestinal lipid uptake and CM secretion (Bietrix et al., 2006; Béaslas et al., 2009; Hayashi et al., 2011).

The Syrian golden hamster has been used extensively in our laboratory due to its similarities to the human lipid and lipoprotein profile. First, Syrian golden hamsters possess tissue-specific editing of apoB meaning that, just like in humans, their liver produces only apoB100 and their intestine produces only apoB48 (Federico et al., 2006; Guo et al., 2005; Xiao et al., 2011). This phenomenon is not observed in mice or rats. Secondly, the plasma CHOL is primarily carried in LDL in Syrian golden hamsters, making them LDL-animals. This is another feature of hamster lipoprotein metabolism that is shared with humans but not found in rats and mice.

However, there are limitations in the use of Syrian golden hamsters, particularly the lack of available tools and reagents. Very little work has been done in terms of generating genetically modified hamsters as models of disease. In this respect, mice still provide a favorable model. In studying intestinal physiology, another limitation exists in the form of a lack of relevant intestinal cell lines. Currently, the intestinal cell line of choice is the Caco-2 cell line, which has been used extensively. However, this is a colonic cell line with a number of limitations and it is difficult to study intestinal physiology in the context of insulin resistance in vitro.

Therefore, in order to study the role of intestinal SR-BI in a physiologically relevant animal model of lipid metabolism and diet-induced insulin resistance, Syrian golden hamsters were used. In order to avoid genetic manipulations, a small molecular SR-BI inhibitor known as block lipid transport 1 (BLT-1) was employed. Based on published and preliminary findings from
our laboratory, we have observed that intestinal SR-BI mRNA and protein levels are upregulated in the small intestine of hamsters and mice with diet-induced insulin resistance (Hayashi et al., 2011). These findings are consistent with another study that also showed increased mRNA and protein expression of intestinal SR-BI in mice fed a high-fat diet (Velde et al., 2008). Our findings suggest that there might be a correlation between intestinal SR-BI expression and CM production. Indeed, we found that over-expression of SR-BI in Caco-2 cells led to an increase in apoB48 secretion (Hayashi et al., 2011). Conversely, knocking down SR-BI expression by siRNA led to a decrease in apoB48 secretion (Hayashi et al., 2011). Consistently, another group using transgenic mice over-expressing intestinal SR-BI demonstrated an increase in both TG and CHOL absorption following a fat load (Bietrix et al., 2006).

These findings suggest a potential role for intestinal SR-BI in CM production. Therefore, it is my hypothesis that intestinal SR-BI plays a role in CM production in normal and insulin resistant states and that inhibition of intestinal SR-BI function will lead to reduced CM production. To test this hypothesis, two approaches were taken: (1) CM production was assessed in normal and insulin resistant Syrian golden hamsters and Sprague-Dawley rats treated with BLT-1 as an inhibitor of intestinal SR-BI function; and (2) CM production was assessed in SR-BI/- mice. The major aim of these studies was to determine, in vivo, whether intestinal SR-BI plays a role in CM production in normal and insulin resistant states.

1.7.1 Hypothesis and Specific aims

**Hypothesis:** Intestinal SR-BI plays a role in CM production in normal and insulin resistant states and that inhibition of intestinal SR-BI function will lead to reduced CM production.

**Aim I:** Inhibition of intestinal SR-BI with BLT-1 in Syrian golden hamsters and rats.

To assess the role of SR-BI in CM production and TG accumulation in plasma, Syrian golden hamsters and rats were administered BLT-1, an inhibitor of SR-BI, followed by a fat load. Plasma was collected for up to two hours and analyzed for TG, CHOL, and CM levels.
**Aim IIa:** Prolonged inhibition of SR-BI with BLT-1 in a model of diet-induced insulin resistance.

To assess the role of intestinal SR-BI in insulin resistance, Syrian golden hamsters were fed a high-fat, high-fructose, and high-cholesterol diet (FFC) for 10 days and were administered 1 mg/kg BLT-1 or vehicle twice-daily. Weights and food consumption were recorded and, upon feeding completion, fasting and postprandial lipemia was assessed by measuring fasting and postprandial TG, CHOL, and CM levels in plasma.

**Aim IIb:** BLT-1 toxicity in hamsters following prolonged BLT-1 treatment.

To determine if prolonged BLT-1 treatment caused toxicity to hamsters, intestine, liver, and kidney tissues were sectioned and stained for H&E and Oil Red O, to determine structural morphology and lipid content. Plasma parameters for kidney and liver injury were also measured.

**Aim IIIa:** CM secretion in SR-BI/-/ and PDZK1/-/ mice.

To confirm our findings that inhibition of SR-BI with BLT-1 leads to reduced CM secretion, fat tolerance tests will be performed on C57B6/J WT and SR-BI/-/ mice and postprandial TG accumulation will be assessed.

**Aim IIIb:** Effect of GLP-2 on CM production in SR-BI/-/ mice.

To determine whether GLP-2 can induce CM overproduction in the absence of SR-BI, SR-BI/-/ mice were administered GLP-2 followed by fat tolerance test.

**Aim IIIc:** Effect of GLP-2 on CM production in PDZK1/-/ mice.

To determine whether GLP-2 can induce CM overproduction in the presence of SR-BI but in the absence of PDZK1 (and thus in the absence of SR-BI signaling) fat tolerance tests were performed in PDZK1/-/ mice treated with or without GLP-2.
2 Materials and methods

2.1 Chemicals and reagents

Bovine Serum Albumin (BSA), glycerol, polyoxyethylenesorbitam monolaurate (Tween 20), β-mercaptoethanol and formaldehyde were purchased from Sigma Aldrich (St. Louis, MO). Methanol and ethanol were purchased from Caledon Laboratories (Georgetown, ON). Complete, mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany).

Anti-SR-BI antibody was purchased from Novus Biologicals (Littleton, CO). Antibodies specific to ABCG5, ABCG8 and FATP4 were purchased from Santa Cruz (Santa Cruz, CA). Antibody specific to β-actin was purchased from Sigma Aldrich (St. Louis, MO). Horse radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Biosciences (Pittsburgh, PA).

Tris(hydroxymethyl)-aminomethane (TRIS), glycine, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N,N’,N’-tetramethyl-ethylenediamine (TEMED), 40% acrylamide/bis solution (29:1 with 3.3%C), blocking-grade non-fat dry milk and Bradford-based protein assay kits were purchased from Bio-Rad (Mississauga, ON). Polyvinylidene fluoride (PVDF) transfer membranes were purchased from Perkin Elmer (Boston, MA). Enhanced chemiluminescence (ECL) detection reagents for Western blot were purchased from Amersham Biosciences (Pittsburgh, PA).

2.2 Laboratory supplies and apparatus

Serological pipettes (5 mL, 10 mL and 25 mL), micropipette tips (10 μL, 200 μL and 1000 μL), microfuge tubes (1.5 mL and 2.0 mL), cryo-vials (2 mL) and 96-well tissue culture plates were purchased from Sarsedt Inc. (Montreal, PQ).

Optimum cutting temperature (O.C.T.) buffer and cassettes were purchased from Sakura Finetek (Torrence, CA). Syringes (1mL and 3mL), needles (21G, 23G, 25G and 30G) and sterile
surgical blades were purchased from BD Biosciences (Franklin Lakes, NJ). Electrophoresis and transfer units, as well as cassette holders, sponges and pads for SDS-PAGE and immunoblotting were purchased from Bio-Rad (Mississauga, ON). Surgical equipment (scissors, forceps and probes) as well as oral gavage feeding needles were purchased from Fine Science Tools (Vancouver, BC).

2.3 Animals

Male Syrian golden hamsters (100-120 g) and Sprague Dawley rats (180-200 g) were purchased from Charles River (Montreal, PQ) and housed individually in a 12-hour light/dark cycle. All animals were fed regular chow diet ad libitum.

C57B6/J WT, B6/129* WT, SR-BI/- and PDZK1/- mice were a generous gift by Dr. Bernardo Trigatti from McMaster University. The mice were housed in pairs and maintained on a 12-hour light/dark cycle. All mice were fed regular chow diet ad libitum. The SR-BI/- mice were backcrossed 10 generations onto a C57B6/J background. C57B6/J WT mice were originally ordered from Jackson Laboratories (Bar Harbor, ME) and bred in-house. The PDZK1/- mice were originally ordered from Jackson Laboratories (Bar Harbor, ME) and bred in-house on a mixed C57BL6/J:129S6 background. The B6129/J control mice were purchased as F2 hybrids from Jackson Laboratories (Bar Harbor, ME) and served as controls for PDZK1/- mice.

All procedures were carried out in compliance with the guidelines of the Hospital for Sick Children Animal Ethics Committee, the Canadian Council on Animal Care and the Institutional Ethics Committee at McMaster University.
2.4 Assessment of postprandial TRL production *in vivo* in hamsters

Hamsters were anaesthetized using isoflurane. The right jugular vein was exposed and cannulated. The cannula was filled with heparinized saline (40 IU/mL), sealed and exteriorized at the back of the neck, to allow for the collection of blood samples. The hamsters were allowed to recover for 24 hours as per approved protocol, followed by an over-night fast. The following day, fasting blood was collected and was followed by oral gavage of 200 μL of olive oil. At 20 minutes after fat load, 20% Pluronic F-127 (Poloxamer 407, Sigma-Aldrich, St. Louis, MO) was administered at a dose of 500 mg/kg by intraperitoneal (IP) injection, to inhibit TRL catabolism. Blood samples (400 μL) were collected into lithium heparin-coated tubes (BD Biosciences, Franklin Lakes, NJ) at 30, 60, 90 and 120 minutes after fat load. This procedure is also known as a fat tolerance test.

Plasma was isolated by centrifugation of blood samples at 6,000rpm for 5min using an Allegra X22-R benchtop centrifuge (Beckman Coulter). Next, 150 μL of plasma was layered under 4 mL of KBr buffer (d = 1.006 g/mL) in polyallomer Microfuge tubes (Beckman Coulter, Palo Alto, CA) and centrifuged at 35,000 rpm at 10°C for 70 minutes in a SW55Ti rotor using an Optima LE-80K Ultracentrifuge (Beckman Coulter) to isolate the TRL fraction (Sf>400).

