Modulation of hepatic gene expression: implications for lipid metabolism

Hoekstra, M.

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“Wetenschap is het mooiste beroep dat er bestaat. Wat is er immers mooier dan betaald krijgen om te mogen nadenken?”

- Teun Hoekstra -

Aan mijn moeder, broer en zus

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General introduction

1.1 Cardiovascular disease and atherosclerosis

The primary cause of death in the Western world involves cardiovascular diseases (CVD) such as ischemic (coronary) heart disease, angina pectoris, and myocardial and cerebral infarction. In the Netherlands, CVD is the major cause of death accounting for 34% of the mortality rate [1]. The most important cause of CVD and thus death is the phenomenon called atherosclerosis, narrowing of the arteries as a result of arterial lipid deposition.
Atherosclerosis is a progressive disease, characterized by the accumulation of lipids and fibrous elements (atherosclerotic plaque) in the arteries as a result of altered gene expression in the endothelium of the artery. Epidemiological studies have revealed numerous environmental and genetic risk factors for the initiation of atherosclerosis. These include smoking, consuming a high fat diet, low blood antioxidant levels, elevated blood pressure, diabetes, systemic inflammation, male gender, family history, and elevated blood lipid levels [2]. Upon local alteration of the endothelium, the expression of surface adhesion molecules and cytokines is induced, resulting in an increased adherence and subsequent migration of mononuclear cells (e.g. monocytes and lymphocytes) into the subendothelial space, where they then differentiate into macrophages and take up (modified) lipoproteins, forming foam cells (*fatty streak*) [3,4]. Subsequently, smooth muscle cells start to proliferate and migrate to form a fibrotic cap, consisting of extracellular matrix, collagen, and proteoglycans, that covers the lipid or necrotic core that is formed by excessive macrophage cell death (*advanced fibrous lesion*) [5]. A uniformly thick fibrous cap provides stability to the plaque resulting in a stable plaque. However, a thin and nonuniform cap may lead to instability of the plaque and rupture of the plaque, resulting in secondary hemorrhage and thrombosis, and possibly occlusion of the artery (*complicated lesion*) [6,7].

1.2 Lipoproteins and atherosclerosis

Cholesterol is an important molecule for eukaryotic organisms, since it modulates membrane fluidity and is essential for steroid hormones synthesis. In mammals, a complex system regulates biosynthesis and transport of cholesterol between cells in the body. Cholesterol is

transported through the blood circulation by lipoproteins. Lipoproteins are water-soluble protein-lipid complexes, which consist of a hydrophobic core, containing triglycerides and cholesterol esters, and a hydrophilic monolayered shell, composed of phospholipids, free cholesterol, and specific proteins (apolipoproteins). Several different lipoproteins can be distinguished based upon their lipid and apolipoprotein composition, electrophoretic mobility, and size (Table 1) [8].
In more detail, chylomicrons, very low-density lipoprotein (VLDL), and low-density liprotein (LDL) have apolipoprotein B (ApoB) as their primary protein, whilst apolipoprotein A-I (ApoA-I) is the major protein constituent of the high-density lipoprotein (HDL). Chylomicrons and VLDL are triglyceride-rich lipoproteins, whilst LDL and HDL contain relatively high levels of cholesterol esters and phospholipids, respectively. Furthermore, the size of the different lipoproteins is inversely correlated to their density, with VLDL being the biggest lipoprotein and HDL the smallest lipoprotein.

