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Scavenger receptor, class B, type I mediates the uptake and degradation of beta-VLDL particles in tissue culture

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1. Introduction

Atherosclerotic vascular disease is on the way being the major cause of morbidity and mortality worldwide (Lloyd-Jones et al., 2009). Considering this circumstance, every effort in understanding the players and contributors in this disease is of importance. A pathological characteristic of atherosclerosis is foam cell formation rooting in cholesterol accumulation in macrophages within the arterial wall (for review see: Van Eck et al., 2005). Besides LDL (low density lipoprotein) and oxidized lipids, also VLDL plays a role in atherogenesis. The atherogenic form of very low density lipoproteins (VLDLs), the so called beta-VLDL (ß-VLDL) particles is widely-used as a model for chylomicron remnants. The particles accumulate in the plasma of patients with type III hyperlipoproteinemia and in cholesterol-fed animals (Mahley et al., 1977; Brewer et al., 1983). ß-VLDL was shown to induce cholesteryl ester accumulation in macrophages leading to foam cell formation (Bersot et al., 1983; Goldstein et al., 1980). Recently, scavenger receptor, class B, type I (SR-BI) has been linked to ß-VLDL uptake (Van Eck et al., 2008), in addition to its well-established role in high density lipoprotein (HDL) metabolism.

The aim of the present study was to examine the role of SR-BI in ß-VLDL metabolism in tissue culture. Although the LDL-receptor pathway appears to be of major importance for ß-VLDL uptake, it is of interest to characterize the alternative route via SR-BI. We used two different sets of cells. On the one hand we examined cells which express normal levels of the LDL-receptor, namely the human hepatoma cell line HepG2 and Chinese Hamster Ovarian cells (CHO). On the other hand we analyzed CHO cells that lack any functional LDL-receptor named ldlA7, as well as ldlA7 cells overexpressing SR-BI (ldlA7-SRBI). In order to charaterize the function of SR-BI in ß-VLDL processing, uptake and degradation experiments were performed using iodinated ß-VLDL. For a more detailed characterization of ß-VLDL binding, competition experiments were performed using a 10-fold excess of unlabeled lipoproteins. SR-BI knockdown in HepG2 cells was another tool in identifying the receptor´s importance in ß-VLDL cell association. Further, the effect of ß-VLDL

feeding on the cellular lipid pool was assessed using HPLC and the Oil Red O method.

2. Status of Research

2.1 Lipoprotein metabolism

Lipoproteins are required to transport cholesterol and triglycerides in the blood. In the intestine, dietary fat is packaged into chylomicrons, which are large triglyceride-rich lipoproteins (Fig.1). Chylomicrons are transported to peripheral tissues where the triglycerides are hydrolyzed by the lipoprotein lipase (LPL) resulting in free fatty acids entering these tissues (mainly in heart, skeletal muscle and adipose tissue). The chylomicron remnants are then cleared by the liver. The liver loads apolipoprotein-B (apo-B) with lipids and secretes very low density lipoproteins (VLDLs), which form low density lipoproteins (LDLs) after lipolysis by LPL. LDLs are taken up by the liver via binding to recognition sites like the LDL-receptor. High density lipoproteins (HDLs) are assembled by the intestine and the liver through lipid-free apo-AI which assimilates cholesterol from these organs through ATP-binding cassette transporter A1 (ABCA1) forming nascent HDLs. In peripheral tissues nascent and mature HDLs promote cholesterol efflux via ABCA1 and ATP-binding cassette transporter G1 (ABCG1). Mature HDLs emerge from the esterification of free cholesterol by lecithin cholesterol acyltransferase (LCAT). HDLs transport cholesterol to the liver, a process which is known as reverse cholesterol transport, involving SR-BI (Rader et al., 2003).

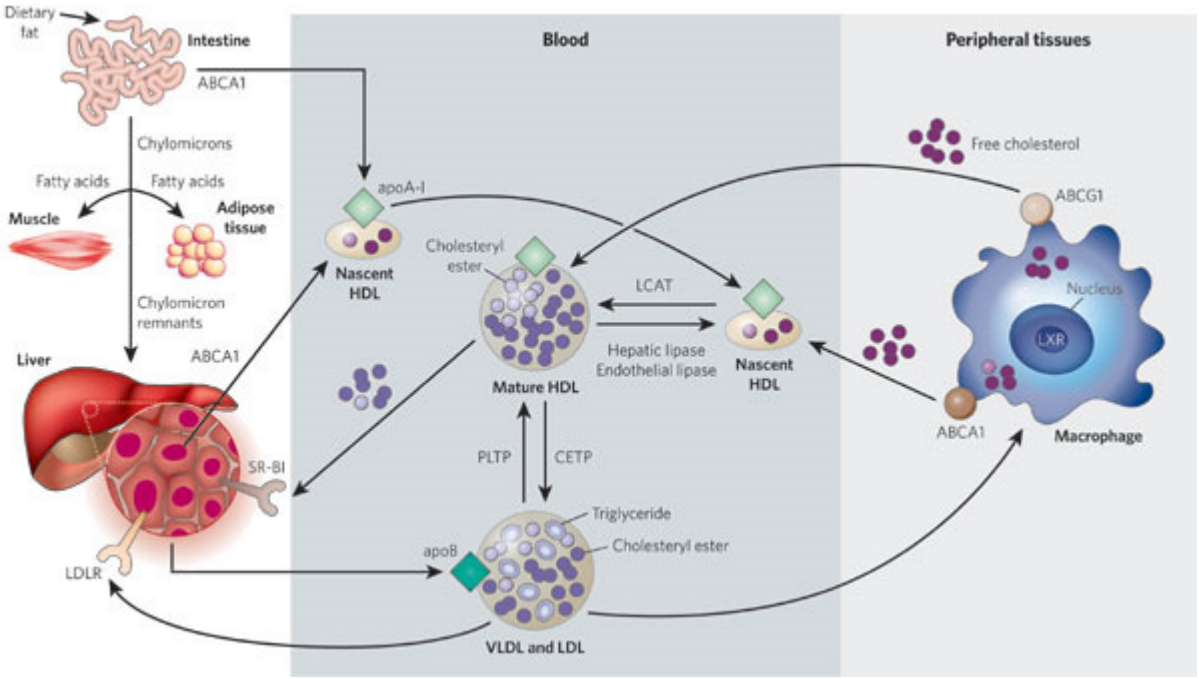


Figure 1: Lipoprotein metabolism (Rader et al., 2008)**.**

2.2 Atherosclerosis

2.2.1 Epidemiology

Atherosclerosis is the underlying cause of most clinical cardiovascular disease (CVD) events. CVD accounted for 35.3% of all deaths in 2005 in the United States. Atherothrombotic diseases are associated with the leading causes of death worldwide, and the prevalence is increasing. They are expected to be the main cause of death in 2020 (Lloyd-Jones et al., 2009). Between 1990 and 2020, the percentage of deaths from CVD is projected to increase from 28.9% to 36.3%. This alarming progression is associated with two trends in developing countries. First, the displacement of malnutrition and infectious disease as primary cause of death, leading to an aging of the population. Second, notably increases in cigarette smoking, one established risk factor of atherosclerosis among others like elevated cholesterol levels or hypertension. Different factors such as obesity, physical inactivity or diabetes are also suggested to be associated with increased risks of

CVD. Besides that, several new atherogenic risk factors, such as homocysteine, are discussed (Hennekens, 1998).

2.2.2 Atherogenesis

Atherosclerotic lesion development, the hallmark of atherosclerosis, ist the initial step contributing to atherogenesis (Fig. 2). Atherosclerotic lesions are thickenings of the intima, the innermost layer of the arteries. Cells (inflammatory and immune cells, endothelial and smooth muscle cells), connective-tissue elements and lipids are the major component of those thickenings. The earliest atherosclerotic lesion, the so-called fatty streak, results from an accumulation of lipid-loaded cells (Hansson, 2005).

These changes of the arterial wall are followed by endothelial activation. Hypercholesterolemia leads to an infiltration of excess LDL in the intima, which causes an inflammatory response. Oxidation or other changes of LDL lead to an activation of endothelial cells. The first response to this activation is platelet adhesion, followed by attachment of other blood cells. The intima then releases chemokines that induce the migration of cells such as monocytes into the subendothelial space (Hansson, 2005)

This migration of cells leads to a further characteristic of atherogenesis, namely foam cell formation. Monocytes that enter the plaque differentiate into macrophages, stimulated by growth factors, such as the macrophage colony-stimulating factor (M-CSF). The focal areas of the arterial walls become populated with differentiated macrophages. The macrophages internalize subendothelial lipoproteins and they accumulate intracellular cholesterol in cytosolic lipid droplets. The lipoproteins enter the subendothelium likely by transcytosis through endothelial cells or through transient gaps between endothelial cells (Tabas, 2000; Hansson 2005). This process involves the LDL-receptor, caveolin-1, ABCA1 and ABCG1 or SR-BI (von Eckardstein et al., 2009). The major storage form of cholesterol is primarily cholesteryl esters. At

this stage, the macrophages are termed foam cells. Especially in advanced atherosclerotic lesions, accumulation of free cholesterol occurs (Tabas, 2000; Hansson 2005).

In addition to foam cell formation, which has an impact on early atherogenesis but also on late lesion development, inflammation plays a key role in atherosclerosis. Immune cells within the atherosclerotic plaque produce inflammatory cytokines (interferon-γ, interleukin-1 and tumor necrosis factor). This activation in the cytokine cascade results in increased levels of interleukin-6 and C-reactive protein. Furthermore, antiinflammatory cytokines such as interleukin-10 and transforming growth factor ß (TGF-ß) are involved.
The progression of atherosclerosis is mainly influenced by the equilibration between pro- and antiinflammatory processes (Hansson, 2005).

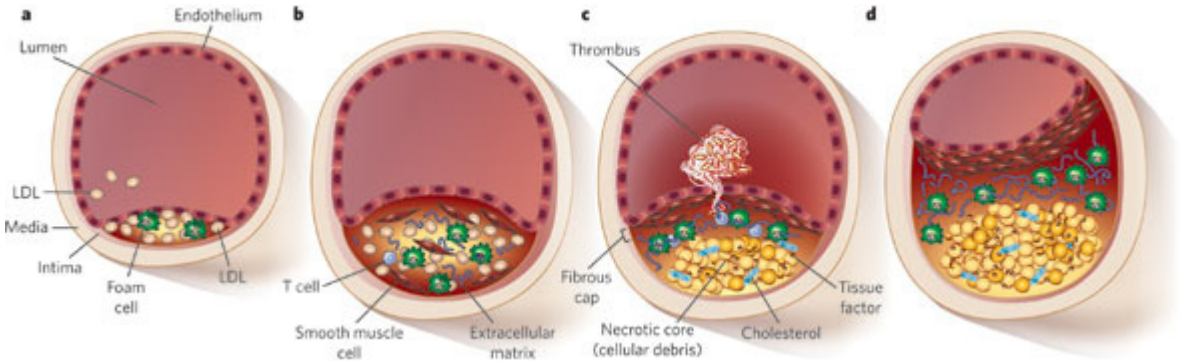


Figure 2: Progression of atherosclerosis. A lesion begins as a fatty streak (a) and can develop into an intermediate lesion (b), and then into a lesion that is vulnerable to rupture (c) and, finally, into an advanced obstructive lesion (d) (Rader et al., 2008).

2.3 Cholesterol homeostasis

Cellular cholesterol homeostasis can be maintained by three possible mechanisms: cholesterol synthesis, uptake and efflux.

2.3.1 Intracellular cholesterol transport and regulation of the cellular cholesterol content

In the endoplasmic reticulum (ER), cholesterol is synthesized by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Another source of cholesterol is the internalization of lipoprotein particles. Cholesteryl esters, delivered by apo-B/E containing lipoproteins via the LDL-receptor, are hydrolyzed in late endosomes and lysosomes. Free cholesterol is then exported to other organelles (Maxfield et al., 2002).

Cellular cholesterol levels are tightly regulated by the LDL-receptor and HMG-CoA reductase. The storage of cholesterol in its cholesteryl ester form is controlled by the esterifying enzyme acyl CoA:cholesterol acyltransferase (ACAT). Cholesterol depleted cells express high levels of the LDL-receptor and HMG-CoA reductase but low levels of ACAT. When cellular cholesterol levels increase, the process is reversed, activating ACAT and lowering LDL-receptor and HMG-CoA reductase activity (Brown et al., 1986).