Total plasma and TRL plasma fraction samples were analyzed for TG and CHOL content using colorimetric assay kits (Randox, Crumlin, UK). TRL plasma apoB48 was determined by immunoblotting. TRL plasma (20 μL) was separated by SDS-PAGE (6% gels) overnight at 30V and transferred onto PVDF membranes using the Bio-Rad wet transfer system at 100V for 2 hours at 4°C. Next, the membranes were blocked with 5% fat-free milk solution, followed by incubation with anti-human apoB serum (1:800) for 1 hour. After washing with TBST buffer, the membranes were incubated with peroxidase-conjugated goat secondary antibody (1:40,000) for 1 hour. Finally, membranes were incubated with ECL and exposed to Hyperfilm. Films were scanned and densitometrically quantified using AlphaEaseFC™ software. The values obtained by densitometry were normalized to baseline plasma values and expressed as percent baseline.
2.5 Assessment of postprandial TRL production *in vivo* in rats

Rats were anaesthetized and jugular cannulation surgery was performed as described in section 2.2. The animals were allowed to rest then fasted overnight. The following day, fasting blood samples were collected and fat tolerance tests were performed as described in section 2.2. Rats were then sacrificed for collection of liver and jejunum, which were flash-frozen for mRNA and protein analysis. To isolate the jejunum, the entire length of the small intestine was excised. To remove the duodenum, a cut was made at the ligament of Treitz. To remove the ileum, a 5cm fragment from the distal portion of the small intestine was cut. Two more cuts at the proximal and distal end of the middle fragment were made, to ensure that there was no duodenum or ileum remaining in the jejunum fragment.

2.6 Assessment of postprandial TRL production *in vivo* in mice

Following an overnight fast, baseline blood samples were collected by tail-vein bleed, followed by 200 μL olive oil gavage. Administration of oral fat load was designated time 0 minutes. At 20 minutes after fat load, poloxamer was administered at 500 mg/kg by IP injection. At 30, 60 and 90 minutes, 50 μL of blood was collected by tail bleeds into EDTA-coated blood collection tubes (Sarsedt, Montreal, PQ). Plasma glucose was measured at every time-point using an Aviva Accu-Chek® glucometer purchased from Roche (Laval, QC). At 120 minutes, a final glucose measurement was made before mice were anaesthetized.

The final blood sample collection was performed under anaesthesia by cardiac puncture, to allow collection of a larger volume of blood (up to 1 mL). Mice were then sacrificed for collection of tissues.

Liver, duodenum, jejunum, ileum, epididymal fat pads and muscle tissues were excised. Tissues were flash-frozen in liquid nitrogen. Liver and jejunum segments were also cryo-preserved in O.C.T. buffer, or fixed in formalin.
2.7 Acute treatment of hamsters and rats with 1 mg/kg BLT-1

The acute BLT-1 treatment experiments were designed as a modification of the fat tolerance test. Briefly, jugular cannulation surgery was performed on mice or rats as described in section 2.2. After an overnight fast, baseline blood samples were collected, followed by oral administration of 1 mg/kg BLT-1 by gavage. Olive oil gavage was given 30 minutes after BLT-1, to allow it to reach the jejunum and inhibit SR-BI prior to olive oil administration. BLT-1 was diluted in PBS, which was used as vehicle for control animals. The fat tolerance test was then carried out as described in section 2.2.

Following the fat tolerance test, animals were sacrificed for tissue collection. Liver and jejunum was collected and flash-frozen in liquid nitrogen for measurement of mRNA levels by qRT-PCR and protein levels by immunoblot.

2.8 Prolonged BLT-1 treatment in a hamster model of diet-induced insulin resistance

Hamsters were fasted for 5 hours, anaesthetized and fasting blood was collected by intra-orbital bleed. Hamster weights were recorded and fasting plasma TG and CHOL were determined as described in section 2.2. Hamsters were then normalized by weight, TG and CHOL levels and split into two groups: one group to be treated with vehicle, the other with BLT-1. The following day, the regular chow diet was taken away and replaced with high-fructose, high-fat, high-cholesterol (FFC) diet (40%, 30%, and 0.25% respectively). The FFC diet was purchased from Dyets Inc. (Bethlehem, PA).

During the 10 day feeding, hamsters were weighed prior to administration of 1 mg/kg BLT-1 or vehicle twice-daily. Vehicle and BLT-1 were administered orally by gavage, under anesthesia, so as to minimize stress or injury to the animals. The FFC diet came as a powder and was placed in metal dishes within the hamster cages. Food was replaced every two days and weighed, in order to keep track of food consumption.

At the end of the feeding, hamsters were anaesthetized and jugular cannulation surgery was performed as described in section 2.2. The animals were allowed to rest and fasted.
overnight. The following day, fat tolerance tests were performed as described in section 2.2. At the end of the fat tolerance test, animals were sacrificed for tissue collection.

Liver, jejunum and epididymal fat pads were excised and weighed to determine any changes in wet weight. Tissues were then flash-frozen in liquid nitrogen for measurement of mRNA and protein expression levels.

2.9 Assessment of toxicity in hamsters following prolonged BLT-1 treatment

To determine if prolonged dosing of hamsters with 1 mg/kg BLT-1 led to injury or toxicity to key tissues, the prolonged BLT-1 treatment of hamsters on FFC diet was repeated as described in section 2.6.

Prior to commencement of feeding, fasting blood samples were collected by intra-orbital bleed under anesthesia. Fasting blood plasma was analyzed for TG and CHOL, as well as liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) which are known markers of liver injury (Hanley et al., 2004; Hanley et al., 2005). Plasma creatinine levels were also measured, as they are markers of kidney injury. Hamsters were normalized by weight and fasting TG and CHOL levels and placed on FFC diet for 10 days. Vehicle or 1 mg/kg BLT-1 was administered twice-daily by oral gavage.

At the end of the feeding, hamsters were anaesthetized and underwent jugular cannulation surgery. Following an overnight fast, fat tolerance experiments were performed as described in section 2.2. After the fat tolerance test, animals were sacrificed for tissue collection.

2.10 Histology and tissue staining

The liver, jejunum, epididymal fat pad and kidney were excised and weighed to determine wet tissue weight. Kidney, liver and jejunum sections were submerged in 10% formalin for 24 hours, then washed with PBS and placed in ethanol for preservation. Segments of
liver and jejunum were also submerged in O.C.T. buffer, frozen in liquid nitrogen and stored at -80°C.

Formalin-fixed tissues were sent to the Toronto Centre for Phenogenomics (TCP) for Haematoxylin and Eosin (H&E) staining, to visualize the morphology and structure of liver, jejunum and kidney sections. Tissues frozen in O.C.T. buffer were also sent to TCP for Oil Red O staining.

2.11 Assessment of postprandial TRL production \textit{in vivo} in mice administered GLP-2

Following an overnight fast, baseline blood samples were collected by tail-vein bleed, followed by 200 µL olive oil gavage. At 20 minutes after fat load, poloxamer was administered by IP injection (500 mg/kg). Immediately after poloxamer injection, 0.25 mg/kg GLP-2 or vehicle (PBS) was administered by IP injection. At 30, 60 and 90 minutes, 50uL of blood was collected by tail bleeds as described in section 2.6.

Mice were anaesthetized for collection of 120 minute blood sample by cardiac puncture and were then sacrificed for tissue collection. Liver, duodenum, jejunum, ileum and epididymal fat pads were excised. Tissues were flash-frozen in liquid nitrogen. Liver and jejunum segments were also cryopreserved in O.C.T. buffer, or fixed in formalin.

2.12 Assessment of tissue lipid content

Jejunum was collected and snap-frozen in liquid nitrogen and stored at -80°C until further use. Mortar and pestle were kept on dry ice and a fragment of frozen of jejunum was placed into the mortar. Liquid nitrogen was also poured into the mortar and the jejunum was ground into a powder carefully, as the liquid nitrogen evaporated. Jejunum powder was then weighed to obtain 300 mg, which was placed in 20 volumes of 2:1 chloroform:methanol mixture, in 15 mL falcon tubes. The tubes were left in the fume hood with lids off for 2 days to evaporate.
Tissue was then removed and 0.2 volume of 0.9% NaCl was added and the mixtures were centrifuged at 2,000 rpm for 3 minutes. The upper layer was suctioned off and the remainder was left to evaporate over-night or over several nights as necessary. Lipids would form a film at the bottom of the tubes which would be dissolved in 100% EtOH. TG and CHOL were measured by colorimetric assay kits purchased from Randox (Crumlin, UK).

2.13 SDS-PAGE and immunoblotting in liver and jejunum tissue samples

Tissues were snap-frozen and stored at -80°C until further use. For SDS-PAGE, tissues were ground into a powder using mortar and pestle as described in section 2.12. Tissue powder was then placed into 1.5 mL microfuge tubes. Zirconium oxide beads (0.5 mm in diameter) were purchased from Next Advance Inc. (Averill Park, NY). Zirconium oxide beads were added to the tissue powder and dissolved into 1 mL of solubilizing buffer and homogenized using a Bullet Blender purchased from Next Advance Inc. (Averill Park, NY), for 5 minutes. After homogenizing, samples were spun at 13,000 rpm for 10 minutes and the supernatant was collected.

Protein concentration was determined using the Bio-Rad protein assay kit (Mississauga, ON). Proteins were separated using SDS-PAGE. Different amounts of total protein were loaded onto gels depending on the specific protein. Following SDS-PAGE, protein was transferred onto PVDF membranes for immunoblotting. Proteins were visualized using the ECL detection method and exposed onto film. Films were scanned and bands were quantified by densitometry using the AlphaEaseFC™ software.

2.14 Statistical Analysis

Depending on the nature of the data to be analyzed, Student’s t-tests and one-way or two-way analysis of variance (ANOVA) were performed using GraphPad Prism software. P-values lower than 0.05 were considered significant.
3 Results

3.1 BLT-1 lowers postprandial plasma TG, TRL TG and apoB48 accumulation in dose-dependent manner

To determine the effective dose of BLT-1 in hamsters, a dose-response experiment was designed. Syrian golden hamsters maintained on regular chow diet (CF) were orally administered vehicle, 0.5 mg/kg BLT-1, 1.0 mg/kg BLT-1 or 2.0 mg/kg BLT-1 (Fig. 1). It was determined that total plasma TG, TRL TG and apoB48 accumulation decreased with increasing amounts of BLT-1 (Fig. 1A, C, D). Total plasma CHOL was only reduced with 2.0 mg/kg BLT-1 (Fig. 1B). The 1 mg/kg BLT-1 dose lead to reduced total and TRL plasma TG and a decrease in apoB48 accumulation (Fig. 1A, C, D).