Table 1
Physical properties and composition of human plasma lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Diameter (nm)	75-1200	30-80	19-25	5-12
Density (g/ml)	<0.96	0.96-1.006	1.019-1.063	1.063-1.210
Mw (X 10^6 Da)	400	10-80	2.3	0.17-0.36
Mobility[1]	origin	Pre-β	β	α
Lipid composition[2]				
Triglyceride	80-95	45-65	18-22	2-7
Free cholesterol	1-3	4-8	6-8	3-5
Cholesterol ester	2-4	6-22	45-50	5-20
Phospholipid	3-6	5-20	18-24	26-32
Apolipoproteins	A-I, A-II, A-IV	-	-	A-I, A-II, A-IV
	B48	B100	B100	-
	C-I, C-II, C-III	C-I, C-II, C-III	-	C-I, C-II, C-III
	E	E	-	E

[1]According to the electrophoretic mobility of plasma α- and β-globulins on agarose gel electrophoresis
[2]The values given for composition are expressed as percentage of total weight

Importantly, multiple studies have indicated a strong correlation between serum levels of the different lipoproteins and the incidence of atherosclerosis and cardiovascular diseases. In fasting participants with normal triglyceride levels (<4.52 mmol/L), a 1.03 mmol/L higher baseline total non-HDL or LDL cholesterol was associated with a 30% to 35% higher cardiovascular heart disease event rate [9]. Data from the Lipid Research Clinics Program Follow-up Study, a mortality study, have shown that the non-HDL cholesterol level is a good predictor of cardiovascular disease mortality [10]. Furthermore, in a 15.5-year prospective study the non-fasting serum triglyceride level predicted the incidence of coronary heart disease among Japanese men and women who possessed low mean values of total cholesterol [11]. These combined findings suggest a strong positive correlation between a high level of (small) ApoB-containing lipoproteins and the risk for cardiovascular diseases. In contrast, Miller et al. have observed a lower HDL level in subjects with existing clinical ischemic heart disease

than in healthy subjects in the same population [12]. Several studies have indicated that there consists a strong negative correlation between serum HDL cholesterol levels and the risk for cardiovascular diseases. In accordance, a rise in HDL cholesterol levels leads to a reduction in the CVD risk at all plasma total cholesterol levels (Fig.1) [13].

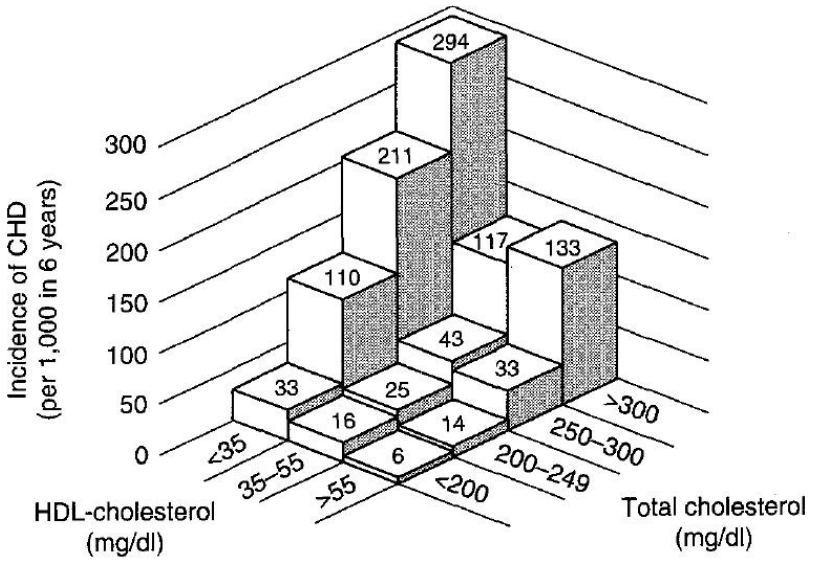

Fig.1. Incidence of cardiovascular heart disease per 1000 subjects over a 6-year period according to total cholesterol and HDL cholesterol levels.