The expression of the LDL-receptor and enzymes involved in cholesterol biosynthesis is regulated by a family of transcription factors, the so called sterol responsive element binding proteins (SREBPs). SREBPs are membrane-bound transcription factors that modulate the transcription of enzymes involved in cholesterol biosynthesis. SREBPs need to be released from the ER-membrane by proteases to function. In sterol-depleted cells, the transcription factors are released and enhance expression of genes encoding enzymes such as HMG-CoA reductase and the transcription of the LDL-receptor. An increasing cellular sterol content blocks the release of SREBPs leading to a decline in the transcription of the target genes (Brown et al., 1999).

2.3.2 Reverse cholesterol transport and cholesterol efflux

The transport of cholesterol from extrahepatic tissues, including arterial wall macrophages, to the liver for excretion as free cholesterol and bile acids is described as reverse cholesterol transport (RCT) (Ohashi et al., 2005). This removal of lipoprotein derived excessive peripheral cholesterol is of major importance in the prevention of atherosclerosis. The atheroprotective function of HDL is mostly ascribed to its participation in RCT.
HDL is also involved in the initial step of RCT referred to as cholesterol efflux. This is a process in which cholesterol is transferred to HDL from peripheral cells. Several receptors mediate the flux of cholesterol to lipoproteins such as ABCA1, ABCG1 and SR-BI (for review see: Tall et al., 2008). SR-BI mediates this bidirectional transport of unesterified cholesterol between lipoproteins and cells in tissue culture. In this context, a correlation of SR-BI expression and cholesterol efflux rates was reported (Ji et al., 1997).

2.3.3 Retroendocytosis

The retroendocytosis mechanism involves receptor binding, endocytosis and resecretion of the lipoprotein particle. HDL retroendocytosis was first described by Bierman et al. (1974) in rat aortic smooth muscle cells. In macrophages HDL was internalized through receptor binding to a non-lysosomal compartment followed by secretion (Schmitz et al., 1985). Further studies by DeLamatre et al. (1990) provided similar results in rat hepatoma cells. Resecreted HDL particles were depleted of cholesteryl esters and the size distribution was modified, indicating lipoprotein catabolism and remodelling. HDL retroendocytosis even occured in the presence of inhibitors of intracellular cholesterol transport, such as monensin (Pagler et al., 2007).

2.3.4 Selective cholesteryl ester uptake

Selective uptake is a process in which cholesteryl esters are transferred to cells without degradation of the lipoprotein particle (Glass et al., 1983). This is in contrast to the LDL-receptor pathway where the lipoproteins are endocytosed through clathrin coated pits and degraded. Selective uptake is thought to be a two-step mechanism: an initial lipoprotein binding step is followed by a lipid transfer step (Trigatti et al., 2003). The cholesteryl esters are first transported to a reversible plasma membrane pool and further transferred to an irreversible intracellular compartment (Rinninger et al., 1993).

SR-BI was shown to mediate selective cholesteryl ester uptake from HDL and is highly expressed in liver and steroidogenic tissues, where this form of uptake ist important (Acton et al., 1996).

Further studies found increased HDL cholesterol levels in SR-BI knockout mice, likely due to decreased selective cholesterol uptake. These results suggest an important role of SR-BI in regulating plasma lipoprotein cholesterol levels (Rigotti et al., 1997).

Fluourescently labeled HDL (DiI-HDL) was used to assess the cellular distribution of HDL derived lipid. LDL-receptor positive CHO cells showed a punctuate staining pattern, typical for receptor-mediated endocytosis, whereas LDL-receptor lacking but SR-BI overexpressing cells exhibited a completely different diffuse staining pattern that was linked to selective uptake (Acton et al., 1996). Silver et al. (2001) reported that HDL particles are transported to the endosomal recycling compartment. This is in contrast to findings that endocytosis is not required for selective cholesteryl ester uptake via SR-BI (Nieland et al., 2005).

2.3.5 Cholesterol metabolism in hepatocytes

Liver cell lines provide a good model for the investigation of the function of SR-BI in lipoprotein metabolism for two reasons. First, highest expression mass of SR-BI is found in liver (for review see: Krieger, 1999). Second, the liver plays a major role in the regulation of the cholesterol homeostasis of the whole body. There exist three sources of hepatic cholesterol: endogenous synthesis, receptor-mediated endocytosis of LDL and internalization of chylomicron remnants.

Once accumulated in hepatocytes, cholesterol has multiple possible fates, according to metabolic need. The cholesterol can be incorporated into the cell membranes in hepatocytes. Further it is packaged into VLDL particles in its cholesteryl ester form. Another possibility is the secretion into the bile, either directly or after metabolization to bile acids (Liscum et al., 1980).
The regulation of hepatic SR-BI expression is regulated by many dietary, metabolic, hormonal and pharmalogical factors (Trigatti et al., 2003).

2.3.6 Cholesterol metabolism in macrophages

As cytosolic accumulation of cholesterol in lipid droplets is involved in atherosclerotic lesion development, cholesterol metabolism in macrophages is of great interest.
The uptake pattern and intracellular processing of cholesterol from LDL and ß-VLDL seems to be different (Maxfield et al., 2002).
It was reported in mouse peritoneal macrophages that human LDL and rabbit ß-VLDL are both internalized via receptor-mediated endocytosis but the intracellular fate differs. LDL is rapidly delivered to lysosomes in the perinuclear area, where it is degraded and cholesteryl esters are hydrolyzed. On the opposite side ß-VLDL, which was found in vesicles widely distributed throughout the cytoplasm. Additionally, compared to LDL, protein degradation and cholesteryl ester hydrolysis were retarded (Tabas et al., 1990).
Internalized LDL is delivered to late endosomes, where cholesteryl ester hydrolysis takes place. This source of cholesterol is limited by down-regulation of the LDL-receptor. This is in contrast to ß-VLDL-derived cholesterol, which becomes intracellulary esterified by ACAT resulting in accumulation in cytosolic storage droplets. A possible explanation could be the higher cholesterol content of the larger ß-VLDL particles (Maxfield et al., 2002).

2.4. Scavenger receptor class B, type I (SR-BI)

2.4.1 Structure and ligands of SR-BI

Scavenger receptor, class B, type I (SR-BI) is a cell surface receptor of 82 kDa molecular mass (Fig.3). The receptor consists of two transmembrane domains and two cytoplasmic N- and C-terminal tails. The cysteine-rich carboxy-terminal is important for SR-BI signalling. The large extracellular region has multiple potential N-glycosylation sites but its structure is unknown so far (for review see: Rhainds et al., 2004).

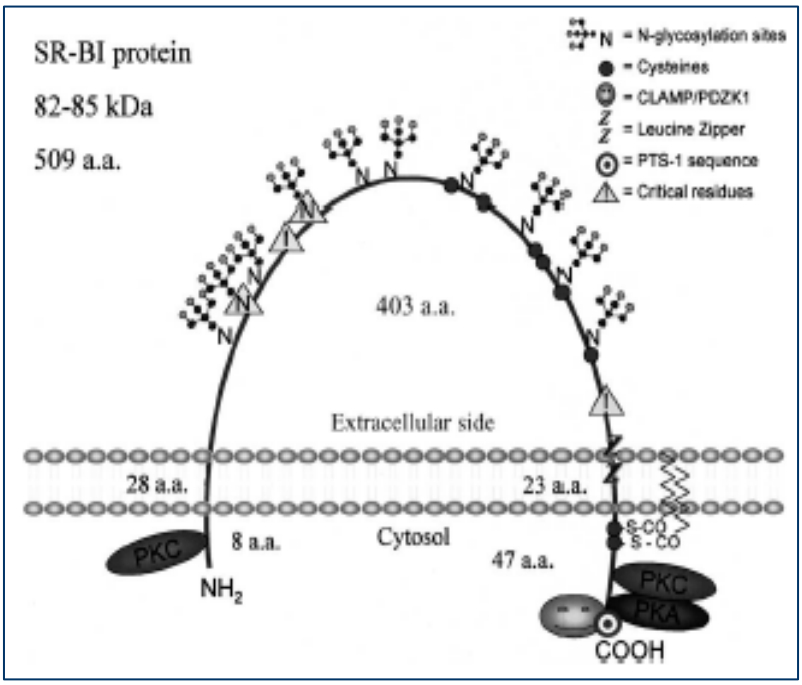


Figure 3: SR-BI protein (Rhainds et al., 2004)**.**

SR-BI was first identified as an HDL-receptor in 1996 (Acton et al.). The class B scavenger receptors comprise SR-BI, SR-BII, CD36 and croquemort (Terpstra et al., 2000). Scavenger receptors typically bind a large spectrum of ligands. Besides HDL several other ligands of SR-BI were identified in the last decade. The receptor does not only bind native lipoproteins, such as HDL, and LDL, but also modified LDL (Calvo et al., 1997), anionic phospholipids (Rigotti et al., 1995), apoptotic cells (Murao et al., 1997), advanced glycation end products (Ohgami et al., 2001) and even virus particles (Scarselli et al., 2002). SR-BI was also shown to mediate the uptake of VLDL and ß-VLDL (Calvo et al., 1997; Herijgers et al., 2000).

Higher expression levels of SR-BI are found in liver, where it is involved in RCT, and in steroidogenic tissues (adrenal gland, ovary), where it delivers sterol for hormone synthesis (Acton et al., 1996).

2.4.2 The role of SR-BI in the development of atherosclerosis

The anti-atherogenic function of SR-BI is largely ascribed to its contribution in the hepatic uptake of cholesteryl esters from HDL. It was shown that disruption of SR-BI in wild-type mice led to increased risk for development of atherosclerotic lesions (Rigotti et al., 1997).
SR-BI and apolipoprotein E deficiency was implicated in premature death and severe cardiac dysfunction in mice fed a normal chow diet (Braun et al., 2002; Trigatti et al., 1999).
Liver-specific overexpression of SR-BI in transgenic mice resulted in a decrease of HDL levels and an increase in reverse cholesterol transport (Wang et al., 1998).
Other studies provided evidence for an anti-atherogenic potential through lowering of VLDL and LDL cholesterol levels due to hepatic overexpression of SR-BI (Arai et al., 1999).
Those findings together indicate an antiatherogenic function of SR-BI in mice.
Several other potential mechanisms of atheroprotective effects of SR-BI were proposed. SR-BI is expressed in macrophage foam cells in atheroscerotic plaques and may therefore influence the flux of cholesterol between HDL and cells. Other possible effects of SR-BI include the involvement in α-tocopherol-mediated protection mechanisms or a potential impact on arterial oxygen supply (Trigatti et al. 2003).
Another protein which mediates cholesterol flux to HDL is ABCA1, which is a member of a group of transmembrane proteins that use ATP to transport various molecules through the plasma membrane. ABCA1 is ubiquitously expressed and transfers lipids to apo-AI resulting in the formation of nascent HDL. High expression levels are found in macrophages, stimulated by cholesterol loading (Van Eck et al., 2005).
In contrast to its atheroprotective role, SR-BI facilitated fatty streak development in LDL-receptor $^{-/-}$ mice. The property of SR-BI to bind atherogenic lipoproteins, like ß-

VLDL or oxidized LDL, is suggested to induce foam cell formation, while the efflux of intracellular cholesterol to HDL will prevent this event (Fig.4) (for review see: Van Eck et al., 2005). Although several studies indicate an atheroprotective function of SR-BI, it possibly plays a dual role in macrophages.