![Graphs showing dose response plasma TG and CHOL](image)

![Graphs showing dose response TRL TG and apoB48](image)

Figure 3. BLT-1 dose-response experiment in CF hamsters. After an over-night fast, hamsters were treated orally with vehicle, 0.5 mg/kg BLT-1, 1.0 mg/kg BLT-1 or 2.0 mg/kg BLT-1, followed by oral fat load (N=1). Plasma was collected at 30, 60, 90 and 120 minutes after fat load. BLT-1 lead to reduced accumulation of TG in total plasma (A) and in TRL plasma fraction (C) as well as reduced apoB48 accumulation (D) in a dose-dependent manner. Total plasma CHOL was decreased with 2.0 mg/kg BLT-1 but not with the other doses (B).
3.2 Single dose treatment of CF hamsters with 1 mg/kg BLT-1 lowers postprandial TG accumulation

Following an over-night fast, hamsters were administered a single dose of vehicle or 1 mg/kg BLT-1 30 minutes prior to fat load. Plasma was collected for up to 2 hours and analyzed for TG and CHOL content. Total plasma TG accumulation following fat load was significantly lower in hamsters treated with 1 mg/kg BLT-1 compared to vehicle at 2 hours (Fig. 2A). No changes were observed in total plasma and TRL CHOL in hamsters treated or not with 1 mg/kg BLT-1 (Fig. 2B, D). TRL TG was also significantly reduced in hamsters treated with 1 mg/kg BLT-1 at 2 hours after fat load (Fig. 2C). Therefore, BLT-1 lead to a reduction in postprandial TG accumulation in total plasma and TRL plasma in hamsters.

Figure 4. Acute treatment of CF hamsters with 1 mg/kg BLT-1. A single dose of 1 mg/kg BLT-1 was administered by oral gavage 30 minutes prior to oral fat load. Pluronic F-127 was used to block lipolysis. Plasma was collected every 30 minutes for up to 2 hours and analyzed for total plasma TG (A) and CHOL (B). TRL plasma fraction was isolated and analyzed for TRL TG (C) and TRL CHOL (D). Results were analyzed using 2-way ANOVA (*p<0.05, **p<0.01,). Graphs (A-D) represent an N=5-6.
3.3 Single dose treatment of CF rats with 1 mg/kg BLT-1 lowers postprandial TG accumulation

To reproduce the observations made in hamsters, Sprague Dawley rats were administered orally with vehicle or 1 mg/kg BLT-1 30 minutes prior to fat load. Plasma was collected at 30, 60, 90 and 120 minutes after fat load and analyzed for TG and CHOL content. Total plasma TG accumulation was significantly reduced at 2 hours in rats treated with 1 mg/kg BLT-1 (Fig. 3A). TRL TG accumulation was also significantly reduced at 2 hours in rats treated with 1 mg/kg BLT-1 (Fig. 3B). A mild but insignificant reduction in TRL CHOL was observed at 2 hours, possibly due to reduced CM secretion (Fig. 3D). No change in total plasma CHOL was observed in rats treated with 1 mg/kg BLT-1 (Fig. 3C). Therefore, BLT-1 reduced postprandial TG accumulation in total plasma and TRL plasma fractions, in accordance to the observations made in hamsters.

Figure 5. Acute treatment of CF rats with 1 mg/kg BLT-1. Rats were administered single dose of either vehicle or 1 mg/kg BLT-1 orally, followed by fat load after 30 minutes. Plasma was collected for up to 2 hours and analyzed for total plasma TG (A) and CHOL (C). TRL plasma fraction was isolated and analyzed for TRL TG (B) and TRL CHOL (D). Results were analyzed using 2-way ANOVA (*p<0.05, ***p<0.001). Graphs (A-D) represent an N=5-6.
3.4 Prolonged BLT-1 treatment of hamsters on FFC diet

Syrian golden hamsters were placed on FFC diet for 10 days. During the feeding, 1 mg/kg BLT-1 was orally administered twice-daily for 10 days. Hamster weights and food consumption were recorded daily (Fig. 4A, B). Fasting plasma lipids were measured before and after feeding (Fig. 4C, D). At the end-point, hamsters were fasted over-night and the acute BLT-1 treatment followed by fat tolerance test was performed. Plasma was collected for up to two hours and assessed for TG, CHOL and apoB48 (Fig. 5). Hamsters with prolonged BLT-1 treatment demonstrated reduced weight-gain compared to controls (Fig. 4A) despite no changes in food consumption (Fig. 4B). Fasting plasma TG was significantly reduced in hamsters following prolonged BLT-1 treatment compared to controls (Fig. 4C) while plasma CHOL levels were elevated in both groups (Fig. 4D).

Figure 6. Prolonged BLT-1 treatment of hamsters fed FFC diet. Hamsters were treated twice-daily with vehicle or 1 mg/kg BLT-1 for 10 days while on the FFC diet. Weights (A) and food consumption (B) were recorded daily. Fasting plasma was collected by intra-orbital bleed at day the start and end of feeding. Fasting plasma was analyzed for TG (C) and CHOL (D). Results were analyzed using t-test (*p<0.05). Graphs (A-D) represent an N=8-10.
Fat tolerance tests were performed in hamsters from the prolonged BLT-1 treatment experiments. Prolonged BLT-1 treatment led to significant reductions in TG accumulation in total and TRL plasma (Fig. 5A and C, respectively). Total plasma CHOL levels were unchanged (Fig. 5B) but TRL CHOL levels were significantly reduced with BLT-1 treatment (Fig. 5D). That can be explained by the dramatic reductions in both fasting and postprandial apoB48 levels. Immunoblotting for apoB48 in TRL plasma fractions revealed visible reductions in apoB48 levels in hamsters with prolonged BLT-1 treatment (Fig. 5F). Quantification of immunoblots revealed significant reductions in apoB48 levels with prolonged BLT-1 treatment (Fig. 5E and 5G).

**Figure 7.** Plasma lipids in hamsters following FFC feeding and prolonged treatment with vehicle or 1 mg/kg BLT-1. After FFC feeding and prolonged BLT-1 administration, fat tolerance test was performed on hamsters. A single dose of BLT-1 was administered orally 30 minutes prior to fat tolerance test and plasma was collected every 30 minutes for up to 2 hours. Total plasma TG (A) and CHOL (B) were measured. TRL plasma fraction was isolated and analyzed for TRL TG (C) and TRL CHOL (D). TRL plasma was immunoblotted for apoB48 (F) to determine apoB48 accumulation (E). Results were analyzed using 2-way ANOVA (*p<0.05, **p<0.01, ***p<0.001). Graphs (A-E) represent an N=5-6.
3.5 Hamster jejunum and liver lipid content after single dose treatment with 1 mg/kg BLT-1

To further explore the lipid-lowering effects of BLT-1, Oil Red O staining was performed on jejunum and liver tissue of hamsters treated acutely with 1 mg/kg BLT-1 followed by fat load. Two hours after fat load, hamsters were anaesthetized, and the liver and jejunum were excised and frozen in O.C.T. buffer. Oil Red O staining was performed in jejunum and liver sections (Fig. 6). Oil Red O staining appeared weaker in jejunum and liver sections of hamsters treated with 1 mg/kg BLT-1.

Figure 8. Oil Red O staining of hamster jejunum following acute treatment with vehicle or 1 mg/kg BLT-1. A single dose of 1 mg/kg BLT-1 was administered by oral gavage, 30 minutes prior to fat tolerance test. Following that, hamsters were anaesthetized, jejunum and liver sections were collected, submerged in O.C.T. buffer and frozen in liquid nitrogen. Oil Red O staining was performed on jejunum (A and B) and liver (C and D) sections. All images are at 20x magnification.
3.6 Hamster jejunum and liver lipid content after prolonged treatment with 1 mg/kg BLT-1

Hamsters were fed FFC diet for 10 days and treated with vehicle or 1 mg/kg BLT-1 twice-daily. After an over-night fast, hamsters were treated acutely with 1 mg/kg BLT-1, followed by fat load. Two hours after fat load, hamsters were anaesthetized, and the liver and jejunum were excised and frozen in O.C.T. buffer. Oil Red O staining was then performed on jejunum and liver sections (Fig. 7). Oil Red O staining was quantified using Volocity® software and expressed as a percentage of surface area stained red over total tissue surface area (Fig. 8A, B). Results were analyzed by t-test and revealed no significant differences. Interestingly, a mild reduction in jejunum Oil Red O staining was observed (Fig. 8A). A mild increase in hepatic Oil Red O staining was also observed (Fig. 8B). To confirm those findings, jejunum and liver tissue was homogenized and TG and CHOL content was determined as previously described. Fecal TG and CHOL were also assessed. Results were analyzed by t-test and revealed no statistical differences. Interestingly, a decrease in TG was observed in jejunum while no changes in TG content of liver and feces were observed (Fig. 8C). Similarly, a decrease in jejunum CHOL content was observed; no changes in liver CHOL content and a minor elevation in fecal CHOL content were also observed (Fig. 8D).

![Figure 9](image)

**Figure 9.** Oil Red O staining of hamster liver following prolonged treatment with vehicle or 1 mg/kg BLT-1. Oil Red O staining was performed in jejunum isolated from hamsters after
prolonged administration with vehicle (A) or BLT-1 (B). Liver sections were also collected for Oil Red O staining (C and D). All images are at 20x magnification.

**Figure 10.** Tissue lipid content of FFC-fed hamsters 1 mg/kg with prolonged BLT-1 treatment. Oil Red O stained slides were scanned and quantified from jejunum (A) and liver (B) samples. Quantification was performed by measuring total red pixels and expressing that value as a percentage of total tissue surface area. To complement Oil Red O staining, tissue lipids were quantified by colorimetric assay kits. Jejunum, liver and feces were analyzed for TG (C) and CHOL (D) content. Results were analyzed t-test. Graphs (A-D) represent an N=5-6. Oil Red O staining images were quantified using Volocity® software.
3.7 Protein expression of lipid transporters in hamster jejunum and liver after FFC-feeding and prolonged treatment with BLT-1

After the 10-day FFC-feeding and prolonged treatment with vehicle or 1 mg/kg BLT-1 twice-daily, hamsters were anaesthetized for collection of jejunum and liver. Tissues were snap-frozen in liquid nitrogen, followed by homogenization and protein extraction. Protein levels of select lipid transporters (SR-BI, FATP4, NPC1L1, ABCG5 and ABCG8) were determined by immunoblot and quantified using ImageJ® software. SR-BI was found to be mildly but significantly downregulated in hamster jejunum following prolonged BLT-1 treatment (Fig. 9A). No significant changes were observed in the rest of the transporters measured in the jejunum (Fig. 9A). Interestingly, hepatic SR-BI protein expression was significantly increased in hamsters following prolonged BLT-1 treatment (Fig. 9B). ABCG5 was also found to be increased, but no other changes in hepatic lipid transporter protein expression were observed (Fig. 9B).