1.3 Lipoprotein metabolism

1.3.1 Chylomicron metabolism

Dietary triglycerides and cholesterol esters that are hydrolysed in the intestine by pancreatic lipase and cholesteryl esterase, respectively, are absorbed by the intestinal epithelium and subsequently repackaged into chylomicrons. Lipid absorption is a complex process which involves coordinated gastric, intestinal, biliary and pancreatic function [14]. Nascent triglyceride-rich chylomicrons contain ApoB48, ApoA-I, and ApoA-IV as its primary aplipoproteins and are secreted into the lymph. Once secreted into the blood circulation, the chylomicrons loose ApoA-I and partly ApoA-IV, and acquire ApoC-I, ApoC-II, ApoC-III, and ApoE. In the circulation lipoprotein lipase (LPL) hydrolyses the triglycerides in the chylomicron core [15], which is accompanied by the transfer of phospholipids by phospholipid transfer protein and apolipoproteins to HDL [16]. This results in the formation of smaller triglyceride-depleted cholesterol ester-rich chylomicron remnants, which are rapidly cleared from the circulation by the liver. In the past, Mahley et al. have shown that initial rapid clearance by the liver

begins with sequestration of the remnants within the space of Disse, where apolipoprotein E secreted by hepatocytes enhances remnant binding and uptake. Heparan sulfate proteoglycans (HSPG), which are also abundant in the space of Disse, mediate this enhanced binding. Next, the remnants undergo further processing in the space of Disse by hepatic and lipoprotein lipases, which may also serve as ligands mediating remnant uptake. The final step, endocytosis by the hepatocytes, appears to be mediated, at least in part, by the LDL receptor and by the LDL receptor-related protein (LRP1). In addition, cell-surface HSPG are suggested to play a critical role in remnant uptake, not only in the important initial sequestration or capture step in the space of Disse, but also as an essential or integral component of the HSPG-LRP1 pathway (reviewed by Mahley and Ji [17]). Recently, Out et al. have shown that scavenger receptor class B type I (SR-BI) on liver parenchymal cells predominantly mediates the hepatic *association* of chylomicron remnants, since the association of chylomicron remants is >70% reduced in SR-BI deficient mice as compared to controls [18]. Subsequently, several recognition sites for ApoE, such as the LDL receptor and the LDL receptor-related protein (LRP1) that are present on the parenchymal cells mediate the whole particle uptake/internalization of the chylomicron remnants. However, the exact mechanism of the interaction between the primary association of chylomicron remnants by SR-BI and the subsequent secondary uptake via the ApoE-mediated LDL receptor/LRP1 process is still under investigation.

1.3.2 VLDL and LDL metabolism

VLDL is produced in the liver from cholesterol and triglycerides derived from de novo synthesis or lipoprotein uptake [19,20]. Human nascent VLDL contains a single copy of ApoB100 as well as newly synthesized ApoE and ApoC's. Upon its secretion into the circulation triglyceride-rich VLDL particles, like chylomicrons, acquire additional ApoE and ApoC. The triglycerides in the core of VLDL are subject to lipolysis by LPL [21], resulting in the formation of VLDL remnants, which can in part be cleared via the same mechanism as proposed for chylomicron remnants. The part of the VLDL remnants that is not cleared via the LDL receptor/LRP1 pathway can be further processed and converted into LDL. LDL only contains the apolipoprotein ApoB100, which serves specifically as a ligand for recognition by the LDL receptor. Importantly, a fraction of the LDL formed is not taken up by liver but is used as a source of cholesterol for the synthesis of membranes and steroids in cells of steroidogenic tissues. Moreover, a fraction of the LDL becomes modified in the circulation and is subsequently removed via uptake by macrophage scavenger receptors (reviewed by Van Berkel et al [22]).