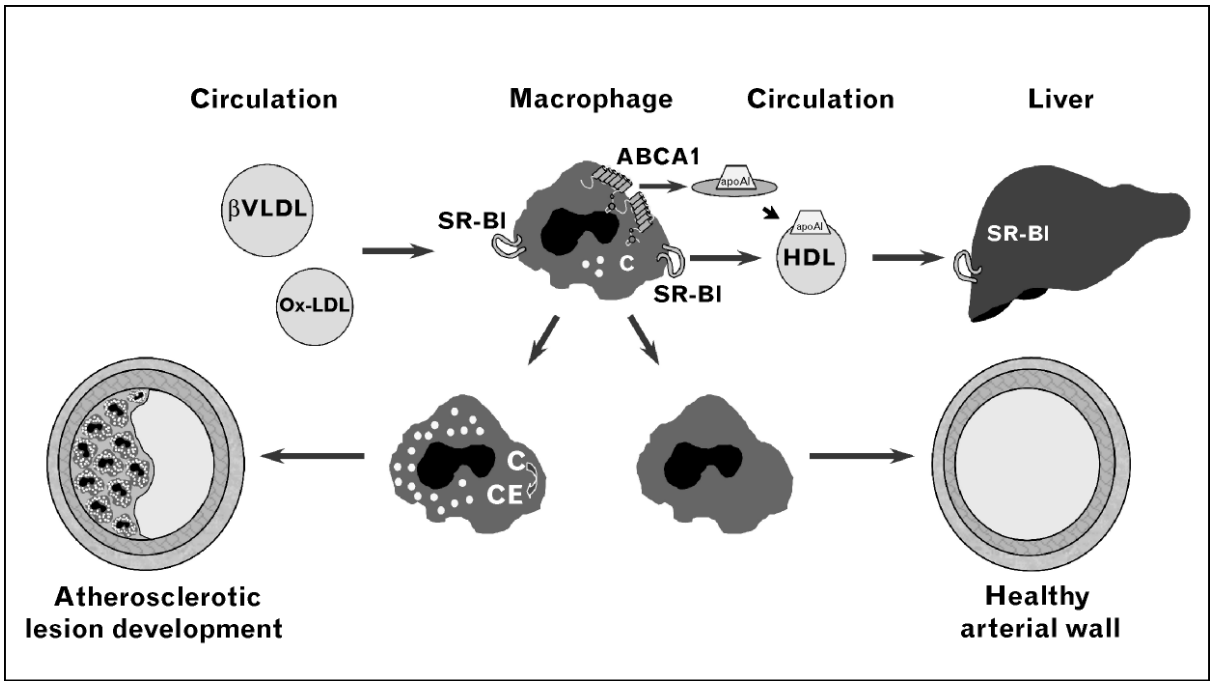


Figure 4: The role of macrophage SR-BI in atherosclerotic lesion development (Van Eck et al., 2005)**.**

2.4.3 SR-BI localization in caveolae

Another theory that has been examined is the involvement of caveolae in selective cholesteryl ester uptake via SR-BI.

Caveolae were identified as caveolin-containing membrane rafts where SR-BI is concentrated. They represent a reversible plasma membrane pool of cholesteryl esters (Graf et al., 1999). Matveev et al. (2001) suggested that caveolin-1 is a negative regulator of SR-BI mediated selective cholesteryl ester uptake in CHO cells overexpressing SR-BI and caveolin-1. However, caveolin-1 overexpression did not affect selective lipid uptake from HDL in human embryonic kidney cells and Fischer

rat thyroid cells. Transient expression of SR-BI did not alter this result either (Wang et al., 2003).
Further studies analysed the localization of SR-BI in HepG2 cells. The results indicate that caveolin-1 does not form caveolae in this liver cell line, as it was primarily detected intracellularly. However, SR-BI was found to be concentrated in lipid rafts (Rhainds et al., 2004).

2.5 ß-VLDL

2.5.1 Characteristics of ß-VLDL

ß-VLDL accumulates in the plasma of patients with type III hyperlipoproteinemia and in cholesterol-fed animals (Mahley et al., 1977; Brewer et al., 1983). The characteristics of the particle include a density < 1,006 g/ml and ß-electrophoretic mobility. The markedly elevated ß-VLDL levels in rabbits fed a high cholesterol diet are linked to saturation of hepatic clearance mechanisms. The decreased ß-VLDL clearance is also thought to be due to supression of hepatic lipoprotein receptors (Kovanen et al., 1981).
ß-VLDL is relatively enriched in cholesteryl esters compared to normal VLDL. The protein part of ß-VLDL consists of multiple apo-E molecules and one apo-B (Fainaru et al.,1982), in contrast to VLDL, which mainly consists of apo-B.
Being the ultimate VLDL remnant, ß-VLDL is widely-used as a model for chylomicron remnants and allows investigation of SR-BI in VLDL metabolism independent of factors remodelling VLDL particles (such as lipolysis) (Van Eck et al., 2008).

2.5.2 Atherogenic character of ß-VLDL

The lacking autoregulation of ß-VLDL receptors on macrophages is an important aspect concerning atherogenesis. The number of receptors is not sufficiently suppressed in response to increasing cellular cholesteryl ester levels, as it is typical for the LDL-receptor (Goldstein et al., 1980). ß-VLDL isolated from patients with atypical dysbetalipoproteinemia was shown to cause cholesteryl ester accumulation

in mouse peritoneal macrophages, thereby converting them into foam cells. The lysosomal degradation of internalized ß-VLDL could be inhibited by chloroquine indicating a receptor-mediated uptake mechanism (Bersot et al., 1983).
In mouse peritoneal macrophages, the formation of cholesteryl esters induced by ß-VLDL was proportional to its amount taken up. Internalized ß-VLDL is hydrolized in lysosomes and liberated cholesterol is re-esterified. Other lipoproteins from hyperlipidemic dogs slightly increased stimulation of cholesteryl esterification, whereas normal VLDL and other lipoproteins did not show an effect on cholesteryl ester accumulation (Goldstein et al., 1980).

2.5.3 SR-BI and ß-VLDL/VLDL uptake: in vitro results

Particles containing apo-E, like VLDL, ß-VLDL or chylomicrons, bind to several recognition sites.
The major apo-E recognizing receptors comprise the LDL-receptor, the LDL-receptor related protein (LRP) and the VLDL-receptor, but also heparan sulfate proteoglycans (HSPG´s) and SR-BI (Hu et al., 2008).
The LDL-receptor plays a major role in ß-VLDL metabolism. In order to identify its relative importance, several studies were performed using LDL-receptor deficient systems (Herijgers et al., 2000; Van Lenten et al., 1985; Perrey et al., 2001). Van Lenten et al. (1985) showed that macrophages lacking the LDL-receptor are still able to metabolize ß-VLDL.
Further studies revealed that ß-VLDL induces cholesteryl ester accumulation in the absence of the LDL-receptor in macrophages, though to a lesser extent than in LDL-receptor $^{+/+}$ macrophages (Herijgers et al., 2000; Van Lenten et al., 1985).
These results demonstrate the importance of the LDL-receptor in ß-VLDL metabolism, but also suggest an alternative recognition site for ß-VLDL on macrophages.
Among several common binding sites for lipoproteins, such as LRP and proteoglycans, the data pointed to SR-BI being responsible for ß-VLDL metabolism in LDL-receptor $^{-/-}$ macrophages (Herijgers et al., 2000).

Furthermore, cell association of ß-VLDL was assessed using hepatocytes from SR-BI $^{-/-}$ mice. ß-VLDL cell association was reduced in the absence of SR-BI and no specific accumulation of degradation products occured (Van Eck et al., 2008).

2.5.4 SR-BI and ß-VLDL/VLDL uptake: in vivo results

Hu et al. (2008) demonstrated that hepatocytes from LRP $^{-/-}$, LDL-receptor $^{-/-}$ and VLDL-receptor $^{-/-}$ mice are still able to bind VLDL-like emulsion particles. Oxidized LDL is an established inhibitor of SR-BI and was therefore used to assess the involvement of SR-BI. Heparin releases heparin binding proteins from HSPG´s. The cell association of the particle could be inhibited by both oxidized LDL and heparin, indicating the involvement of SR-BI and HSPG´s.
To analyze the role of SR-BI in VLDL metabolism, hepatic uptake and plasma clearance of ß-VLDL were assessed in SR-BI knockout and wildtype mice (Van Eck et al., 2008).
Serum VLDL cholesterol levels were elevated in SR-BI $^{-/-}$ mice, on chow diet (4.3% fat) as well as on Western-type diet (15% fat, 0.25% cholesterol) and high-cholesterol diet (15% fat, 1% cholesterol, 0.5% cholate). This increase was not due to impaired lipolysis, decreased hepatic LDL-receptor and LRP-1 expression or increased VLDL production. The authors could also observe a delayed serum decay and liver uptake of ß-VLDL in SR-BI $^{-/-}$ mice. Interestingly, there was no detectable SR-BI mediated selective uptake of cholesteryl esters from ß-VLDL (Van Eck et al., 2008).

3. Material and Methods

3.1 Tissue Culture

3.1.1 Media

DHG : 1:1 mixture of DMEM and Hams F-12 medium
MEM: Minimal essential medium (Euro lone)
DMEM: Dulbecco's minimal essential medium (PAA laboratories GmbH)

3.1.2 Cell Lines

HepG2:

HepG2 are human hepatoma cells that were contained in MEM (minimal essential medium) supplemented with 1% penicillin/streptomycin, 10% FCS, 1% non essential amino acids and 1% glutamine. HepG2 cells were plated at a density of 300.000 cells/well in 12-well plates for experiments.

CHO:

Chinese Hamster Ovarian cells were kept in DHG supplmented with 1% penicillin/streptomycin and 5% fetal calf serum (FCS). CHO cells were plated at a density of 80.000 cells/well in 12-well plates for experiments.

ldlA7:

ldlA7 are CHO cells that lack any functional LDL receptor but express normal levels of LRP. ldlA7 cells were kept in DHG supplemented with 1% penicillin/streptomycin and 5% FCS.

ldlA7-SRBI:

ldlA7-SRBI are ldlA7 cells that were stably transfected with SRBI. ldlA7-SRBI cells were kept in DHG medium supplemented with 1% penicillin/streptomycin, 5% FCS and 0.25 mg/ml geneticin (G418).

3.2 Buffer

Phosphate buffered saline (PBS):

- 0.8 % NaCl
- 0.02 % KCl
- 0.29 % $Na_2HPO_4*12H_2O$
- 0.02 % KH_2PO_4
- pH=7.4

TRIS buffer:

- 1.2 % TRIS
- 0.9 % NaCl
- pH=7.4

TRIS buffered saline (TBS):

- 0.24 % TRIS
- 0.84 % NaCl
- pH=7.4

Sample buffer (4x):

- 90 µl Glycerol
- 45 µl TRIS (8 % SDS, pH=6.8)
- 5 % Mercaptoethanol
- Bromphenol blue

TRIS-Glycin buffer:

- 0.3 % TRIS
- 1.4 % Glycin
- 10% SDS solution (10%)

Transfer buffer:

- TRIS-Glycin buffer
- 20 % methanol

3.3 Bradford protein assay

The protein concentration of lipoproteins as well as of cell lysates was estimated using the Bradford protein assay (Bradford MM, 1976).
This method is based on an absorbance shift of the Coomassie dye when it gets bound to protein.
The assay was performed using microtiter plates. 20µl Bradford reagent (Biorad) and 2-20µl sample (depending on the expected concentration) were filled up with distilled water to a volume of 100µl. Within the same set-up 0, 1, 2, 5 and 10 µg BSA (bovine serum albumin) were used for the standard curve. After 10 minutes, the reaction was finished. The absorption was measured spectrophotometrically at 595nm using a plate reader (Biorad Model 550). The standard curve was used to calculate the protein concentration.

3.4 Isolation of Lipoproteins

3.4.1 Rabbit ß-VLDL

ß-VLDL was obtained from New Zealand white rabbits that had received a high cholesterol diet, containing 2% cholesterol and 10% olive oil for 3 days (Kovanen PT et al., 1981). Fasting blood was collected and plasma was obtained by centrifugation (3000rpm, 4°C, 30min). The density of ß-VLDL (d=1.006) was adjusted by KBr.

$$g\ KBr = \frac{V(plasma) * (final\ density - initial\ density)}{1-(0{,}312 * final\ density)}$$

The ß-VLDL fraction was isolated by ultracentrifugation according to Mahley et al. (1980).
Ultracentrifuge tubes were filled with the plasma and centrifuged (Beckmann ultracentrifuge, rotor=55.2 Ti) at 51000 rpm, at 4°C for 20 hours. The tubes were then

sliced to obtain the ß-VLDL containing plasma at the top of the tubes. The ultracentrifugation step was repeated a second time.
The ß-VLDL containing plasma was transferred into dialysis tubes and kept in dialysis buffer (0.9% NaCl, 1% EDTA, pH=7.4) to remove the KBr. After the buffer had been changed twice, tubes were maintained within overnight.
Finally the concentration of ß-VLDL was determined by Bradford protein assay.