Figure 11. Jejunum and liver protein expression of key lipid transporters in 1 mg/kg FFC-red hamsters with prolonged BLT-1 treatment. Tissue protein expression of select lipid transporters was determined by immunoblot in jejunum (A) and liver (B) samples from hamsters following prolonged BLT-1 administration. Immunoblots were quantified using ImageJ® software. Results were analyzed using 1-way ANOVA (*p<0.05, **p<0.01). Graphs (A, B) represent an N=5-6.
3.8 Prolonged BLT-1 treatment did not lead to hepatic or renal toxicity in hamsters

To determine whether prolonged BLT-1 treatment caused toxicity to liver, jejunum and kidney tissues, the prolonged BLT-1 treatment experiment was repeated. Hamster weights were recorded daily and again it was observed that prolonged BLT-1 treatment led to a reduction in weight-gain (Fig. 10A). Fasting plasma creatinine, AST and ALT were measured prior to and after the feeding and BLT-1 treatment. No changes were observed in plasma creatinine following prolonged BLT-1 treatment, indicating no kidney toxicity (Fig. 10B). No changes were observed in plasma AST and ALT following prolonged BLT-1 treatment, indicating no liver toxicity (Fig. 10C and D respectively).

Figure 12. Plasma creatinine, AST and ALT levels in 1 mg/kg FFC-fed hamsters with prolonged BLT-1 treatment. The prolonged BLT-1 experiment was repeated, to determine if there was any toxicity caused by BLT-1. Fasting plasma was collected prior to and after the feeding and was analyzed for creatinine (B), as well as AST (C) and ALT (D) levels. Results were analyzed using 2-way ANOVA. Graphs (A-D) represent an N=5-6.
3.9 Acute or Prolonged BLT-1 treatment did not lead to changes in H&E staining in jejunum, liver or kidney

To further explore if BLT-1 is toxic, tissue integrity and morphology were studied by H&E staining of jejunum, liver and kidney tissue sections from hamsters following acute and prolonged treatment with BLT-1. At 2 hours after fat load (2.5 hours after BLT-1 administration) hamsters were anaesthetized and the jejunum, liver and kidneys were excised. Tissues were fixed in formalin for paraffin embedding and H&E staining was performed on tissue sections (Fig. 11). There were no changes in jejunum tissue morphology in hamsters treated following acute or prolonged treatment with BLT-1 compared to vehicle. Villi appeared normal and there was no visible detachment of enterocytes or any structural abnormalities in the jejunum of hamsters treated with BLT-1 compared to vehicle (Fig. 11A-D). Similarly, no changes were observed in liver tissue structure and integrity in hamsters treated with BLT-1 compared to vehicle, in hamsters with acute (Fig. 11E, F) and prolonged BLT-1 treatment (Fig. 11G, H). Finally, no changes in kidney tissue morphology or structure were observed in hamsters following acute and prolonged BLT-1 administration (Fig. 11I-L). Based on the H&E stains, BLT-1 does not appear to cause toxicity to hamsters following acute or prolonged treatment.
Figure 13. Tissue morphology visualized by H&E stain, following acute or prolonged BLT-1 treatment. Jejunum, kidney and liver tissues were collected from hamsters following acute or prolonged BLT-1 treatment. Tissues were fixed in formalin for H&E staining. Slides were scanned and visualized using Mirax Viewer software. Magnification of images is 20x for jejunum (A-D), 10x for liver (E-H) and 10x kidney (I-L) sections.
3.10 Postprandial lipid accumulation in plasma is significantly reduced in SR-BI +/- mice

To further explore the importance of intestinal SR-BI in postprandial CM production, fat tolerance tests were performed in C57BL/6 WT and SR-BI +/- mice. Similar to the experiments performed in hamsters, mice were administered oral fat load and plasma was collected by tail-vein bleed every 30 minutes for up to two hours after fat load. Plasma glucose, TG and CHOL were measured during the experiment. At the endpoint, plasma was collected by cardiac puncture for isolation of TRL plasma fraction and subsequent measurement of TRL TG and CHOL. Interestingly, there was a significant reduction in plasma glucose response in SR-BI +/- mice compared to WT (Fig. 12A, B). At 30 minutes after fat load, glucose levels reached (11.98 mM±1.36 mM) in WT mice compared to (7.85 mM±1.16 mM; p<0.01) in SR-BI +/- mice (Fig. 12A). Most importantly, in accordance with the data obtained by the BLT-1 inhibition experiments in hamsters and rats, a significant reduction in plasma TG accumulation was observed at 90 minutes and at 2 hours in SR-BI +/- mice (17.05±5.83 mmol/L) compared to WT (29.15±1.80 mmol/L; p<0.001) (Fig. 12C). Not surprisingly, SR-BI/- mice exhibited significantly elevated plasma CHOL levels compared to WT mice (Fig. 12D). That finding has been previously shown and is thought to be caused by the absence of hepatic SR-BI, leading to a reduction in reverse-cholesterol transport and consequently reduced clearance of cholesterol from the body. Interestingly, despite elevated plasma CHOL, TRL CHOL was actually lower in SR-BI +/- mice (0.29±0.20 mmol/L) compared to WT mice (0.55±0.13 mmol/L) (Fig. 12F). That points to reduced CM production in SR-BI +/- mice. Additionally, TRL TG was significantly lower in SR-BI/- mice (1.07±0.69 mmol/L) compared to WT mice (3.46±1.15 mmol/L; p<0.05) (Fig. 12E). This data suggest that intestinal SR-BI plays a role in postprandial lipid metabolism, and is in accordance with the observations made in hamsters and rats treated with the SR-BI inhibitor, BLT-1.
Figure 14. Plasma glucose and lipid levels in WT and SR-BI/- mice following oral fat load. Fat tolerance tests were performed in WT and SR-BI/- mice to determine postprandial lipid response. Plasma glucose was measured during the fat tolerance test (A). Plasma was collected every 30 minutes for up to 2 hours and analyzed for total plasma TG (C) and CHOL (D). TRL plasma fraction was isolated and analyzed for TRL TG (E) and TRL CHOL (F). Results were analyzed using 2-way ANOVA (A, C and D) or t-test CB, E and F) (*p<0.05, **p<0.01, ***p<0.001). Graphs (A-F) represent an N=4.
3.11 GLP-2 significantly enhanced postprandial lipid accumulation in WT but not in SR-BI -/- mice

It is known that GLP-2 causes postprandial hypertriglyceridemia and enhances CM production. To determine the importance of SR-BI in intestinal CM production, SR-BI/- and WT mice were treated with 0.25 mg/kg GLP-2 following an oral fat load. Blood was collected every 30 minutes for up to 2 hours and analyzed for TG, CHOL and glucose (Fig. 13). At the endpoint, blood was collected by cardiac puncture for TRL plasma isolation and subsequent measurement of TRL TG and TRL CHOL (Fig. 14). Treatment of WT and SR-BI/- mice did not lead to any changes in postprandial glucose levels (Fig. 13A and B, respectively). Postprandial TG accumulation in the plasma of WT mice treated with 0.25 mg/kg GLP-2 was significantly increased at 2 hours (35.41±1.98 mmol/L) compared to vehicle (29.15±1.80 mmol/L; p<0.001) (Fig. 13C). This difference was however smaller than expected and increasing the number of mice is required in order to determine if the observed increase in postprandial CM secretion by GLP-2 is reproducible and functionally important. Interestingly, there was no significant difference in postprandial TG accumulation in the plasma of SR-BI/- mice treated with GLP-2 or vehicle (Fig. 13D). Similarly, there was no difference in postprandial CHOL accumulation in the plasma of WT or SR-BI/- mice (Fig. 13E and F, respectively) following treatment with vehicle or GLP-2. TRL plasma CHOL was also not increased in SR-BI/- mice treated with GLP-2 compared to vehicle (Fig. 14D), but a mild elevation in TRL CHOL was observed in WT mice treated with GLP-2 (0.80±0.06 mmol/L) compared to vehicle (0.55±0.13 mmol/L) (Fig. 14C). Reflective of the observations made in total plasma, TRL plasma TG was significantly elevated in WT mice treated with GLP-2 (6.14±1.11 mmol/L) compared to vehicle (3.46±1.15 mmol/L) (Fig. 14A). In SR-BI/- mice however, GLP-2 did not lead to any changes in TRL TG 2 hours after fat load.
Figure 15. Plasma glucose and lipid levels in WT and SR-BI/- mice following oral fat load and treatment with either vehicle or 0.25 mg/kg GLP-2. Fat tolerance tests were performed in WT and SR-BI/- mice. At 20 minutes post-fat load, 0.25 mg/kg GLP-2 was administered by IP injection. Plasma glucose was measured during the fat tolerance test (A and B). Plasma was collected every 30 minutes for up to 2 hours and analyzed for total plasma TG (C and D) and CHOL (E and F). Results were analyzed using 2-way ANOVA (***p<0.001). Graphs (A-F) represent an N=4.
Figure 16. TRL plasma lipids in WT and SR-BI-/− mice following oral fat load and treatment with vehicle or 0.25 mg/kg GLP-2. Fat tolerance tests were performed in WT and SR-BI-/− mice receiving vehicle or 0.25 mg/kg GLP-2 by IP injection. At 2 hours, mice were anaesthetized and blood was collected by cardiac puncture. TRL plasma fraction was isolated and analyzed for TRL TG (A and B) and TRL CHOL (C and D) levels. Results were analyzed using t-test (*p<0.05). Graphs (A-D) represent an N=4.
3.12 GLP-2 significantly enhanced postprandial lipid accumulation in WT but not in PDZK1 -/- mice