1.3.3. HDL metabolism

HDL is produced primarily by the liver through an interaction of lipid-poor ApoA-I with the ATP-binding cassette transporter A1 (ABCA1), which mediates lipidation of ApoA-I, resulting in the formation nascent pre-β HDL

particles [23]. The nascent HDL particles subsequently take up free cholesterol from peripheral cells via an ABC mediated efflux system, which are converted to cholesterol esters by lecithin:cholesteryl acyltransferase (LCAT), leading to the formation of small spherical HDL_3. HDL_3 is subsequently converted into large α-migrating mature HDL_2 by acquirement of phospholipids and apolipoproteins that are released during lipolysis of triglycerides in chylomicrons or VLDL. The mature circulating HDL is then transported back to the liver, where it is bound to SR-BI with high affinity. Upon binding, SR-BI mediates the uptake of cholesterol esters into the liver without internalisation and degradation of the HDL particle (selective cholesterol ester uptake) [24]. The complete process of peripheral cholesterol efflux to HDL and subsequent transport and uptake of HDL cholesterol esters by the liver is called reverse cholesterol transport [25,26] and is considered to be a very important anti-atherogenic system in the body. A second route of hepatic HDL cholesterol clearance is through the enrichment of HDL with ApoE by either extrahepatic tissues or in the circulation and the subsequent whole particle uptake via the LDL receptor/LRP1 uptake system [27]. The third route of HDL cholesterol delivery to the liver is via the transfer of cholesterol esters from HDL to VLDL and LDL by cholesteryl ester transfer protein (CETP) [28] and subsequent hepatic uptake of these lipoproteins as described earlier. Importantly, rodents do not express CETP, which excludes this pathway for HDL cholesterol removal in these animals.

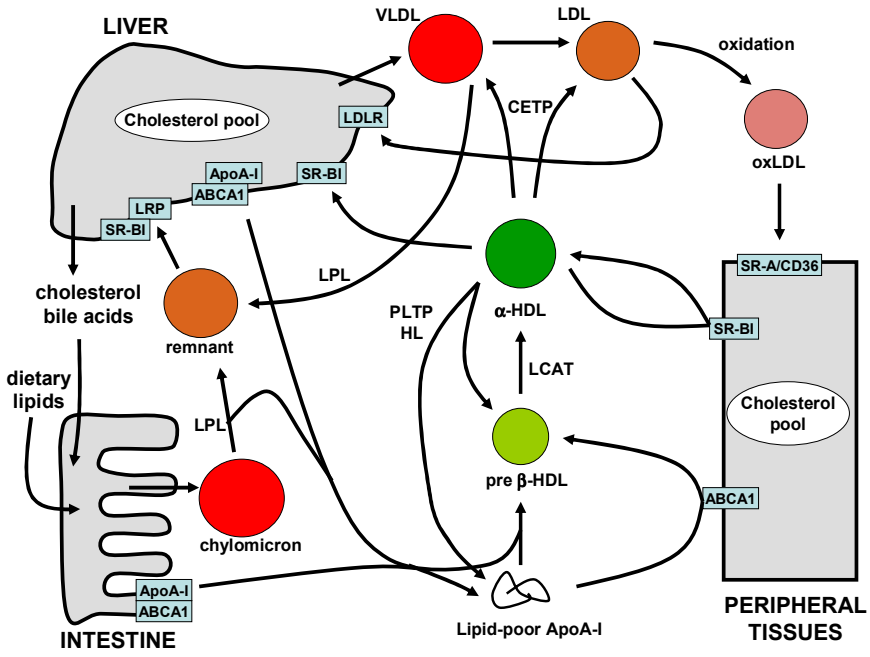

Fig.2. Schematic representation of the pathways involved in human lipoprotein metabolism.