3.4.2 Human VLDL, LDL and HDL

VLDL, LDL and HDL were isolated from human blood. Fasting blood was collected, 7.2 mM EDTA was added. The further preparation steps were similar to ß-VLDL isolation, except for the density adaptation. First, the density of VLDL (d=1.019) was adjusted. After an ultracentrifuge step and removement of VLDL containing plasma, the density of LDL (d=1.07) was adapted, followed by another cetrifuge step and separation of LDL rich plasma. Ultimately, the density of HDL (d=1.22) was adjusted and after a last centrifuge step, HDL was obtained. The concentrations of the lipoproteins were estimated using the Bradford protein assay.

3.5 Iodination of lipoproteins

Lipoproteins were labeled with sodium (^{125}) iodide (Hartmann Analytic) using the Pierce IODO-BEADS iodination reagent kit.
In every labeling procedure, the lipoprotein solution (750µg ß-VLDL, 250µg HDL, 250µg LDL in PBS) was incubated with a iodo-bead and 1 mCi sodium (^{125}) iodide for 20 minutes at room temperature. Labeled lipoproteins were seperated from free sodium (^{125}) iodide by gel chromatography (Pierce D-Salt Dextran Desalting Column). First, the storage solution containing 0,02% sodium azide was decanted. The column was then equilibrated with 5 column volumes PBS (25ml).
The lipoprotein solution was applied on the gel after the 20 minutes incubation period. The lipoprotein was eluted by five steps with first 750µl and then four times 500µl PBS. The fractions were collected and the radioactivity (1µl) was measured

using a gamma-counter. Finally the protein content was quantified by the Bradford protein assay. The ß-VLDL labeling resulted in a specific activity of ~1000 cpm/ng. For experiments, labeled ß-VLDL was diluted ~1:5 (~200 cmp/ng) with unlabeled ß-VLDL.

3.6 Retroendocytosis experiments

3.6.1 Association experiments

Cells were seeded on 12-well plates (ldlA7, ldlA7-SRBI, CHO: 80.000 cells/well; HepG2: 300.000 cells/well) and grown for 2 days. On day 2 the cells were washed twice with sterile PBS and medium was changed overnight to DHG + 5% lpds (lipoprotein deficient serum) for ldlA7/ldlA7-SRBI cells and to MEM + 10% lpds for HepG2 cells.
On day 3 the experiment was performed:
First, medium was removed and cells were washed twice with PBS (37°C). Medium [0.5 ml DHG + 2mg/ml faf BSA (fatty acid free bovine serum albumin)] was added. A 40-fold excess of unlabeled lipoprotein was added to every fourth well to calculate unspecific binding. Labeled lipoprotein was added in an amount of 10µg/ml. Aliquots from the medium (10µl) were collected to count the specific radioactivity.
After one hour of incubation at 37°C, the association medium was removed and saved for degradation experiments. Cells were washed twice with 50mM TRIS + BSA (2 mg/ml) and twice with 50mM TRIS, both ice cold.
For lysis 0.5 ml 0.1 M NaOH per well were added for 30 minutes at room temperature. The radioactivity was counted in aliquots (300 µl) of the cell lysate. The amount of protein in the lysates was determined by Bradford protein assay.
Calculation of specific binding [ng lipoprotein/mg cell protein]:

$$\frac{(\text{Counts-blank})*(\text{factor/specific activity})}{\text{total protein}}$$

3.6.2 Displacement experiments

Association experiments were proceeded by a displacement procedure. The association media were removed and saved for degradation experiments. Cells were washed on ice twice with PBS + BSA (2 mg/ml) and twice with PBS ice cold. Next, 0.5 ml DHG + BSA (2 mg/ml) and a 50 fold excess of unlabeled lipoprotein per well were added. Additionally it was necessary to add HEPES (5µl; 1 M) because of the long period out of the incubator. The displacement process took two hours at 0°C. Cells were washed twice with 50mM TRIS + BSA (2 mg/ml) and twice with 50mM TRIS, both ice cold.
Cells were lyzed by addition of 0.5 ml 0.1 M NaOH per well for 30 minutes at room temperature. The radioactivity was counted in aliquots (300 µl) of the cell lysate. The amount of protein in the lysates was determined by Bradford protein assay.

3.6.3 Chase experiments

Chase experiments started after removal of the displacement media. Cells were washed on ice twice with PBS + BSA and twice with ice cold PBS. Then cells were incubated for 30 minutes at 37°C with 0.5 ml DHG + BSA (2 mg/ml) and a 20 fold excess of unlabeled lipoprotein. Cells were washed twice with 50mM TRIS + BSA (2 mg/ml) and twice with 50mM TRIS, both ice cold.
For lysis 0.5 ml 0.1 M NaOH per well were added for 30 minutes at room temperature. The radioactivity was counted in aliquots (300 µl) of the cell lysate. The amount of protein in the lysates was determined by the Bradford protein assay.

3.7 Degradation experiments

The media from association experiments were collected, and the protein (400µl) was precipitated with 50% trichloroacetic acid. The samples were kept at least 1 hour at 4°C. Next, the TCA soluble degradation products were seperated from the pellet. After centrifugation (4°C, 10 min, 4000 rpm) 400µl of the supernatant were

transferred into glass tubes. In order to remove free iodide, 40% potassium iodide (10µl) and 30% hydrogen peroxide (40µl) were added. Samples were mixed and after 5 minutes chloroform (1 ml) was added to extract the supernatant. Samples were again mixed and after ~10 minutes, 150µl of the upper aqueous phase were counted to assess the degradation activiy. The amount of acid-soluble products (mainly [^{125}I] iodotyrosine) is expressed in ng/mg cell protein.

3.8 Oil Red O Staining

The Oil Red O solution was prepared according to Ramirez-Zacarias JL et al. (1992). Cells were grown in 12-well plates and cholesterol depleted overnight. On day 3 they were incubated with 0, 5 or 10 µg/ml ß-VLDL overnight. Lipoproteins were therefore sterile filtrated. On day 4, cells were washed twice with PBS and then incubated with 500µl/well Oil Red O solution at room temperature. After 2 hours, cells were washed twice with PBS and the dye was extracted by adding 1 ml/well isopropanol for 10 minutes. The absorption was estimated photometrically in the supernatant at 510nm against isopropanol as blank value. The cells were lyzed with 1ml/well 0,1 M NaOH for 1 hour at room temperature. The protein concentration was measured using the Bradford protein assay.
The values were normalized to total cell protein.

3.9 Western Blot analysis

3.9.1 Preparation of cell lysates

Cells were grown in 6-well plates and washed twice with PBS on ice. Lysis buffer (62.5 mM Tris, 8 M Urea, 20 mM EDTA, 20 mM EGTA, 10 % Glycerol, 1 % SDS, pH 6.8) (50µl/well) was added, containing 1% protease inhibitor (Sigma). After 10 minutes of incubation on ice, the cell lysates were transferred into tubes and homogenized by 3 seconds ultrasonic treatment followed by 1 minute in liquid nitrogen. The cell lysates were then centrifuged by 12000 rpm for 10 minutes at 4°C.

The supernatants were collected and the protein content was quantified photometrically at 280nm. The lysates were stored at -20°C.

For the Western blot analysis the concentrations of the cell lysates were adjusted with PBS. The lysates were mixed with sample buffer in a ratio of 1:4. This sample mixture was heated for 30 minutes at 40°C.

3.9.2 Preparation of the gel

Glass plates were assembled for a gel of 0.75 mm thickness. The gels were prepared according to the table. The resolving gel was poured and sealed with isopropanol.
When the resolving gel had set, the stacking gel was poured and the comb (10 or 15 slots according to sample number) was inserted.

	Resolving Gel (8%)	Stacking Gel (4%)
Distilled water	1.6 ml	1.1 ml
3 M TRIS (pH 8.45; 0.3% SDS)	1.1 ml	0.3 ml
30% Acrylamid	1.3 ml	0.7 ml
10% APS	30 µl	20 µl
TEMED	3 µl	2 µl

3.9.3 Running the gel

The electrophoresis chamber was filled with running buffer (Tris Glycin) and the gel was inserted. The slots were flushed with buffer and loaded with the samples (10 slots: 20µl; 15 slots: 12µl).
A prestained protein standard (Fermentas) was also applied (~2 µl) to one lane.

The electrophoresis was run with constant voltage of 180 V with a current of ~60 mA per gel for 80 minutes.

3.9.4 Membrane transfer

The sandwich was assembled as follows: sponge – filter paper – nitrocellulose membrane (Amersham, Hybond-C) – gel – filter paper – sponge. The filter papers, sponges and the membrane were prewetted in tranfer buffer. The sandwich was inserted in the electrophoresis chamber that was filled with transfer buffer. The electrophoresis was run with constant current of 400 mA and voltage >100 V for 45 minutes.

The membrane was stained with Ponceau Red (0.1 g Ponceau S were dissolved in 5 ml acetic acid and filled up to 100 ml with distilled water) to visualize the protein bands. The membrane was then washed several times with a washing buffer (TBS, 0.1 % Tween). In order to reduce unspecific binding the membrane was incubated with blocking buffer (TBS, 5 % milk powder) for 1 hour. Thereafter the membrane was washed three times for 10 minutes with washing buffer.
The specific antibody (SR-BI/BII rabbit antibody, Novus biologicals) was added to a solution of washing buffer (3 % BSA, sodium azid) at a dilution of 1:1000. Beta-Actin (beta-Actin mouse antibody, Biorad) was used as a loading control. The membrane was incubated with the antibodies overnight at 4℃.
On day 2 the membrane was again washed by three steps.
The second antibody (rabbit anti-goat, Pierce; mouse anti-goat, Sigma) was added at a dilution of 1:10000 to the buffer solution (TBS, 5 % milk powder). After one hour of incubation the membrane was washed three times.
Finally, the membrane was incubated for 10 minutes with a chemiluminescent substrate (Super Signal, Pierce) and then analyzed in a chemiluminescent imager.

3.10 Inhibitors of intracellular cholesterol transport

Several inhibitors blocking intracellular cholesterol transport were used to analyze their effect on ß-VLDL derived cholesterol.

3.10.1 Chloroquine

Chloroquine is a weak base and raises the intracellular pH. This impairs several pathways that require a low pH, such as the intracellular cholesterol transport. It blocks the cholesterol transport from lysosomes to the endoplasmic reticulum by inhibiting the lysosomal acidic hydrolase (De Duve et al., 1974). LDL accumulates in lysosomes as a consequence of this process.

3.10.2 U18666A

U18666A inhibits the cholesterol transport from both the plasma membrane and the lysosomes to the endoplasmic reticulum (Liscum et al., 1992).

3.10.3 Imipramine

Imipramine is a hydrophobic amine as well as U18666A. It blocks the transport of cholesterol out of the lysosome (Liscum et al., 1992).

3.10.4 Monensin

Monensin is a monovalent carboxylic ionophore that interferes with vesicular transport. It disrupts LDL endocytosis through coated pits (Basu et al., 1981) and the transit of membrane vesicles from the Golgi apparatus to the plasma membrane (Tartakoff et al., 1983). The recycling of the LDL receptor to the plasma membrane is impaired.

Association experiments were performed to analyse the effect of the mentioned inhibitors on ß-VLDL cell association. Therefore cells were preincubated for 15 minutes with 50 µM chloroquine, monensin, imipramine or U18666A.

3.11 SR-BI knockdown in HepG2 cells

The SR-BI knockdown was performed using a lipid-based agent for reverse transfection (siPORT^{TM} NeoFX^{TM} Transfection Agent, Applied Biosystems).
A HepG2 cell suspension was prepared containing ~10^6 cells in 2.3 ml MEM medium. Next, 5 µl siPORT were diluted into 100 µl OPTI-MEM I medium (Invitrogen) and incubated at room temperature for 10 minutes. After that 5 µl siRNA (5 µmol/L) were also diluted into 100 µl OPTI-MEM I medium. The two solutions were then combined and incubated for 10 minutes at room temperature. The mixture was dispensed into cell culture plates (6-well plates) and overlayed with the prepared cell suspension. The transfected cells were then incubated under normal cell culture conditions. The medium was exchanged the next day. On day 2, medium was changed to MEM with 10 % lpds. After overnight cholesterol depletion an association experiment was performed.