The mechanism by which GLP-2 leads to enhanced postprandial CM production is thought to be mediated by an increase in eNOS activity in enterocytes, leading to increased circulation to the gut. It is known that SR-BI adaptor protein PDZK1 mediates signaling initiated upon the binding of SR-BI to its substrate, leading to activation of eNOS. To further examine the role of SR-BI in CM production and whether SR-BI signaling is required for GLP-2 to induce CM over-production, WT and PDZK1-/− mice were treated with 0.25 mg/kg GLP-2 or vehicle following fat load. Blood was collected by tail-vein bleed every 30 minutes for up to 2 hours and analyzed for TG, CHOL and glucose (Fig. 15). At the endpoint, blood was collected by cardiac puncture for TRL plasma isolation and subsequent analysis of TRL TG and TRL CHOL (Fig. 16). GLP-2 did not lead to changes in postprandial plasma glucose in WT or PDZK1-/− mice (Fig. 16A and B, respectively). Postprandial TG accumulation in the plasma of WT mice was significantly elevated at 2 hours, following treatment with 0.25 mg/kg GLP-2 (26.86±3.58 mmol/L) compared to vehicle (17.95±2.40 mmol/L; p<0.001) (Fig. 15C). There was no change in postprandial TG accumulation in the plasma of PDZK1-/− mice treated with GLP-2 or vehicle (Fig. 15D). Similarly, no changes were observed in postprandial CHOL levels in WT and PDZK1-/− mice following treatment with vehicle or GLP-2 (Fig. 15E and F, respectively). TRL TG was significantly elevated in WT mice treated with 0.25 mg/kg GLP-2 (5.06±0.62 mmol/L) compared to vehicle (1.94±0.53 mmol/L; p<0.01) (Fig. 16A). A mild elevation in TRL TG was observed in PDZK1-/− mice treated with GLP-2 (3.83±0.47 mmol/L) compared to vehicle (2.74±0.43 mmol/L) (Fig. 16B). No change was observed in TRL CHOL in WT and PDZK1-/− mice treated or not with GLP-2 (Fig. 16C and D, respectively).
Figure 17. Plasma glucose, TG and CHOL in PDZK1-/- and WT mice treated or not with 0.25 mg/kg GLP-2 following oral fat load. Fat tolerance tests were performed in WT and PDZK1-/- mice administered with vehicle or 0.25 mg/kg GLP-2 by IP injection. Plasma glucose was monitored during the experiment (A and B). Plasma was collected every 30 minutes for up to 2 hours and analyzed for total plasma TG (C and D) and CHOL (E and F). Results were analyzed using 2-way ANOVA (*p<0.05, **p<0.01, ***p<0.001). Graphs (A-F) represent an N=4.
**Figure 18.** TRL plasma lipids of PDZK1−/− and WT mice following fat load and treated with vehicle or 0.25 mg/kg GLP-2. Fat tolerance tests were performed in WT and PDZK1−/− mice receiving vehicle or 0.25 mg/kg GLP-2 by IP injection. At 2 hours, mice were anaesthetized and blood was collected by cardiac puncture. TRL plasma fraction was isolated and analyzed for TRL TG (A and B) and TRL CHOL (C and D) levels. Results were analyzed using t-test (*p<0.05, **p<0.01, ***p<0.001). Graphs (A-D) represent an N=4.
4 Discussion

4.1 Inhibition of intestinal SR-BI with BLT-1

The function of intestinal SR-BI has yet to be elucidated. Originally discovered as the HDL receptor in the liver, it was proposed to play a role in intestinal CHOL transport (Mardones et al., 2001). There have been a number of studies that have examined the role of intestinal SR-BI, yielding inconclusive and often contradictory results. In an early study, SR-BI was over-expressed in Chinese hamster ovary (CHO) cells and it was observed that CHOL absorption was increased and could be inhibited by ezetemibe, an inhibitor of CHOL uptake (Altmann et al., 2002). At the time, it was not known that ezetemibe inhibits NPC1L1, making the results irrelevant in the context of SR-BI. The authors believed that their findings suggested that SR-BI is involved in intestinal CHOL uptake, which was not the case.

However, once the authors performed CHOL absorption studies in SR-BI/-/- mice, it was observed that ezetemibe reduced CHOL absorption in both WT and SR-BI/-/- mice, suggesting SR-BI did not play a role in intestinal cholesterol uptake in vivo (Altmann et al., 2002). This can also be explained by the fact that ezetemibe acts on NPC1L1 and not SR-BI (Garcia-Calvo et al., 2005). Consistently, another study was also able to show that SR-BI/-/- mice did not have impaired cholesterol uptake (Mardones et al., 2001), furthering the notion that intestinal SR-BI is not involved in cholesterol uptake (Altmann et al., 2002; Mardones et al., 2001). Therefore, it remains unclear whether intestinal SR-BI really plays a role in CHOL uptake.

Our laboratory studies lipoprotein metabolism in normal and insulin resistant states. One area of focus is the small intestine, where dietary lipid uptake and CM production occurs. Based on preliminary findings from our laboratory, protein and mRNA expression of intestinal SR-BI was up-regulated in insulin resistance and correlated with the observed increase in apoB48 secretion in insulin resistant subjects (Hayashi et al., 2011). Furthermore, over-expression of SR-BI in Caco-2 cells led to an increase in apoB48 secretion, whereas inhibition of SR-BI by siRNA led to reduced apoB48 expression in Caco-2 cells (Hayashi et al., 2011).
These findings are consistent with observations made in another study where authors described SR-BI as being a lipid sensor in Caco-2 cells (Béaslas et al., 2008). In this study the authors treated Caco-2 cells with postprandial micelles (PPM) containing hydrolyzed TG products or inter-prandial micelles (IPM) containing no TG. It was found that SR-BI interacts with PPM which was shown by co-immunoprecipitation experiments. Upon binding of PPM to SR-BI, it was observed that there was a subsequent de-localization of sub-apical apoB48 pools towards the ER, a process which was successfully inhibited by SR-BI inhibitors like BLT-1, agonists (HDL) and by knock-down of SR-BI expression in Caco-2 cells (Béaslas et al., 2008). This was the first time that SR-BI was shown to play a role in apoB48 production, although apoB48 secretion was not measured. These findings suggest that SR-BI is involved in dietary lipid uptake and sensing and may be responsible for relaying a signal stimulating apoB48 synthesis and secretion.

Therefore, the first aim of the present thesis was to determine the role of SR-BI in postprandial CM secretion. This is the first study to use BLT-1 as an SR-BI inhibitor in vivo and in order to target the small intestine I used oral gavage as the method of delivery. A dose response experiment was performed to determine the minimum effective dose that caused any changes in postprandial lipid levels. Syrian golden hamsters were administered 1 mg/kg BLT-1 30 minutes before fat load, to allow the SR-BI inhibitor to reach the jejunum.

The first observation made was that acute BLT-1 treatment led to a significant reduction in postprandial TG but not CHOL accumulation in plasma (Fig. 2A and B, respectively). The acute BLT-1 treatment experiment was repeated in rats and it was also observed that BLT-1 reduced postprandial TG but not CHOL accumulation in plasma for up to 2 hours following fat load (Fig. 3A and C, respectively). These findings are consistent with the observations made in SR-BI/- mice in previous studies (Mardones et al, 2001).

To determine CM secretion into plasma in the presence or absence of BLT-1, the TG-rich lipoprotein (TRL) plasma fraction containing CM was isolated from all plasma samples for subsequent analysis of TG and CHOL content. It was observed that acute treatment with 1 mg/kg BLT-1 led to reduced postprandial TG accumulation in TRL plasma fraction, indicating reduced CM secretion by the intestine upon inhibition of SR-BI (Fig. 2C).
The same was observed in the TRL TG accumulation in rats (Fig. 3B). No changes in postprandial accumulation of CHOL in the TRL fraction were observed in hamsters and rats (Fig. 2C and 3D, respectively). Most importantly, acute BLT-1 treatment led to significant reductions in the accumulation of TG in TRL plasma of hamsters treated acutely with BLT-1 (Fig. 2C). Together, these data demonstrate that SR-BI inhibition with BLT-1 leads to reductions in postprandial TG and CM accumulation in plasma.

This is the first time a functional role has been described for intestinal SR-BI in CM metabolism in vivo using BLT-1 as an inhibitor of SR-BI function. These findings suggest that intestinal SR-BI is involved in postprandial TG and CM secretion by the intestine and not in CHOL uptake as was once thought. Consistent with our findings, Bietrix et al observed that transgenic mice over-expressing intestinal SR-BI showed an increase in both TG and CHOL secretion into plasma (Bietrix et al., 2006). Interestingly, they observed a greater increase in TG secretion compared to CHOL in the SR-BI transgenic mice, indicating the increase could be attributed to an increase in CM secretion, since CM contain primarily TG but also some CHOL (Bietrix et al., 2006).

However, their study was performed in mice and one of the limitations in working with mice is the inability to differentiate between VLDL and CM as they are of similar size and apoB100 and apoB48 can be found in both CM and VLDL. This is why the hamster model was ideal to use, since it allowed us to measure CM secretion into plasma directly, by blotting for apoB48 in the TRL plasma fraction. In summary, acute treatment of hamsters with an SR-BI inhibitor followed by a fat tolerance test showed reductions in postprandial TG and CM but not CHOL levels. That suggests that intestinal SR-BI may play a role in postprandial CM and TG secretion into plasma, rather than a role in CHOL uptake.

4.2 Prolonged BLT-1 treatment of diet-induced insulin resistant Syrian golden hamsters

Following our initial observations on the function of intestinal SR-BI in CM production, we sought to determine whether prolonged inhibition of SR-BI led to changes in lipid metabolism in a model of insulin resistance. Our laboratory employs a diet-induced model of
insulin resistance whereby Syrian golden hamsters are fed a high-fat, high-fructose, high-cholesterol (FFC) diet for 10 days, making the hamsters obese, hyperlipidemic and insulin resistant (Adeli & Lewis, 2008; Dekker et al., 2010; Federico et al., 2006; Haidari et al., 2002; Lewis et al., 2005; Rutledge & Adeli, 2007).

During the 10-day FFC-feeding, hamsters were orally administered 1 mg/kg BLT-1 twice-daily and it was immediately observed that hamsters receiving BLT-1 gained weight at a slower rate than controls while no differences in food consumption were noted (Fig. 4A and B, respectively). Fasting plasma TG and CHOL levels were measured prior to and after the feeding. Control hamsters became hypertriglyceridemic as their fasting plasma TG levels increased dramatically after the feeding, while fasting plasma TG levels were significantly lower in hamsters following prolonged BLT-1 treatment (Fig. 4C).