1.4 Hepatic lipid metabolism

The liver plays an essential role in the removal of lipid from the blood circulation by mediating the uptake of lipoproteins [29,30]. Subsequently, cholesterol can be secreted into the bile or converted to bile acids coupled to excretion into the bile [31,32]. The liver primarily aims to control its intra-hepatic cholesterol homeostasis, by maintaining an appropriate balance between the regulatory free cholesterol and the more inert cholesterol ester pool. Several key processes are involved in the intra-hepatic cholesterol balance. These include: 1) the uptake free and esterified cholesterol from the lipoproteins, VLDL, LDL, and HDL, and 2) the de novo synthesis of free cholesterol from acetyl-CoA, which induce an increase in the intra-hepatic free cholesterol level, and 3) the esterification of free cholesterol for storage in the inert cholesterol ester pool, 4) the efflux of cholesterol to ApoA-I for the production of nascent HDL, 5) the catabolism of cholesterol to bile acids for excretion into the bile, and 6) the direct biliary efflux of free cholesterol (Fig.3). Importantly, each process is mediated through a complex interaction between different proteins, which all have their specific role in the process.

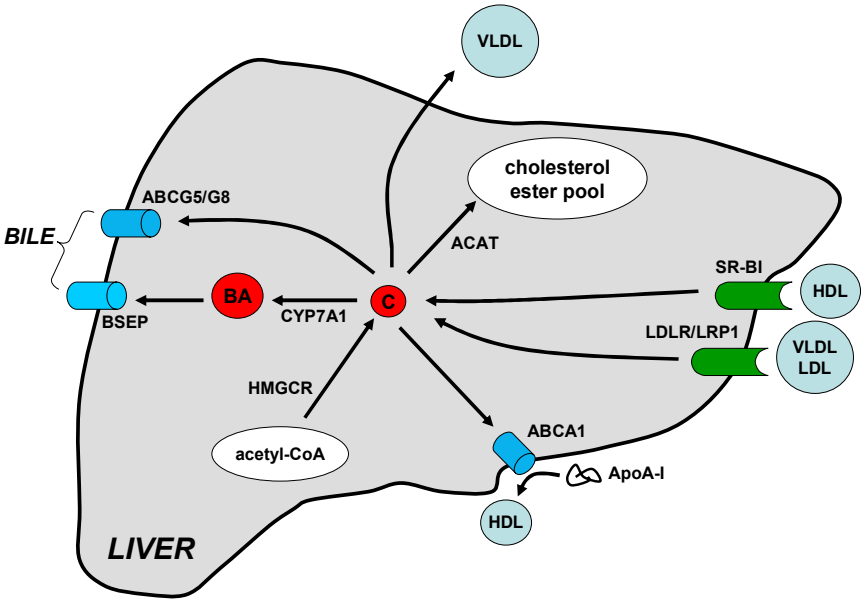

Fig.3. Pathways involved in the maintenance of the intra-hepatic cholesterol balance.
C = free cholesterol; BA = bile acids.

1.4.1 Genes involved in increasing the hepatic free cholesterol level

Hepatic uptake of cholesterol from lipoproteins via the LDL receptor, the LDL receptor-related protein, and scavenger receptor BI is an important mechanism via which the liver can *increase* its intra-hepatic cholesterol level. In addition, de novo synthesis of cholesterol via the action of the enzyme HMG-CoA reductase is a second important mechanism through which the liver is able to increase its intra-hepatic cholesterol level.

1.4.1.1 Low-density lipoprotein receptor (LDL receptor)

The LDL receptor is the prototype lipoprotein receptor of the LDL receptor family, which is highly expressed in tissues that utilize lipoproteins, such as the liver and adrenals [33]. The mature LDL receptor protein is a 160 kDa protein, which is formed in the Golgi from the 120 kDa LDL receptor precursor protein synthesized in the endoplasmatic reticulum [30]. At a cellular level, hepatic LDL receptors are confined to the basolateral (sinusoidal) surface of the parenchymal liver cells on microvilli and not to the apical (bile canalicular) surface [34]. Upon expression on the cell surface, the LDL receptor binds cholesterol-rich lipoproteins that contain ApoB and/or ApoE and mediates their endocytic uptake [35]. Particles internalised via the LDL receptor are subject to lysosomal degradation in which the apolipoproteins are broken down into amino acids while the lipids are released from the lysosomes into the cytosol. The uptake of lipoproteins by the LDL receptor serves a dual role in lipid metabolism. It delivers essential lipids required for the maintenance of cellular functions, and it regulates the concentration of cholesterol-rich lipoproteins in the blood. The importance of the latter function is underscored by pathological abnormalities observed in patients with LDL receptor gene defects. These patients suffer from a syndrome called familial hypercholesterolemia (FH), which is associated with increased serum cholesterol levels and premature atherosclerosis and coronary heart disease [36,37].