3.12 High performance liquid chromatography (HPLC)

Cells were grown in 6 cm dishes for 1 day at a density of 80.000 cells/dish (ldlA7, ldlA7-SRBI) and 300.000 cells/dish (HepG2). On day 2, they were depleted of cholesterol overnight. On day 3, ldlA7 and ldlA7-SRBI cells were incubated with 0, 5 or 10 µg/ml sterile filtrated ß-VLDL overnight, HepG2 cells were incubated for 6 hours. After the incubation period, cells were washed twice with PBS+BSA (2 mg/ml) and afterwards twice with PBS. Cells were then incubated with 3 ml hexane:isopropanol (3:2) for 30 minutes to extract the lipids. In order to quantify the intracellular cholesterol, an internal standard of ergosterol (12 µg/dish, dissolved in hexane:isopropanol) was added. The extracted solution was transferred into glass tubes and cells were washed with another 3 ml hexane:isopropanol, which was also transferred into the glass tubes.
After extraction of the lipid content, cells were lyzed with 0.1 M NaOH and the protein was quantified using the Bradford protein assay.

The collected extract (6 ml) was separated into two aliquots. For analysis of free cholesterol (FC), one aliquot was dried under N_2 and taken up in 1 ml ethanol:chloroform (8:2). For analysis of total cholesterol (TC), the cholesteryl esters (CE) were saponified at 70 °C for one hour using 5 ml ethanolic KOH (3 %). The samples were mixed every ten minutes during the saponification. After the tubes had reached room temperature, 5 ml distilled water were added and the lipids were extracted with 10 ml hexane:isopropanol. The upper organic phase was collected after vortexing for 30 seconds. The lower phase was extracted a second time with 10 ml hexane:isopropanol. The upper phase was again removed and combined with the first extract. The solution was dried under N_2 and taken up in 1 ml ethanol:chloroform. Both aliquots were filtrated through a syringe filter (sterile Acrodisc, 0.45 µm, Gelman Sciences) into HPLC screw neck vials and applied to HPLC analysis.
HPLC analysis was performed on a separation module (Waters 2695, Waters) using a Nova-Pack C184 µm column (3.9 x 150 mm) and a dual absorbance detector (Waters 2487, Waters) at a wavelenght of 210 nm. Acetonitril:isopropanol (80:20) was used as running solution at a constant flow of 1 ml/minute. Every sample was run for 30 minutes.

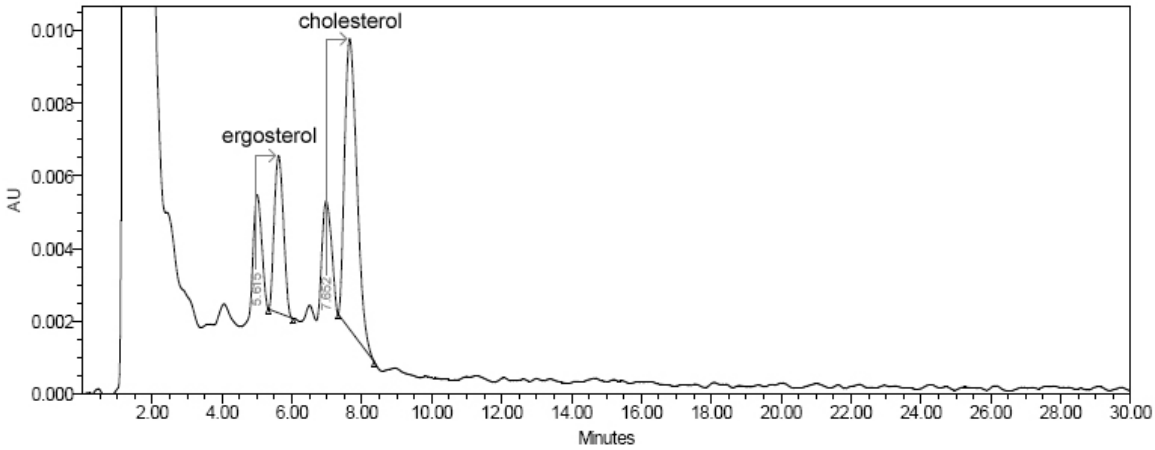


Figure 5: Representative HPLC-peaks of ergosterol and cholesterol.

Ergosterol was detected after ~5.6 minutes, cholesterol after ~7.6 minutes.
The areas under both peaks were measured for quantification. The cholesterol peak area was related to the ergosterol peak area and to the protein content for calculation

of the total cholesterol content and the free cholesterol content respectively. The cholesteryl ester content was calculated as total cholesterol content minus free cholesterol content.

The HPLC column was flushed with distilled water for 1 hour to remove excess acetonitril and with 50% methanol for several hours at a column temperature of 50℃ to remove the residual lipids from the column.

4. Results and Discussion

The aim of the study was to assess the function of SR-BI in ß-VLDL metabolism. Several lines of evidence indicate a major role of the LDL-receptor in ß-VLDL uptake (Herijgers et al., 2000; Van Lenten et al., 1985; Perrey et al., 2001). Therefore, ß-VLDL metabolism was analyzed in two different sets of cells. First, we used Chinese Hamster Ovarian cells (CHO) and as a more physiological model, the human liver cell line HepG2, which both express normal levels of the LDL-receptor and LRP. Second, we analyzed CHO cells that lack the LDL-receptor termed ldlA7 as well as SR-BI overexpressing ldlA7-SRBI cells.

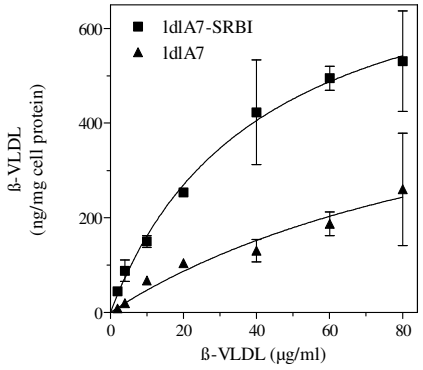
4.1 Cell association and competition experiments

4.1.1 ß-VLDL cell association in ldlA7 and ldlA7-SRBI cells

Recent studies demonstrated reduced ß-VLDL cell association in hepatocytes from SR-BI knockout mice compared to wildtype mice. Furthermore in vivo results suggest an impact of SR-BI in ß-VLDL metabolism (Van Eck et al., 2007).
SR-BI overexpressing cells were used to evaluate the role of SR-BI in ß-VLDL metabolism.
ß-VLDL cell association was measured after 1 hour of incubation with increasing concentrations of [^{125}I]-ß-VLDL in ldlA7 and ldlA7-SRBI cells (Fig. 6). The aim of this experiment was to assess whether SR-BI overexpression increases ß-VLDL cell association.
Both cell lines showed a dose dependent increase in ß-VLDL cell association. As expected, the dose response in SR-BI overexpressing cells was much higher. Incubation with 20 µg/ml iodinated ß-VLDL resulted in a cell association of 254 ± 7 ng ß-VLDL/mg cell protein in ldlA7-SRBI cells compared to 105 ± 4 ng ß-VLDL/mg cell protein in ldlA7 cells. These results confirm the contribution of SR-BI in ß-VLDL cell association, which was more than doubled due to SR-BI overexpression. The fact that ldlA7 cells still show a considerable amount of ß-VLDL association raises the

question whether other receptors might contribute to ß-VLDL binding. One possible binding site could be LRP, as ldlA7 cells express normal levels of this receptor. Besides the LDL-receptor family, several other uptake mechanisms have been discussed for VLDL and chylomicrons. Ioka et al. (2003) identified the 22kDa protein glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1). It was first described to mediate the binding of HDL (Ioka et al., 2003). Further studies (Beigneux et al., 2007) analyzed its role in the lipolytic processing of chylomicrons. GPIHBP1 $^{-/-}$ mice exhibited chylomicronemia on normal chow diet and GPIHBP1 was shown to bind chylomicrons as well as LPL, suggesting an important role in the metabolism of triglyceride-rich particles. These findings make GPIHBP1 an alternative candidate for ß-VLDL uptake, though it could not be detected in tissue culture cells (Beigneux et al., 2007).
Another receptor that was shown to mediate chylomicron remnant uptake is ß-chain ATPase (Beisiegel et al., 1988). It might therefore play a role in ß-VLDL metabolism.

Figure 6: [^{125}I]-ß-VLDL cell association in ldlA7 and ldlA7-SRBI cells.
ldlA7 and ldlA7-SRBI cells were seeded into 12-well plates at a density of 80000 cells/well. Cells were grown for 2 days followed by cholesterol depletion overnight with DHG supplemented with 1% Penicillin/Streptomycin and 5% lpds. On day 3, cells were washed twice with PBS and fed with DHG containing 1% Penicillin/Streptomycin and 2 mg/ml faf BSA. After incubation with increasing concentrations of [^{125}I]-ß-VLDL for 1 hour, cells were washed and lyzed as described in the methods section. Aliquots from the cell lysates were counted and specific cell association was calculated.



The data represent mean ± SD of two independent experiments.
The results are expressed in ng ß-VLDL/mg cell protein.

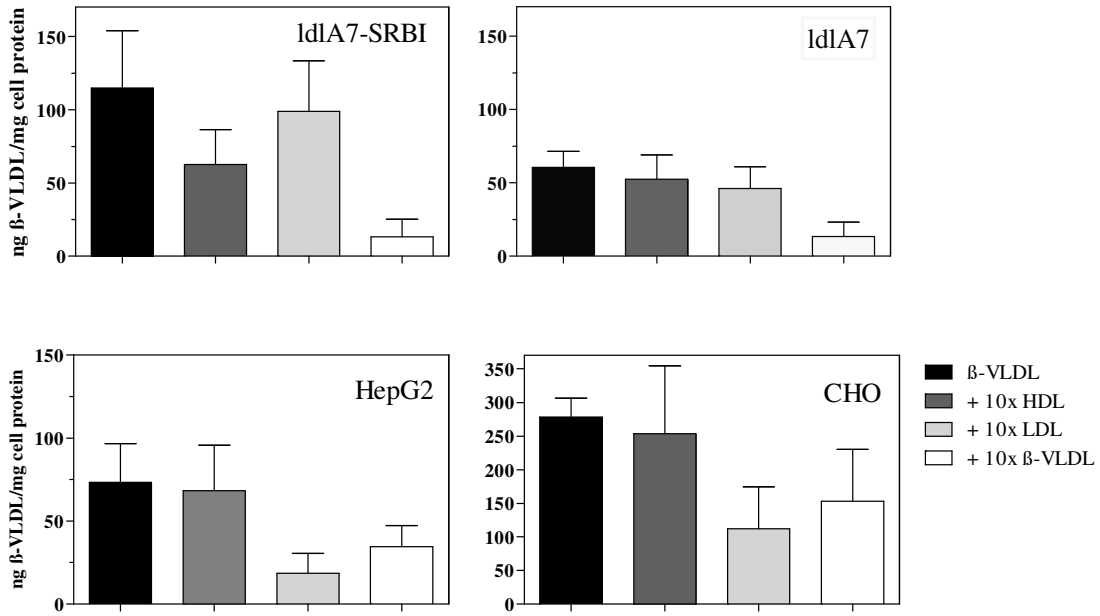
ß-VLDL (µg/ml)	*2*	*4*	*10*	*20*	*40*	*60*	*80*
ldlA7-SRBI	*45 ± 8*	*88 ± 23*	*150 ± 13*	*254 ± 7*	*423 ± 110*	*495 ± 26*	*531 ± 106*
ldlA7	*9 ± 1*	*20 ± 4*	*68 ± 3*	*105 ± 4*	*131 ± 23*	*188 ± 25*	*260 ± 119*

4.1.2 Association of [^{125}I]-ß-VLDL competed by HDL, LDL and ß-VLDL

For a more detailed characterization of ß-VLDL binding, competition experiments were performed.
Van Eck et al. (2007) performed competition experiments with a similar set-up in hepatocytes from SR-BI $^{+/+}$ and SR-BI $^{-/-}$ mice. They could show a decrease in ß-VLDL cell association after addition of a 10-fold excess of ß-VLDL (by ~80-90 %) , HDL (by ~55 %) and LDL (by ~30 %) in both cell types. Interestingly, HDL competed effectively for ß-VLDL binding also in the absence of SR-BI.
Competition experiments were performed by coincubating 10µg/ml [^{125}I]-ß-VLDL with a 10-fold excess of either ß-VLDL, HDL or LDL (Fig. 7).
Cell association of ß-VLDL was about 2-fold higher in SR-BI overexpressing cells compared to the parental cell line ldlA7. In CHO cells, which exhibit a functional LDL receptor, cell association was about 5-fold higher.
There was a considerable reduction of ß-VLDL association in ldlA7 and ldlA7-SRBI cells due to addition of a 10-fold excess of unlabeled ß-VLDL, indicating specific binding. The effect of a 10-fold excess of unlabeled HDL or LDL was much lower.
CHO and HepG2 cells showed a similar competition pattern. ß-VLDL association was well competed with LDL and ß-VLDL, but not with HDL. These differences can be ascribed to the contribution of the LDL receptor in CHO and HepG2 cells.
The competition pattern demonstrates that ß-VLDL is taken up differently by the cells. In HepG2 and CHO cells the particle is mainly internalized by the LDL-receptor, whereas in ldlA7 and ldlA7-SRBI cells it is preferentially taken up via SR-BI.