The increase in fasting TG in control hamsters fed FFC diet has already been shown and is consistent with previous studies from our laboratory (Adeli & Lewis, 2008; Federico et al., 2006). The reduction in fasting plasma TG in hamsters following prolonged BLT-1 treatment was a novel finding, suggesting that SR-BI not only plays a role in CM secretion but in overall lipid metabolism and energy balance. This finding is also consistent with our previous findings that SR-BI is involved in CM production.

On the other hand, fasting plasma CHOL levels were not affected by prolonged BLT-1 treatment (Fig. 4D). This finding appears to be consistent with our initial observation that acute BLT-1 treatment of hamsters did not lead to changes in postprandial CHOL levels (Fig. 2B and D).

After the prolonged BLT-1 treatment experiment, hamsters were fasted over-night and the acute BLT-1 treatment followed by fat tolerance test was performed as described in section 2.8. Total and TRL plasma TG levels were significantly lower at 2 hours after fat load in hamsters treated with BLT-1 (Fig. 5A and C, respectively). Total plasma CHOL was not affected, but TRL plasma CHOL was significantly reduced in BLT-1 treated hamsters (Fig. 5B and D, respectively). A dramatic reduction in CM secretion was observed in BLT-1 treated hamsters as determined by immunoblotting for apoB48 in TRL plasma fractions (Fig. 5F).
Fasting apoB48 levels were significantly higher in controls, while hamsters with prolonged BLT-1 treatment exhibited much lower fasting apoB48 levels (Fig. 5F). Postprandially, the accumulation of apoB48 in TRL plasma was significantly lower in BLT-1 treated hamsters compared to controls (Fig. 5E and F, respectively). The reduced TG and CM accumulation was consistent with our initial findings that BLT-1 reduced CM and TG accumulation in hamster plasma. Total plasma CHOL was not affected by prolonged BLT-1 treatment, however TRL CHOL was significantly reduced. This can be attributed to the significant reduction in CM secretion, since CMs also contain some CHOL. Moreover, the primary carriers of CHOL (HDL and LDL) are not protected by Pluronic F-127, so the postprandial plasma accumulation of lipids is largely attributed to CM.

The findings in the controls are consistent with previous studies, showing an increase in fasting plasma TG and CHOL, as well as increased postprandial lipemia following FFC-feeding (Federico et al., 2006). The findings that prolonged BLT-1 treatment lead to reduced fasting and postprandial plasma TG and CM levels is novel and suggests an important role for intestinal SR-BI in CM production.

To explain these findings, it is important to keep in mind that what we are observing is not necessarily a lipid uptake defect. The measurements made in this experimental design, can only directly assess CM and TG accumulation in plasma. Therefore, it can be inferred that CM production is slowed down leading to the observations that CM and TG accumulation in plasma is reduced when intestinal SR-BI is inhibited. This can be due to impaired dietary lipid uptake, reducing substrate availability for CM synthesis. Alternatively, this can be due to defects in intracellular events governing apoB48 trafficking as well as CM assembly and secretion.

A number of studies have recently implicated SR-BI in intracellular lipid trafficking. In one study, a novel role was proposed for intestinal SR-BI, suggesting that SR-BI acts as a lipid sensor (Béaslas et al., 2009). Upon stimulation with postprandial micelles (PPM) consisting of hydrolyzed TG, mimicking luminal contents of the intestine following a meal, the sub-apical pool of apoB48 re-localized to the ER, whereas BLT-1 inhibited that effect (Béaslas et al., 2009). Therefore, it is possible that our observations are due to intracellular events preventing apoB48 secretion. These findings are consistent with our in vivo studies.
There is another study that provides some evidence regarding the role of intestinal SR-BI in postprandial lipid metabolism. In this study, the authors obtained electron micrographs of pig enterocytes following an oral fat load. They observed that SR-BI localized primarily on the apical side of enterocytes and that upon fat ingestion, SR-BI re-localized to newly-formed large cytosolic lipid droplets (Hansen et al., 2003). Moreover, SR-BI was not found to be localized in apoAI-containing lipid droplets, showing no association of SR-BI with nascent CMs (Hansen et al., 2003). This indicates that SR-BI may not directly interact with CM particles. However, SR-BI was detected in intracellular lipid droplets, suggesting that it participates in lipid uptake and or lipid trafficking. These findings are consistent with the idea that SR-BI plays a role in lipid trafficking and uptake which can explain our observations that inhibition of SR-BI results in reduced CM secretion.

In another study, transgenic mice were generated to over-express intestinal SR-BI primarily in the apical or brush-border surface of the jejunum. Briefly, the authors placed the mouse SR-BI gene under the control of the intestine-specific apoCIII enhancer coupled to the apoAIV promoter (Bietrix et al., 2006). Fat tolerance tests were performed using Triton to block CM and VLDL lipolysis (similar to Pluronic F-127 used in our laboratory). They observed an increase in postprandial TG and CHOL plasma levels compared to controls (Bietrix et al., 2006). They also performed TG and CHOL uptake studies and observed an increase in TG and CHOL uptake in the SR-BI transgenics (Bietrix et al., 2006).

The authors were surprised to find that increased intestinal SR-BI levels correlated with an increase in TG uptake and secretion, as they were expecting to observe an increase in CHOL uptake. An increase in CHOL uptake was in fact observed, however it was much smaller compared to the increase in TG uptake, pointing towards an increase in CM secretion. Our findings suggest that SR-BI is involved in CM secretion, which may explain the finding that SR-BI over-expression led to an increase in postprandial TG observed by Bietrix et al.

The next aim of the present thesis was to determine the role of intestinal SR-BI on the development of insulin resistance and the associated hyperlipidemia. As we previously published, intestinal SR-BI was up-regulated in states of insulin resistance in mice and hamsters (Hayashi et al., 2011). This expression pattern of SR-BI correlates well with the observed
increase in apoB48 production in insulin resistant animals as well as in humans (Adeli & Lewis, 2008; Federico et al., 2006; Haidari et al., 2002; Hayashi et al., 2011).

Prolonged BLT-1 treatment experiments revealed that prolonged inhibition of SR-BI led to reductions in weight-gain (Fig. 4A), normalization of fasting TG levels (Fig. 4C), as well as reductions in postprandial lipemia (Fig. 5), in our diet-induced hamster model of insulin resistance.

Our findings that SR-BI may be involved in lipid metabolism and insulin resistant are consistent with human studies that have shown SR-BI polymorphisms affecting insulin sensitivity (Pérez-Martínez et al., 2005). Briefly, SR-BI variants in humans have been associated with changes in BMI, HDL-c and LDL-c as well as TG metabolism (Acton et al., 1999; Pérez-Martínez et al., 2005). In our study we observed that prolonged inhibition of SR-BI using BLT-1 led to improvements in lipid metabolism as well as in amelioration of diet-induced weight-gain and hyperlipidemia in insulin resistance. These findings are consistent with human studies linking SR-BI to overall lipid metabolism.

4.3 Effects of prolonged BLT-1 treatment on tissue lipid content

Following the observations that acute and prolonged BLT-1 treatment led to marked reductions in fasting and postprandial lipid levels in hamsters, we sought to determine the effect of BLT-1 on tissue lipids. To characterize the effects of acute and prolonged BLT-1 administration in hamsters, Oil Red O staining was performed in jejunum and liver tissue sections. Following acute BLT-1 treatment, hamster liver and jejunum sections showed no changes in neutral lipid staining. On the other hand, prolonged BLT-1 treatment resulted in mild reductions in neutral lipid content in the jejunum as determined by quantifying for total surface area stained with Oil Red O (Fig. 8A). Conversely, neutral lipid staining was moderately increased in the liver of hamsters following prolonged BLT-1 treatment (Fig. 8B).

While Oil Red O staining provides information regarding the overall neutral lipid content of tissues, it is impossible to distinguish between tissue TG and CHOL content. To address this issue, lipid extraction was performed in jejunal hepatic and fecal samples obtained from hamsters.
following acute or prolonged treatment with BLT-1. No changes were observed in liver TG and CHOL content (Fig. 8C and D, respectively). Consistent with Oil Red O staining, jejunum TG and CHOL content was found to be reduced with prolonged BLT-1 treatment (Fig. 8C and D, respectively). No changes in fecal TG or CHOL content were observed (Fig. 8C and D, respectively), suggesting that lipid uptake was not impaired.

These findings demonstrate that at 2 hours after a fat load, hamsters with acute or prolonged BLT-1 treatment did not have any differences in hepatic, jejunal and fecal TG and CHOL content. This suggests that lipid uptake, at two hours after fat load is not impaired by BLT-1. However, in order to determine the effect of BLT-1 on intestinal lipid uptake, more detailed analysis of lipid uptake is warranted, in the form of TG and CHOL absorption experiments, using radio-labeled TG and CHOL to determine if BLT-1 alters their rate of appearance in plasma.

What is apparent however is that prolonged BLT-1 administration, although significantly reducing fasting and postprandial TG and CM levels, does not affect jejunal, hepatic and fecal lipid content. One possible interpretation is that SR-BI inhibition may impair lipid uptake in the intestine but not to the extent that it depletes intracellular lipid stores. Also, staining with Oil Red O and determination of tissue lipid content by colorimetric kits does not differentiate between the distinct sub-populations of lipid droplets and lipid stores present in enterocytes. Therefore, it is also possible that BLT-1 does not affect uptake, but rather affects the organization of intracellular lipids or their trafficking towards the ER.

The latter scenario appears attractive since the ER is the site of apoB48 lipidation, the rate-limiting step of CM production. As discussed earlier, there is evidence of SR-BI being present in lipid droplets (Hansen et al., 2003). Furthermore, SR-BI has recently been proposed to be a lipid sensor, capable of interacting with PPM and resulting in a displacement of apoB48 towards the ER, the site of apoB48 lipidation.

Previous findings from our laboratory also support the notion that SR-BI may be involved in trafficking of lipid droplets towards the ER. We observed that upon insulin stimulation of Caco-2 cells transfected with EGFP-tagged SR-BI, SR-BI re-localized towards the perinuclear region. This occurred in a PI3K-mediated process that was inhibited by PI3K inhibitors wortmannin and LY294002 (Hayashi et al., 2011). This suggests that SR-BI is
sensitive to insulin signaling and not only its function, but its sub-cellular localization may be affected by insulin.

In summary, acute and prolonged BLT-1 treatment does not affect TG, CHOL and neutral lipid content in the liver, jejunum and feces of hamsters. Therefore, the observation that SR-BI inhibition leads to reduced postprandial CM production may not be attributed solely to defects in lipid uptake. It is possible that the role of SR-BI in CM production is more complicated and involves the intricate organization and trafficking intracellular lipid droplets.