1.4.1.2 Low-density lipoprotein receptor-related protein (LRP1)

LRP1 (a.k.a. α2-macroglobulin receptor) is a largely conserved high molecular weight (Mw=600 kDa) multifunctional receptor of the LDL receptor family that is expressed in many different tissues and on numerous cell types, such as fibroblasts, smooth muscle cells, monocytes/macrophages, and in particular in liver parenchymal cells [38-40]. LRP1 is a heterodimer composed of a 515 kDa extracellular and an 85 kDa membrane-anchored subunit [41]. Both subunits are generated by proteolytic cleavage from a 600 kDa precursor and remain non-covalently associated to form the mature receptor on the cell surface. Like the LDL receptor, LRP1 is confined to the basolateral surface of the parenchymal liver cells and not to the apical surface. LRP1 has diverse biological roles, since it is able to recognize numerous ligands, including ApoE-enriched remnant lipoproteins, LPL, α2-macroglobulin-protease complexes,

plasminogen activator-inhibitor complexes, and tissue-type plasminogen activator [42,43]. Evidence that LRP1 is involved in the in vivo clearance of ApoE-containing lipoprotein remnants came from studies using transgenic mice deficient for LRP1 or receptor-associated protein (RAP), a chaperone protein and a strong inhibitor of ligand binding to LRP1 [44,45]. Furthermore, these studies demonstrated that in vivo chylomicron remnant uptake proceeds by a dual hepatic lipoprotein receptor system consisting of the LDL receptor and LRP1. Either receptor is able to assure virtually normal chylomicron remnant clearance when the other receptor pathway is defective. Furthermore, in dual receptor deficient mice (LDL receptor/LRP1 double knockout mice) the initial capture of chylomicron remnants by the liver is not affected.

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

SR-BI (a.k.a CD36 and lysosomal integral membrane protein-II analogous-1 [CLA-1]) is expressed in many tissues and cell types, including brain, lung, intestine, placenta, smooth muscle cells, macrophages, and endothelial cells. However, its highest expression has been detected in tissues that need cholesterol for bile acid (liver) and steroid hormone (adrenals) synthesis [46-50]. SR-BI is an 82 kDa cell surface membrane protein that consists of a heavily N-linked glycosylated and fatty acylated protein backbone, containing a large extracellular loop, two transmembrane domains, and short cytoplasmic N-terminal and C-terminal domains [51]. Under normal conditions, SR-BI in the liver is predominantly expressed on the sinusoidal membrane of parenchymal cells. However, SR-BI protein expression has also been detected in liver endothelial and Kupffer cells (tissue macrophages) [52], and on the canalicular membrane of parenchymal cells [53]. SR-BI in the plasma membrane has been suggested to facilitate lipid transport, since SR-BI it able to mediate the selective uptake of cholesterol esters from HDL, the process in which cholesterol esters from the HDL core are taken up without the endocytic uptake and degradation of the HDL particle/protein. Direct evidence for a role of SR-BI in the selective uptake was obtained from studies using genetically manipulated mice. Mice deficient in SR-BI accumulated cholesterol-rich HDL particles that are heterogenous in size and are enriched with ApoE, resulting from a hampered cholesterol ester uptake. In addition, these mice have a decreased hepatic biliary cholesterol secretion rate and their adrenal glands are depleted of cholesterol [54,55]. Moreover, on a Western type diet SR-BI deficient mice have an increased susceptibility to atherosclerosis as compared to their wild-type littermates [56], suggesting that the SR-BI mediated selective uptake of cholesterol esters from HDL is an essential atheroprotective process.