ß-VLDL cell association was about 2-fold higher in CHO cells compared to SR-BI overexpressing cells, indicating that the LDL receptor plays an important role in ß-VLDL metabolism. These findings are in line with several studies that have elucidated the major role of the LDL-receptor in ß-VLDL metabolism (Herijgers et al., 2000; Van Lenten et al., 1985). The SR-BI pathway might be of particular importance in cases, where the LDL receptor pathway is defective.

Figure 7: Association of [^{125}I]-ß-VLDL competed by HDL, LDL and ß-VLDL.
Cells were grown and treated as described in Figure 6. In order to investigate the influence of lipoproteins on ß-VLDL cell association, a 10-fold excess of unlabeled lipoproteins (HDL, LDL or ß-VLDL) was added. Aliquots of the cell lysates were counted and specific cell association was calculated.



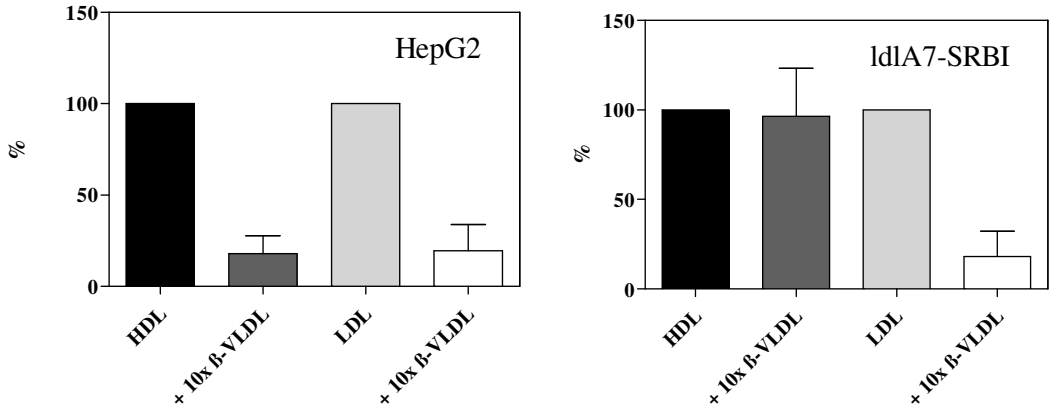
The data represent mean ± SD of three independent experiments (ldlA7-SRBI: n=4). ß-VLDL cell association is expressed in ng ß-VLDL/mg cell protein.

	ß-VLDL (control)	*+ 10x HDL*	*+ 10x LDL*	*+ 10x ß-VLDL*
ldlA7-SRBI	*115 ± 39*	*63 ± 24*	*99 ± 35*	*3 ± 12*
ldlA7	*61 ± 11*	*52 ± 17*	*46 ± 15*	*13 ± 10*
HepG2	*73 ± 23*	*68 ± 28*	*19 ± 12*	*35 ± 13*
CHO	*278 ± 28*	*254 ± 101*	*112 ± 62*	*153 ± 78*

4.1.3 Association of [^{125}I]-HDL or [^{125}I]-LDL competed by ß-VLDL

Cells lacking the LDL-receptor (ldlA7-SRBI) and HepG2 cells were compared with regard to inhibitory effects of ß-VLDL on HDL and LDL cell association (Fig. 8). In ldlA7-SRBI cells no effect of ß-VLDL on HDL cell association was observed, but LDL cell association was reduced by ~80 %. An explanation for the lacking inhibition of HDL association could be the abundance of SR-BI or different binding sites. A different pattern was observed in HepG2 cells. Both HDL as well as LDL association was well competed by ß-VLDL. HDL and LDL cell association were both reduced by ~80 %. HepG2 cells contain few SR-BI compared to ldlA7-SRBI cells which could be the reason for the competitive effect of ß-VLDL on HDL cell association. ß-VLDL preferentially binds to members of the LDL-receptor family, present in HepG2 cells, and thereby impaires LDL cell association.

Figure 8: Association of [^{125}I]-HDL or [^{125}I]-LDL competed by ß-VLDL.
Cells were grown and treated as described in Figure 6. HepG2 cells were plated at a density of 300000 cells/well. On day 3, cells were washed twice with PBS and fed with DHG containing 1% Penicillin/Streptomycin and 2 mg/ml faf BSA. Iodinated Lipoprotein (HDL or LDL) was added right after a 10-fold excess of unlabeled ß-VLDL in a concentration of 10 µg/ml. Control cells were incubated without the addition of ß-VLDL. After one hour of incubation, aliquots from the cell lysates were counted and specific cell association was calculated.



The data represent mean ± SD of three independent experiments.
HDL and LDL cell association competed by ß-VLDL is expressed in % of control.

	HDL + 10x ß-VLDL	*LDL + 10x ß-VLDL*
HepG2	*18.0 ± 9.7*	*19.5 ± 14.3*
LdlA7-SRBI	*96.4 ± 26.8*	*18.1 ± 14.3*

4.2. [^{125}I]-ß-VLDL binding, uptake and resecretion

Next we assessed, whether associated ß-VLDL was metabolized by the cells. Therefore, we performed association experiments followed by extensive washing and incubation with a 50-fold excess of unlabeled ß-VLDL for 2 hours at 0°C (displacement). Displacement experiments were performed to analyze whether associated ß-VLDL was taken up by the cells. There was no alteration in the amount of radioactivity between the association and the displacement lysates observed (Fig. 9). This data indicates an holoparticle uptake of almost all of the associated ß-VLDL. Since it was previously shown (Pagler et al., 2007) that HDL internalized by SR-BI can be resecreted ("retroendocytosis"), we performed the same experiment with ß-VLDL. For resecretion experiments (chase), cells were extensively washed after the displacement and further incubated with a 20-fold excess of unlabeled ß-VLDL for 30 minutes at 37°C to analyze the resecretion of the particle. The radioactivity did not

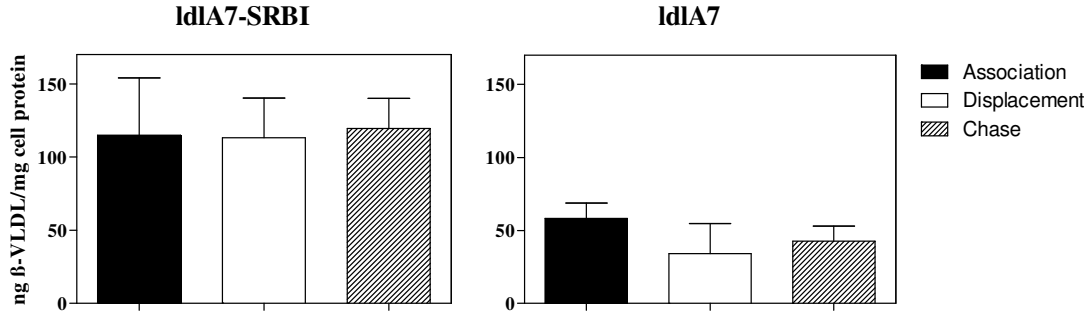
even change in the chase lysate meaning that the particle could not be released back to the media.

Again, cell association in ldlA7-SRBI cells was about 2-fold higher than in ldlA7 cells (compare Fig. 7).

In summary, it can be said that the vast majority of associated ß-VLDL is taken up by the cells and can not be released back to the media.

Figure 9: [^{125}I]-ß-VLDL binding, uptake and resecretion.

Cells were grown and treated as described. After one hour of association with 10µg/ml [^{125}I]-ß-VLDL, cells were washed and bound [^{125}I]-ß-VLDL was displaced for 2 hours at 4°C using a 50-fold excess of unlabeled lipoprotein. To analyse if the particle is released back to the media, internalized [^{125}I]-ß-VLDL was chased for one hour with a 20-fold excess of unlabeled lipoprotein at 37°C. The radioactivity of the cell lysats was counted.



The data represent mean ± SD.

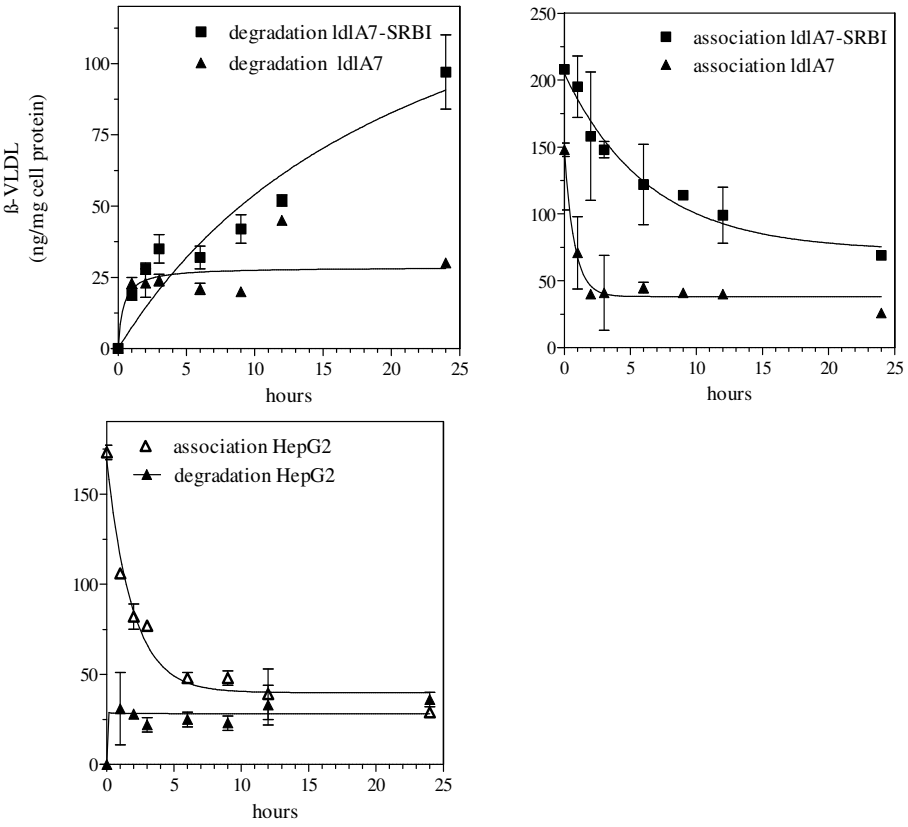
The results are expressed in ng ß-VLDL/mg cell protein.