4.4 Effects of prolonged BLT-1 treatment on protein expression of select lipid transporters

Liver and jejunum tissues were collected from hamsters following prolonged BLT-1 treatment and were analyzed for protein expression of key lipid transporters by immunoblot. There were no significant changes in protein expression of intestinal FATP4, NPC1L1, ABCG5 and ABCG8 (Fig. 9A). Similarly, there were no changes in hepatic FATP4, NPC1L1 and ABCG8, although ABCG5 was surprisingly up-regulated (Fig. 9B). Hepatic ABCG5 has a known role in CHOL efflux, but typically it works together with ABCG8 and changes in expression are generally similar for both. The fact that ABCG8 was not found to be up-regulated puts the observation that ABCG5 is up-regulated with prolonged BLT-1 treatment into question.

 Interestingly, intestinal SR-BI was found to be down-regulated in BLT-1-treated hamsters compared to controls (Fig. 9A), whereas hepatic SR-BI was significantly up-regulated in BLT-1-treated hamsters compared to controls (Fig. 9B). The reduction in intestinal SR-BI protein mass appeared to be statistically significant, but may not have physiological relevance due to the modest reduction observed. However, the importance of this finding is that, as we have previously shown, intestinal SR-BI is significantly up-regulated in insulin resistance (Hayashi et al., 2011), which appears not be the case with prolonged BLT-1-treatment.

Furthermore, it is important to note that SR-BI protein mass does not necessarily correlate linearly with its function in the intestine. As already mentioned, we have shown that insulin causes dramatic changes in the sub-cellular localization of SR-BI and possibly its function, but without changing its protein mass (Hayashi et al., 2011).
On the other hand, hepatic SR-BI protein levels were increased significantly in hamsters following prolonged BLT-1 treatment. This correlates with the mild increase in Oil Red O staining in the liver (Fig. 8B) but not with hepatic TG and CHOL levels (Fig. 8C and D, respectively). From a pathophysiological point of view, the increase in hepatic SR-BI is seen as beneficial because hepatic SR-BI has been shown to be atheroprotective (Chao et al., 2010; Fioravanti et al., 2011; Huby et al., 2005) due to its role in clearance of excess cholesterol via the Reverse Cholesterol Transport (RTC) pathway (Lewis & Rader, 2005)(Hoekstra et al., 2010; Zhang et al., 2005). This could also explain the increase in ABCG5 expression since it is involved in CHOL efflux. However, the fact that ABCG8 was not up-regulated remains puzzling.

In summary, prolonged BLT-1 treatment did not lead to significant changes in protein levels of lipid transporters in jejunum and liver of hamsters. Intestinal SR-BI was modestly but significantly down-regulated, whereas hepatic SR-BI was robustly up-regulated with prolonged BLT-1 treatment. These findings demonstrate that BLT-1 leads to significant changes in SR-BI expression in the liver and intestine and suggest that BLT-1 is specific to SR-BI function.

4.5 Effect of prolonged BLT-1 treatment on intestinal, renal and hepatic tissue function and morphology

Following the observations made in hamsters with prolonged BLT-1 treatment, it was important to assess for any hepatic, renal or intestinal injury. Creatinine is an important marker for kidney function and glomerular filtration rate (Levey et al., 2006). We measured fasting plasma creatinine prior to and after FFC-feeding and observed no differences between control hamsters and hamsters with prolonged BLT-1 treatment (Fig. 10B).

To assess liver function, it is common to measure plasma AST and ALT (Sakka, 2007). Again, there were no differences in plasma AST and ALT following prolonged BLT-1 treatment compared to controls, indicating no liver injury (Fig. 10C and D, respectively). Next, tissue morphology was examined in order to determine if there were any structural abnormalities in hepatic, renal and intestinal tissue of hamsters following prolonged BLT-1 treatment. Tissues were collected and fixed in formalin for H&E staining.
There were no observable gross abnormalities in tissue structure in any of the tissues studies. In the intestine, there villi maintained structural integrity and did not show any signs of damage. There was no apparent enterocyte death, and the apical and basolateral sides of the intestine appeared to be no different between hamsters following prolonged treatment with BLT-1 or vehicle. Similarly in the liver and kidney, there were no signs of dead or inflamed tissue, glomeruli did not appear to be distorted.

Together, these observations do not suggest that BLT-1 causes any injury to liver, jejunum or kidney in hamsters after prolonged BLT-1 treatment. No changes in plasma markers or tissue morphology were observed. More thorough analysis can be performed to assess cell death by TUNEL staining, or inflammation by measuring IL-6, TNF-α or macrophage infiltration by S4/80 staining, so as to determine if BLT-1 causes any adverse effects. However, in our hamster model of diet-induced insulin resistance, prolonged BLT-1 treatment did not lead to injury of liver, kidney or jejunum. Therefore, the observed reductions in weight-gain and lipid levels are probably not due to tissue injury.

4.6 Postprandial plasma TG accumulation in SR-BI-/- mice

Following up on our observations made using BLT-1 to inhibit intestinal SR-BI, it was important to use a different approach to study the role of intestinal SR-BI. As mentioned earlier, intestinal SR-BI has been proposed to play a role in the uptake of dietary cholesterol. Studies have been performed in SR-BI-/- mice which have been well characterized in terms of CHOL metabolism. SR-BI-/- mice are characterized by marked elevations in plasma CHOL and HDL-CHOL levels, due to reduced RCT resulting from the absence of hepatic SR-BI (Mardones et al., 2001). The authors concluded that hepatic SR-BI plays a critical role in RCT and regulation of HDL and biliary cholesterol secretion, whereas intestinal SR-BI does not play a role in intestinal cholesterol uptake (Mardones et al., 2001).

Consistent with literature, we observed that fasting as well as postprandial plasma CHOL was elevated in SR-BI-/- mice compared to WT (Fig. 12D). Fat tolerance tests were performed in SR-BI-/- mice to determine the rate of TG accumulation and CM production in plasma following a fat load. There was a significant reduction in postprandial TG secretion in SR-BI-/- mice (Fig.
12C) which was also reflected by the TRL TG levels in SR-BI/- mice (Fig. 12E). Despite the elevated total plasma CHOL levels observed in SR-BI/- mice, TRL CHOL levels were actually lower in SR-BI/- mice (Fig. 12F) which is due to the fact that TRL plasma does not contain HDL or LDL responsible for carrying CHOL. Surprisingly, SR-BI/- mice also showed reduced glucose response following fat load compared to WT mice (Fig. 12A) suggesting that intestinal SR-BI may also play a role in lipid-sensing and signaling that initiate a normal postprandial response.

TG metabolism has not been well characterized in these mice due to the fact that the majority of studies performed on SR-BI/- mice have focused largely on CHOL metabolism. There is one study that looked at CM metabolism in SR-BI/- mice but their results were contradictory to the observations we made in our hamster model using BLT-1. Here, the authors delivered an oral fat load to SR-BI/- and WT littermate mice and observed that SR-BI/- mice had increased plasma TG accumulation over a 6-hour period (Out et al., 2005).

However, no detergent was administered (such as Triton or Pluronic F-127 used in our experiments) to prevent lipolysis of newly synthesized lipoproteins and their subsequent clearance from the circulation. Moreover, the authors claim to have measured CM secretion, which is technically impossible in mice without the administration of Pluronic F-127 or Triton. The observed increase in TG following a fat load could be due to reduced clearance of CM from the circulation of SR-BI/- mice, or over-production of VLDL from the liver, since CM and VLDL cannot be resolved by FPLC or immunoblot in mice. Their observations are thus difficult to interpret.

The observations made in the present thesis were determined using Pluronic F-127 to block lipolysis of newly synthesized lipoproteins, allowing us to measure postprandial lipid secretion into plasma. Our findings are in stark contrast with the observations made by Out et al, since we observed reduced TG accumulation in plasma following oral fat load in the presence of poloxamer in SR-BI/- mice compared to WT.

On the other hand, in agreement with our observations, Bietrix et al demonstrated that SR-BI transgenic mice over-expressing intestinal SR-BI had increased postprandial TG secretion (Bietrix et al., 2006). Their findings support our observations. Furthermore, our laboratory published recent data demonstrating that SR-BI over-expression in Caco-2 cells results in
increased apoB48 secretion, whereas SR-BI knock-down leads to reduced apoB48 secretion (Hayashi et al., 2011) which supports the findings of the present thesis.

Fat absorption is a multi-step process starting from the uptake of lipids from the intestinal lumen, which is followed by re-synthesis of TG in the ER and the subsequent lipidation, packaging and finally secretion of apoB48-containing CM. It is thus difficult to tease out the function of proteins that are implicated in fat uptake and studies that measure the appearance of radiolabelled lipids in plasma do not differentiate between uptake and secretion.

The aim of this project was to determine whether SR-BI was involved in regulating changes in CM secretion as determined by fat tolerance tests. We consistently observed reductions in postprandial CM secretion in normal hamsters and rats treated with an SR-BI inhibitor, in insulin resistant hamsters with prolonged administration of the SR-BI inhibitor, and now in SR-BI/-/- mice.

These data support our previous observations in hamsters and rats using BLT-1 to inhibit intestinal SR-BI, showing that deletion of SR-BI results in reduced CM secretion. This finding is also consistent with our previous findings in hamsters using BLT-1 to inhibit SR-BI, further demonstrating that intestinal SR-BI plays a role in intestinal CM secretion.

4.7 Postprandial plasma TG accumulation in SR-BI/-/- and PDZK1/-/- mice treated or not with GLP-2

So far, we observed reductions in CM secretion in SR-BI/-/- mice as well as in hamsters by inhibition of SR-BI using BLT-1. In order to explore the mechanism by which SR-BI may be affecting CM secretion, we looked at SR-BI-mediated signaling. Interestingly, SR-BI has been shown to initiate signaling cascades leading to p38, MAPK and eNOS activation, through its adaptor protein, PDZK1 (Al-Jarallah & Trigatti, 2010; Béaslas et al., 2009; Kocher et al., 2011; Kocher et al., 2010; Zhu et al., 2008).