1.4.1.4 HMG-CoA reductase

In addition to the uptake of cholesterol from lipoproteins, the liver can also increase its intra-hepatic cholesterol level via de novo synthesis of cholesterol from acetyl coenzyme A (acetyl-CoA). The biosynthesis of cholesterol (C_{27}) from acetyl-CoA involves the formation of several carbon intermediates, including 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA; C_6), mevalonate (C_6), isopentenyl phosphate (C_5), and squalene (C_{30}). The rate-limiting step in cholesterol biosynthesis is the formation of mevalonate from HMG-CoA by the cytosolic enzyme HMG-CoA reductase. HMG-CoA reductase is therefore considered to be the key rate-limiting enzyme in cholesterol biosynthesis. HMG-CoA reductase is a 97 kDa endoplasmatic reticulum glycoprotein, anchored 7-fold in this membrane. Expression of HMG-CoA reductase has been detected in many organs where cholesterol is being synthesized, such as the intestine [57]. However, the major site for cholesterol biosynthesis in mammals is the liver. Importantly, the expression and activity of HMG-CoA reductase is rapidly reduced by sterols and metabolites derived from mevalonate (negative feedback pathway) [58]. Furthermore, the essential role of HMG-CoA reductase in cholesterol biosynthesis has been evaluated using specific HMG-CoA reductase inhibitors. Treatment of both rats and mice with these inhibitors resulted in a >90% decrease in hepatic sterol synthesis, suggesting an essential role for HMG-CoA reductase in hepatic cholesterol biosynthesis [59,60]. Since inhibition of HMG-CoA reductase activity significantly affects cholesterol biosynthesis rates, specific inhibitors of HMG-CoA reductase have been developed for the primary treatment of dyslipidemia and atherosclerosis. The most potent inhibitors are the statins, which in multiple studies have proven to be effective in lowering serum total and LDL cholesterol (up to 40%) and triglycerides [61], thereby considerably reducing the risk for and incidence of atherosclerosis and thus CVD death.

1.4.2 Genes involved in decreasing the hepatic free cholesterol level

The esterification of free cholesterol to cholesterol esters, the conversion of cholesterol to bile acids and the direct efflux of cholesterol to the serum or bile are important mechanism via which the liver can *decrease* its intra-hepatic cholesterol level. Several proteins involved in these processes have been identified. These include acyl-CoA:cholesterol acyltransferases, cholesterol 7α-hydroxylase, ATP-binding cassette transporter (ABC) A1, ABCG5, and ABCG8.

1.4.2.1 Acyl-CoA:cholesterol acyltransferase (ACAT)

The ACAT gene family consists of several enzymes involved in cholesterol ester synthesis. ACAT1 and ACAT2 function intracellularly to convert free cholesterol to cholesterol esters using an acyl coenzyme A (acyl-CoA) substrate. The tissue distribution of ACAT1 and ACAT2 is quite different, with ACAT2 being confined to sites of lipoprotein particle secretion in liver

hepatocytes and mucosal cells in the intestine, whereas ACAT1 is present in most tissues, with a relatively high expression in cells and tissues that store cholesterol esters in cytoplasmic lipid droplets such as macrophages of hyperlipidemics and adrenal cortical cells [62,63]. Both ACAT1 and ACAT2 are membrane-bound enzymes, which have multiple transmembrane domains, with the N-terminus of the enzyme residing in the cytosol and the C-terminus of the enzyme being situated in the ER lumen [64]. The structures and topology of ACAT1 and ACAT2 are still quite different (only about 60% similar in regions of the putative transmembrane domains), indicating that functional differences between the enzymes might be caused by differences in structure/topology. The important role for ACAT1 and ACAT2 in cholesterol ester synthesis has become evident from specific knockout animals. ACAT2 deficiency results in a reduction in cholesterol ester synthesis in the small intestine and liver, which in turn limits intestinal cholesterol absorption, hepatic cholesterol gallstone formation, and the accumulation of cholesterol esters in the plasma lipoproteins [65]. Total ACAT1 deficiency in LDL receptor deficient mice led to marked alteration in cholesterol homeostasis and extensive deposition of unesterified cholesterol in the skin and brain [66,67].