	Association (n=5)	*Displacement (n=4)*	*Chase (n=2)*
ldlA7-SRBI	*115 ± 39*	*113 ± 27*	*120 ± 21*
ldlA7	*58 ± 11*	*34 ± 21*	*43 ± 11*

4.3. Time course of [^{125}I]-ß-VLDL association and degradation.

In order to investigate the fate of internalized ß-VLDL, we performed a time course experiment measuring association and degradation at several time points.
ß-VLDL cell association and degradation were measured in ldlA7 and ldlA7-SRBI cells over a 24 hour period (Fig. 10).
There was a time dependent decrease of ß-VLDL association and a corresponding increase of degradation in both cell lines. ß-VLDL was degraded at a rate of 6 ng per hour. The catabolism of the particle is slow, possibly due to the particle size.
Similar results were found in HepG2 cells. The time dependent decrease of radioactivity in the cell lysate was accompanied by an increase in TCA-resistant radioactivity in the media.
After 24 hours about 2/3 of the ß-VLDL was degraded. This indicates that ß-VLDL is metabolized faster in HepG2 cells, where the LDL receptor is present.
Previous studies reported that there was no specific accumulation of degradation products detectable in hepatocytes isolated from SR-BI $^{+/+}$ or SR-BI $^{-/-}$ mice (Van Eck et al., 2007). Tabas et al. (1990) found reduced ß-VLDL degradation in peritoneal macrophages compared to LDL, which was degraded more rapidly.
The degradation of ß-VLDL appeared to be very slow, however we could clearly detect degradation products which is in line with the findings of Tabas et al. (1990).

Figure 10: Time course of [^{125}I]-ß-VLDL association and degradation.
Cells were seeded on 6 cm dishes for 2 days. Cells were depleted of cholesterol overnight. On the experimental day, cells were washed twice with PBS and fed with DHG containing 1% Penicillin/Streptomycin, 2 mg/ml faf BSA and 10 µg/ml iodinated ß-VLDL. After one hour media were removed and cells were washed twice with PBS. Next DHG with 2 mg/ml faf BSA was added. From there on cells were washed and lyzed at several time points up to 24 hours. Media were removed to perform degradation experiments.



The data represent mean ± SD of two independent experiments.

The results are expressed in ng ß-VLDL/mg cell protein.

Association				***Degradation***		
Hours	*ldlA7-SRBI*	*ldlA7*	*HepG2*	*ldlA7-SRBI*	*ldlA7*	*HepG2*
0	*208 ± 105*	*148 ± 5*	*173 ± 4*	*0 ± 0*	*0 ± 0*	*0 ± 0*
1	*195 ± 23*	*71 ± 27*	*106 ± 1*	*19 ± 2*	*23 ± 2*	*31 ± 20*
2	*158 ± 48*	*40 ±1*	*82 ± 7*	*28 ± 2*	*23 ± 5*	*28 ± 2*
3	*148 ± 6*	*41 ± 28*	*77 ± 1*	*35 ± 5*	*24 ± 2*	*22 ± 4*
6	*122 ± 30*	*45 ± 4*	*48 ± 3*	*32 ± 4*	*21 ± 2*	*25 ± 4*
9	*114 ± 1*	*41*	*48 ± 4*	*42 ± 5*	*20*	*23 ± 4*
12	*99 ± 21*	*40*	*39 ± 14*	*52 ± 2*	*45*	*33 ± 11*
24	*69 ± 2*	*26*	*29 ± 1*	*97 ± 13*	*30*	*36 ± 4*

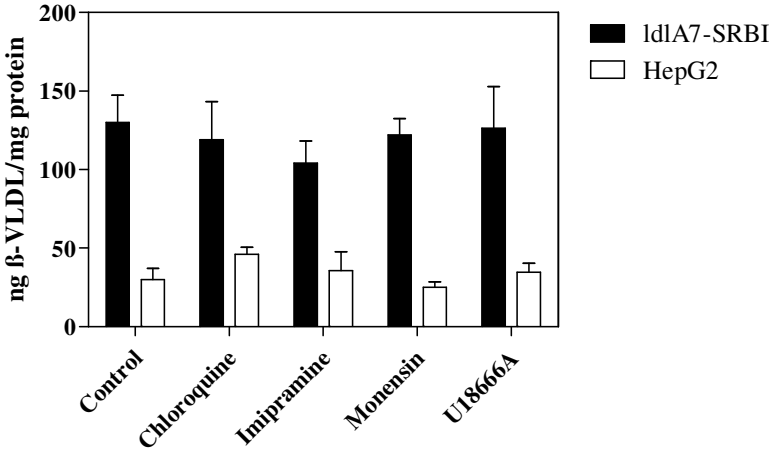
4.4. Effect of inhibitors of intracellular cholesterol transport on [^{125}I]-ß-VLDL cell association

To gain more knowledge of the mechanisms underlying ß-VLDL uptake, the effect of inhibitors blocking intracellular cholesterol transport on ß-VLDL uptake was assessed in HepG2 and ldlA7-SRBI cells (Fig. 11). In order to analyse the cholesterol transport between cellular compartments, inhibitors of different stages of intracellular cholesterol transport are useful instruments, for example hydrophobic amines, such as imipramine or U18666A, that block cholesterol transport out of the lysosomes (Liscum et al., 1992).
Chloroquine, which is a pH-raising agent, inhibits the lysosomal hydrolases (De Duve et al., 1974).
In both cell lines, preincubation with 50µM chloroquine, imipramine, monensin or U18666A did not substantially alter ß-VLDL cell association.
The experiment was repeated several times, but did not provide proper results due to decreased total cell protein levels. However, the radioactivity measurements in the cell lysates also indicated almost no alteration of the cell association compared to the control. This either suggests that there is no influence of the inhibitors on the trafficking of ß-VLDL derived cholesterol or that alternative endocytic pathways are activated.
Treatment of HepG2 cells with chloroquine blocked the hydrolysis of LDL- (by 89 %) and HDL-cholesteryl esters (by 52 %) but did not impair the uptake of cholesteryl esters. This inhibitory effects indicate that LDL is mostly degraded in lysosomes, whereas HDL is partially hydrolyzed in an extralysosomal compartment (Rhainds et al., 1999).
Previously, several inhibitors were used to assess their effect on HDL retroendocytosis in SR-BI overexpressing ldlA7 cells. Retroendocytosis of HDL involves the holo-particle uptake and resecretion of HDL, in contrast to LDL uptake. Chloroquine, imipramine, monensin or U18666A treatment did not significantly alter HDL uptake and resecretion (Pagler et al., 2007) in ldlA7-SRBI cells.

Figure 11: Effects of inhibitors on [^{125}I]-ß-VLDL cell association.

Cells were grown and treated as described.

HepG2 and ldlA7-SRBI cells were preincubated for 15 minutes with 50µM chloroquine, imipramine, monensin or U18666A. Afterwards an association experiment was performed.



The data represent mean ± SD of one representative experiment out of five.

The results are expressed in ng ß-VLDL/mg cell protein.

	Control	*Chloroquine*	*Imipramine*	*Monensin*	*U18666A*
ldlA7-SRBI	*130 ± 17*	*119 ± 24*	*104 ± 14*	*122 ± 10*	*126 ± 27*
HepG2	*30 ± 7*	*46 ± 5*	*35.67 ± 12*	*25 ± 4*	*35 ± 6*

4.5. SR-BI knockdown did not impair [^{125}I]-ß-VLDL cell association

Former studies analyzed the association and degradation of ß-VLDL particles in hepatocytes isolated from SR-BI knockout mice. As a consequence of SR-BI absence, ß-VLDL cell association was reduced and no specific accumulation of degradation products was detected (Van Eck et al., 2007).

SR-BI knockdown is another tool to assess the importance of the receptor in ß-VLDL metabolism.

Western blot analysis of SR-BI knockdown in HepG2 cells revealed a decreased expression of SR-BI of ~50% compared to control cells (Fig. 12).
ß-VLDL cell association was assessed comparing HepG2 control and SR-BI knockdown cells (Fig. 13). The same experiment was performed with HDL cell association (data not shown). Neither ß-VLDL nor HDL cell association was altered in siRNA treated cells. This all together indicates that other pathways might compensate for diminished SR-BI function. These results agree with the data from the competition experiments (compare Fig. 7).
However, hepatocytes from SR-BI knockout mice showed decreased ß-VLDL cell association (1.6 fold lower) compared to SR-BI $^{+/+}$ hepatocytes (Van Eck et al., 2007).

Figure 12: Western blot analysis of SR-BI knockdown cells.
HepG2 SR-BI knockdown and control cells were lyzed and analysed by western blot. 98 µg sample per lane were applied in dublicates. SR-BI was detected using a SRBI/BII antibody. ß-actin was used as a loading control.

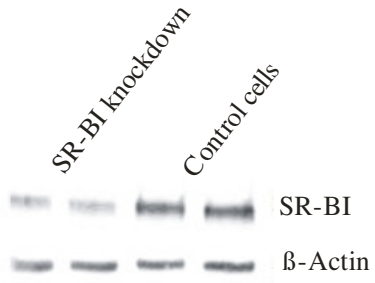
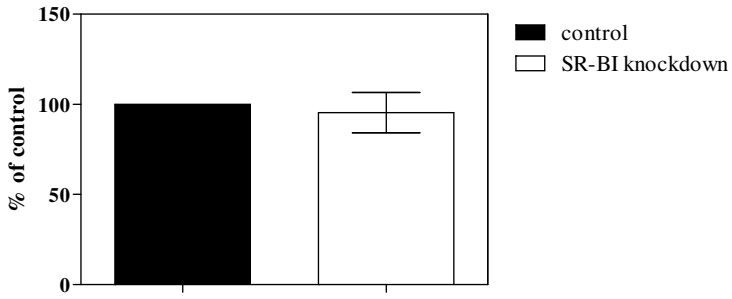


Figure 13: SR-BI knockdown did not impair [^{125}I]-ß-VLDL cell association.
SR-BI was knocked down in HepG2 cells using siRNA as described in the methods section. On day 2, medium was changed to MEM with 10 % lpds. After overnight cholesterol depletion an association experiment was performed.



The data represent mean ± SD of three independent experiments.

The results are expressed in % of control.

	SR-BI knockdown
HepG2	*95.32 ± 11.19*

4.6. ß-VLDL feeding increased the cellular lipid pool

We further questioned whether ß-VLDL derived lipid increases the cellular lipid content. We assessed alterations in the lipid pool of HepG2, ldlA7 and SR-BI overexpressing cells after ß-VLDL feeding, using two different methods. First, we used the Oil Red O method, which allowed a semi-quantitative determination of the cellular lipid content (triglycerides and cholesteryl esters) (Fig. 14). Second, we analyzed the cellular cholesterol content using HPLC (Fig. 15, Fig. 16).

The lipid content increased dose-dependently in both cell lines, ldlA7 and ldlA7-SRBI, after overnight ß-VLDL feeding. Incubation with 10 µg/ml ß-VLDL led to an increase of ~100% compared to control. The dose response was again higher in SR-BI overexpressing cells (Fig. 14, compare Fig. 6).

The total cholesterol content (TC) increased in both cell lines (ldlA7, ldlA7-SRBI) after incubation with increasing concentrations of ß-VLDL overnight. Both, a rise in free cholesterol as well as in the cholesteryl ester content contributed to this increase. The TC more than doubled in both cell lines after incubation with 10 µg/ml ß-VLDL compared to control (Fig. 15). These data are in line with the results from the Oil Red O test and indicate an utilization of ß-VLDL derived cholesterol by the cells. The

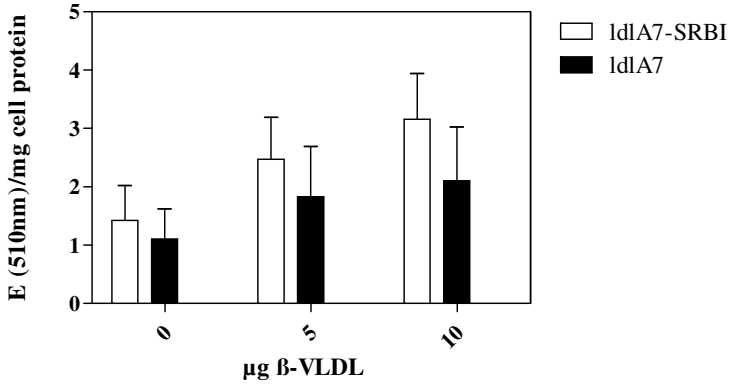
increase in the CE content points to the storage of excess cholesterol as cholesteryl esters.

The increase of the lipid content in HepG2 cells after ß-VLDL feeding was mainly due to an increase in TC and CE. The FC content just increased slightly (Fig. 17). Similar results were obtained from the Oil Red O test. There was just a little increase in the cellular lipid content even after incubation with 10 µg/ml ß-VLDL (Fig. 16). However, cholesterol delivered via ß-VLDL particles reaches the intracellular lipid pool in HepG2 cells.