PDZK1/-/- mice have an interesting phenotype whereby SR-BI expression is reduced by 90% in the liver and by 50% in the intestine (Fenske et al., 2008; Kocher et al., 2003). To gain some insight into the mechanisms by which SR-BI may regulate CM secretion, fat tolerance tests
were performed in SR-BI/- and PDZK1/- animals administered vehicle or 0.25 mg/kg GLP-2. GLP-2 is known to stimulate postprandial CM over-production (Hsieh et al., 2008; J. Hsieh et al., 2009).

In our laboratory, we have shown that upon its secretion by intestinal L-cells, GLP-2 stimulates CM over-production in a CD36-mediated manner (Hsieh et al., 2009; Hsieh & Adeli, 2010). It was also shown that endothelial nitric oxide synthase (eNOS) activity is increased by GLP-2 and mediates the observed increase in CM secretion (Hsieh et al., 2010). SR-BI has also been shown to possess the ability to mediate downstream signaling via its adaptor protein PDZK1, leading to activation of eNOS in endothelial cells (Al-Jarallah & Trigatti, 2010).

PDZK1, which binds to the C-terminus of SR-BI, maintains steady state expression of SR-BI protein levels in liver and intestine (Fenske et al., 2009; Kocher et al., 2011; Kocher et al., 2003; Kocher et al., 2010; Nakamura et al., 2005; Yesilaltay et al., 2005; Yesilaltay et al., 2006) and has been shown to be essential in SR-BI mediated signaling in endothelial cells (Al-Jarallah & Trigatti, 2010; Zhu et al., 2008).

It is therefore important to determine whether PDZK1 and more specifically PDZK1-mediated signaling by SR-BI play an important role in CM production. Furthermore, since we have been able to show that absence or inhibition of SR-BI led to reductions in CM metabolism, GLP-2 was used to test whether CM over-production could occur in both SR-BI/- and PDZK1/- mice.

Fat tolerance tests were performed in SR-BI/-, PDZK1/- and WT mice with or without GLP-2 and it was observed that GLP-2 stimulated CM production in C57BL6/J WT mice but not in SR-BI/- mice (Fig. 13C and D, respectively). Similarly, GLP-2 stimulated CM over-production in B6129 WT mice but not in PDZK1/- mice (Fig. 15D and C, respectively). Interestingly, PDZK1/- mice were fully capable of secreting CM as there were no reductions in postprandial CM secretion in PDZK1/- mice.

This suggests that the relationship between SR-BI expression and CM secretion is not a linear one and that CM secretion does not depend on PDZK1-mediated SR-BI signaling but rather on the physical presence of SR-BI and possibly SR-BI signaling that is independent of PDZK1. Granting support to the observations made in total plasma TG accumulation, the TRL
TG levels at 2 hours post-fat-load were higher in WT mice treated with GLP-2 (Fig. 14A; Fig. 16A) while SR-BI/-/- and PDZK1/-/- mice did not show significant increases in postprandial TRL TG levels with GLP-2 treatment (Fig. 14B; Fig. 16B). The TRL TG data is more important as it only determines the TG content in the CM/VLDL fraction and in our experiments is more representative of CM secretion.

In summary, GLP-2 stimulated CM over-production in WT mice but not in SR-BI/-/- and PDZK1/-/- mice. However, caution must be taken when interpreting these observations since knock-out mice may have alterations to their metabolism and may not respond to a multitude of signals, including GLP-2. What is apparent however, is the fact that absence of PDZK1 does not impair postprandial TG secretion, whereas the absence of SR-BI results in reduced postprandial TG levels. Together with the observations made in the present thesis, there is strong evidence suggesting a role for intestinal SR-BI in CM production and postprandial lipid metabolism.

4.8 Concluding Remarks

The intestine is a complex organ with the ability to absorb and secrete nutrients and is now also being viewed as a regulator of lipid homeostasis and a key player in the development of insulin resistance and T2D. In an effort to better understand intestinal physiology, our laboratory studies intestinal lipoprotein production as well as proteins that are involved in dietary lipid uptake, lipoprotein synthesis and secretion in normal and insulin resistant states.

Due to its ubiquitous expression and its many functions in different tissues, the function of intestinal SR-BI has yet to be elucidated. Originally discovered as the HDL receptor in the liver, SR-BI was thought to be involved in intestinal CHOL uptake. There have been many inconclusive and contradictory studies on intestinal SR-BI, but in recent years, evidence has begun to accumulate pointing towards a role for intestinal SR-BI in CM production.

The present study has provided some key information regarding the function of intestinal SR-BI. By acute and prolonged inhibition of SR-BI, it was shown that postprandial TG and CM, but not CHOL accumulation in plasma was significantly reduced. Similarly, SR-BI/-/- mice showed significant reductions in postprandial TG accumulation following a fat load. Along with
observations made in other studies implicating SR-BI in CM secretion, lipid droplet localization and lipid sensing in enterocytes, we believe that we have determined that the role of intestinal SR-BI is in CM production (Béaslas et al., 2009; Hansen et al., 2003; Hayashi et al., 2011).

We believe SR-BI does this by mediating lipid-trafficking in the enterocyte towards the ER where apoB48 is lapidated. Postprandial CM secretion depends on efficient uptake of fatty acids (FA), the subsequent delivery of those fatty acids towards the ER to facilitate apoB48 lipidation by MTP and finally the secretion of mature CM particles into the lymph (Hsieh et al., 2008). Since no changes were observed in intestinal lipid content following acute or prolonged BLT-1 treatment, it seems that SR-BI may play a role in CM secretion independent of lipid uptake.

The tissue lipid methods used in the present study were limited in their ability to differentiate between cytosolic and endoplasmic lipid droplets, two very distinct intracellular lipid populations with separate fates and roles in the cell. Since the availability of lipid in the ER is rate limiting to apoB48 lipidation and therefore secretion, it is likely that SR-BI plays a role in trafficking lipid droplets towards the ER, making them available for apoB48 lipidation and CM secretion.

In a key study by Hansen et al., the authors looked at the sub-cellular localization of SR-BI in pig enterocytes in the fasting and postprandial state. They observed that SR-BI was primarily localized in the apical brush border membrane of enterocytes in the fasting state, while postprandially SR-BI was found to be internalized by clathrin coated pits and localized to large cytosolic lipid droplets (Hansen et al., 2003). Moreover, SR-BI did not co-localize with apoA-I, a component of nascent chylomicrons (Hansen et al., 2003). In our lab, we have also seen changes in sub-cellular SR-BI localization in response to insulin, in a PI3K-mediated fashion (Hayashi et al., 2011). Together, these data support a role for SR-BI in CM secretion by regulation of lipid droplet trafficking, making lipid available to the ER for lipidation of apoB, leading to secretion of CM.

In conclusion, the present thesis has provided novel findings pertaining to the function of intestinal SR-BI. For the first time, we have observed a functional role for intestinal SR-BI in vivo, using multiple animal models and approaches. SR-BI has been associated with insulin resistance, T2D and CVD and has also been proposed to be a promising immunotherapy target.
for many pathological disorders, including cardiovascular disease and type-2 diabetes (T2D) (Fioravanti et al., 2011). It is important to further delineate the function of SR-BI as well as the effectiveness of BLT-1 and other potential compounds that modulate its function, in an effort to develop treatments targeted at improving dyslipidemia in insulin resistant states.

4.9 Future directions

The present thesis explored the role of intestinal SR-BI in postprandial CM secretion. In vivo experiments using BLT-1 as an inhibitor of SR-BI were performed in hamsters and rats. This permitted the study of intestinal SR-BI in a physiologically relevant model of lipoprotein metabolism as well as insulin resistance. To test this hypothesis directly, knockout models are required. Thus, experiments were performed in in SR-BI/- mice, however there are some shortcomings with this model. First of all, SR-BI/- mice are global knockouts, meaning there are probably other confounding factors as well as changes in metabolism that make it difficult to interpret results. Ideally, future studies should employ a Cre-LoxP mouse model, specific to the intestine that can allow for SR-BI knockout to occur later in life, so as to avoid any developmental complications. This can be achieved by using intestine-specific villin-Cre mice that are commercially available.

The results that were obtained with the prolonged BLT-1 treatment were intriguing, however future experiments are needed to aid in the interpretation of those findings. Postprandial lipids were significantly reduced with prolonged BLT-1 treatment; however hamsters with prolonged BLT-1 treatment also exhibited reduced weight-gain. It becomes unclear whether BLT-1 caused reductions in weight-gain which consequently improved postprandial lipids, or whether BLT-1 caused reductions in postprandial lipids by inhibiting SR-BI, which in turn caused reductions in weight-gain.

There are two experiments that can be performed to address this. First of all, hamsters can be fed the high fructose diet (similar to FFC without CHOL) which has shown to induce insulin resistance without inducing weight-gain in hamsters. Prolonged BLT-1 treatment of fructose-fed hamsters can provide insight into the effects of BLT-1 in the context of a diet that does not induce weight gain. Alternatively, longer feeding studies can be planned to determine if
the reduction in weight-gain persists for a longer period of time. These experiments can provide insight into the function of SR-BI.

Observations made in the SR-BI/- and PDZK1/- mice treated with GLP-2 are currently very preliminary and were limited in numbers. More animals will be needed to add strength to the observations made. Furthermore, it is necessary to determine whether these animals are capable of responding to GLP-2. Our results show that GLP-2 did not stimulate postprandial lipids in SR-BI/- and PDZK1/- mice; however it might also be the case that these mice do not respond to GLP-2 at all. The pathways that GLP-2 signals through and are activated upon GLP-2 administration are known, so it would be important to first determine if GLP-2 had those effects in the SR-BI/- and PDZK1/- mice.

Finally, the observations made in the present thesis suggest an important role for SR-BI in postprandial CM secretion. Based on our present understanding, it appears as though SR-BI has a role in lipid trafficking within the enterocyte, possibly delivering lipids towards the ER for apoB48 lipidation and CM assembly. Our laboratory has experience with confocal microscopy and we have published data showing that SR-BI’s sub-cellular localization is dynamic and can respond to signaling pathways such as the PI3K signaling pathway. More work is needed in determining the signaling pathways that SR-BI initiates and is regulated by, as well as how they affect its function in lipid trafficking and CM secretion.

Intestinal SR-BI is an interesting protein that appears to be very important in postprandial lipid metabolism in healthy as well as insulin resistant animals. Further work is warranted in uncovering its mechanism of action as well as its role in intestinal CM secretion. Furthering our understanding of intestinal SR-BI can add to our knowledge of lipid metabolism and provide new therapeutic targets for the treatment of dyslipidemia, obesity and insulin resistance.
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