Taken together, these results show that ß-VLDL derived lipid is utilized by all three cell lines.

Figure 14: ß-VLDL feeding increased cellular lipid pool in ldlA7 and ldlA7-SRBI cells.

ldlA7 and ldlA7-SRBI cells were seeded on 12-well plates. On day 1, cells were cholesterol depleted overnight. On day 3, cells were incubated with 500µl Oil Red O solution after overnight (18 h) incubation with 0, 5 or 10 µg/ml ß-VLDL. The extinction of the extracted dye was measured at 510 nm and normalized to total cell protein.

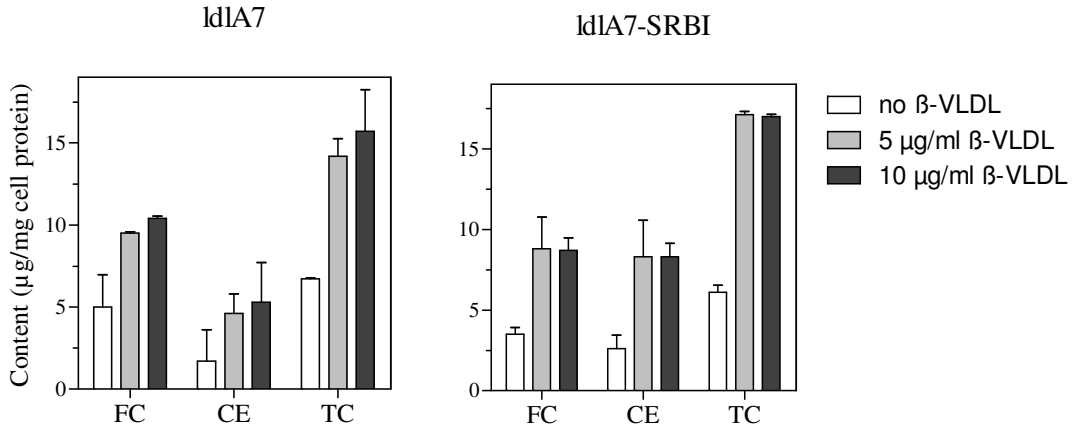


The data represent mean ± SD of three independent experiments.
The results are expressed in extinction (510nm)/mg cell protein.

µg ß-VLDL	*0*	*5*	*10*
ldlA7-SRBI	*1.42 ± 0.6*	*2.47 ± 0.7*	*3.16 ± 0.8*
ldlA7	*1.11 ± 0.5*	*1.83 ± 0.9*	*2.10 ± 0.9*

Figure 15: Analysis of the cellular cholesterol content in ldlA7 and ldlA7-SRBI cells.

ldlA7 and ldlA7 cells were plated in 6 cm dishes at a density of 80.000 cells/dish. After overnight cholesterol depletion, they were incubated on day 2 with 0, 5 or 10 µg/ml ß-VLDL overnight. Free cholesterol (FC) and total cholesterol (TC) content were measured by HPLC. The cholesteryl ester (CE) content was calculated as total cholesterol minus free cholesterol.

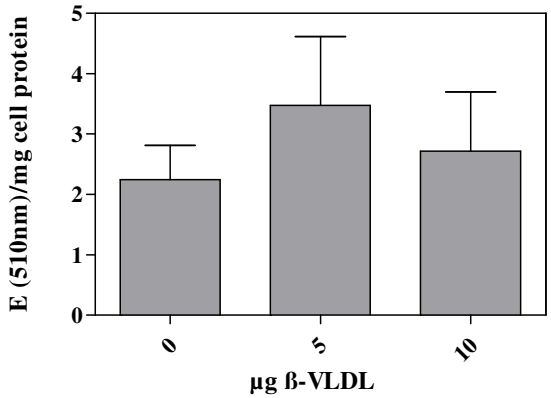


The data represent mean ± SD of triplicate values of one representative experiment out of three.

Results are expressed in µg cholesterol(respectively cholesteryl esters)/mg cell protein.

ldlA7-SRBI	*0 µg/ml ß-VLDL*	*5 µg/ml ß-VLDL*	*10 µg/ml ß-VLDL*
FC	*3.5 ± 0.6*	*8.8 ± 2.8*	*8.7 ± 1.1*
CE	*2.6 ± 1.2*	*8.3 ± 3.2*	*8.3 ± 1.2*
TC	*6.1 ± 0.6*	*17.1 ± 0.3*	*17.0 ± 0.2*
ldlA7			
FC	*5.0 ± 2.8*	*9.5 ± 0.1*	*10.4 ± 0.2*
CE	*1.7 ± 2.7*	*4.6 ± 1.7*	*5.3 ± 3.4*
TC	*6.7 ± 0.1*	*14.2 ± 1.5*	*15.7 ± 3.6*

Figure 16: ß-VLDL feeding increased cellular lipid pool in HepG2 cells.
Cells were treated as described in Fig. 14.



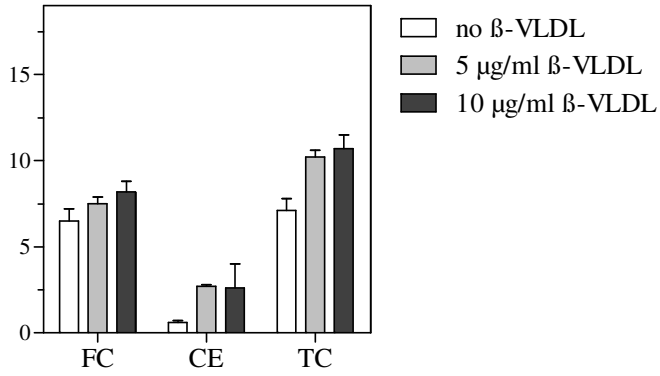
The data represent mean ± SD of three independent experiments. Results are expressed in E (510nm)/mg cell protein.

µg/ml ß-VLDL	*0*	*5*	*10*
HepG2	*2.25 ± 0.6*	*3.48 ± 1.1*	*2.72 ± 1.0*

Figure 17: Analysis of the cellular cholesterol content in HepG2 cells.

HepG2 cells were plated in 6 cm dishes at a density of 300.000 cells/dish. After overnight cholesterol depletion, they were incubated with 0, 5 or 10 µg/ml ß-VLDL for 6 hours. FC and TC content were measured by HPLC. The CE content was calculated as TC minus FC.

HepG2



The data represent mean + SD of triplicate values of one representative experiment out of three. The results are expressed in µg cholesterol (respectively CE)/mg cell protein.

HepG2	*0 µg/ml ß-VLDL*	*5 µg/ml ß-VLDL*	*10 µg/ml ß-VLDL*
FC	*6.5 ± 0.7*	*7.5 ± 0.4*	*8.2 ± 0.6*
CE	*0.6 ± 0.1*	*2.7 ± 0.1*	*2.6 ± 1.4*
TC	*7.1 ± 0.7*	*10.2 ± 0.4*	*10.7 ± 0.8*

5. Conclusion

In this study we found higher ß-VLDL cell association in SR-BI overexpressing CHO cells compared to ldlA7 cells. There was still ß-VLDL cell association in ldlA7 cells, indicating the contribution of other receptors than the LDL-receptor and SR-BI to ß-VLDL binding. Such possible binding sites could be LRP or ß-chain of ATPase that were both shown to mediate chylomicron uptake.

ß-VLDL was utilized by the cells, as indicated by internalization and degradation of the particle over time. Furthermore, analyses of the cellular lipid pool after ß-VLDL feeding substantiate this finding. ß-VLDL derived lipid was delivered to the cells as shown by an increase in their cholesterol and cholesteryl ester content.

Nevertheless, the LDL-receptor plays an important role in ß-VLDL metabolism as indicated by ~ 2-fold higher ß-VLDL cell association in LDL-receptor containing CHO cells compared to LDL-receptor lacking but SR-BI overexpressing ldlA7-SRBI cells. This is supported by results from SR-BI knockdown in HepG2 cells: diminished SR-BI expression did not decrease ß-VLDL cell association.

We could show that SR-BI mediates the uptake and degradation of ß-VLDL particles in tissue culture. The role of SR-BI in the metabolism of particles containing apo-B might have an impact on arterial lesion development.

6. Abstract

The present study was designed to elucidate the role of SR-BI in ß-VLDL metabolism. As SR-BI has recently been implicated in ß-VLDL uptake (Van Eck et al., 2007) it was of interest to further investigate the importance and characteristics of this process.

The utilization of two different sets of cells, LDL-receptor positive HepG2 and CHO cells and LDL-receptor negative ldlA7 and ldlA7-SRBI cells, allowed an evaluation of the contribution of SR-BI and the LDL-receptor in ß-VLDL metabolism.

Experiments illustrated that the majority of associated ß-VLDL is taken up by the cells and internalized particles can not be released back to the media, indicating an holoparticle uptake.

We also found that SR-BI overexpression leads to a ~ 2-fold higher ß-VLDL cell association. The higher ß-VLDL association in CHO cells compared to ldlA7-SRBI cells provides evidence for the relative importance of the LDL-receptor. This is substantiated by the results from SR-BI knockdown in HepG2 cells, that did not impair ß-VLDL cell association. Former studies are in line with the present results concerning the importance of the LDL-receptor in ß-VLDL metabolism (Herijgers et al., 2000; Van Lenten et al., 1985; Perrey et al., 2001).

ß-VLDL was utilized by the cells as indicated by an increase in the cellular lipid content (cholesterol, cholesteryl esters and triglycerides) after ß-VLDL feeding.

The degradation of the particle was low but measurable, which is in accordance with former findings in macrophages (Tabas et al., 1990).

In summary, SR-BI was shown to mediate the binding, uptake and degradation of ß-VLDL particles in tissue culture. However, SR-BI seems to play a subsidiary role, as the LDL-receptor is particularly prominent in ß-VLDL metabolism, that might gain importance in cases where it compensates for defective pathways.

7. Zusammenfassung

Das Ziel dieser Arbeit war, die Rolle von SR-BI im Stoffwechsel von ß-VLDL zu untersuchen. Da SR-BI kürzlich mit ß-VLDL Aufnahme in Verbindung gebracht wurde (Van Eck et al., 2007), war es von Interesse, die Wichtigkeit und Merkmale dieses Prozesses zu analysieren.
Die Verwendung von zwei verschiedenen Zellsystemen, LDL-Rezeptor positive HepG2 and CHO Zellen und LDL-Rezeptor negative ldlA7 und ldlA7-SRBI Zellen, erlaubte eine Bewertung des Einflusses von SR-BI und des LDL-Rezeptors im Metabolismus von ß-VLDL.
Die Experimente zeigten, dass die Mehrheit des assoziierten ß-VLDL von den Zellen aufgenommen wurde und dass die internalisierten Partikel nicht wieder von der Zelle abgegeben werden, was auf eine „Holopartikel"-Aufnahme hindeutet.
Wir haben weiters herausgefunden, dass SR-BI Überexpression zu einer ~ 2-fach höheren ß-VLDL Zellassoziation führt.
Die höhere ß-VLDL Assoziation in CHO Zellen verglichen mit ldlA7-SRBI Zellen untermauert die verhältnismäßige Wichtigkeit des LDL-Rezeptors. Diese wird bestätigt durch die Ergebnisse von SR-BI knockdown in HepG2 Zellen, welcher die ß-VLDL Assoziation nicht beeinträchtigt hat. Vorherige Studien entsprechen diesen Ergebnissen bezüglich der Wichtigkeit des LDL-Rezeptors im ß-VLDL Metabolismus.
ß-VLDL wurde von den Zellen umgesetzt, was anhand des Anstiegs des zellulären Fettgehaltes (Cholesterin, Cholesterol Ester und Triglyceride) nach ß-VLDL Zufuhr gezeigt wurde.
Die Abbaurate des Partikels war gering, aber messbar, was mit früheren Ergebnissen in Makrophagen übereinstimmt.
Zusammenfassend kann gesagt werden, dass SR-BI die Bindung, Aufnahme und den Abbau von ß-VLDL in Zellkultursystemen vermittelt. Dennoch scheint SR-BI eine untergeordnete Funktion zu übernehmen, da der LDL-Rezeptor eine entscheidende Rolle im Metabolismus von ß-VLDL spielt, die aber an Wichtigkeit gewinnen könnte indem er beeinträchtigte pathways kompensiert.

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