1.4.2.2 Cholesterol 7α-hydroxylase (CYP7A1)

In addition to the conversion of free cholesterol to cholesterol esters, the intra-hepatic free cholesterol content can be decreased through catabolism of cholesterol to bile acids. CYP7A1 is a microsomal cytochrome P450 that catalyzes the first step in bile acid synthesis. In agreement with its function in bile acid formation, CYP7A1 is only expressed in the liver [68], the organ involved in the conversion of cholesterol to bile acids. The intracellular distribution profile of CYP7A1 is quite similar to the one found for HMG-CoA reductase, with a confined expression of both enzymes in the smooth, ribosome-poor, endoplasmatic reticulum. The critical role for bile acid synthesis and thus CYP7A1 in the maintenance of cholesterol homeostasis has become clear from studies using transgenic mice. CYP7A1 gene knockout mice, as young adults on a regular chow diet, are hypercholesterolemic due a concomitant decrease in the expression of hepatic LDL receptors [69]. In contrast, mice with transgenic overexpression of human CYP7A1 had decreased plasma cholesterol levels on a regular chow diet [70]. Importantly, recent data have also indicated an important role for CYP7A1 in the maintenance of cholesterol homeostasis in humans; individuals with a homozygous deletion mutation in CYP7A1 resulting in loss of the active site and enzyme function (L413fsX414) have high LDL cholesterol levels, double the normal hepatic cholesterol content, and a markedly deficient rate of bile acid excretion [71].

1.4.2.3 ATP-binding cassette transporter A1 (ABCA1)

ABCA1 is a 240 kDa protein belonging to a large family of conserved transmembrane proteins that use ATP as a source to transport a wide variety of substrates across cellular membranes [72]. ABC transporters consist of two 6-helix transmembrane domains that serve as a pathway for the translocation of substrates across membranes and two nucleotide-binding domains that bind ATP and provide the energy for substrate transport [73,74]. ABCA1 is ubiquitously expressed, with highest expression levels in placenta, fetal tissues, lung, adrenal glands, brain, and liver. In the liver, ABCA1 is expressed on the sinusoidal membrane of parenchymal cells and on Kupffer cells [75]. The recognition that mutations in the human ABCA1 gene are the underlying molecular defect in HDL deficiency syndromes such as Tangiers disease [76-78] has contributed substantially to the understanding of the function of ABCA1. In addition, targeted disruption of ABCA1 in mice results in a virtual absence of HDL cholesterol [79,80], whilst overexpression of ABCA1 in mice increases HDL levels [81,82]. The liver expresses high levels of ABCA1 [75] and secretes lipid-free and lipid poor ApoA-I [83], the primary protein constituent of HDL, suggesting that the liver itself mediates the formation of HDL.

1.4.2.4 ATP-binding cassette transporters ABCG5 and ABCG8

ABCG5 and ABCG8 are, in contrast to ABCA1, half-size ABC proteins belonging to the ABCG or White subfamily of ABC transporters that are intended to dimerize to form active membrane transporters [84]. Among the half-size molecules ABCG proteins have a peculiar domain organization characterized by the nucleotide-binding domain (ATP-binding cassette) at the N-terminus followed by six transmembrane-spanning domains. ABCG5 and ABCG8 are both highly expressed in small intestine and liver. In the liver, mature ABCG5 and ABCG8 are expressed on the apical plasma membrane [84]. Exit of the proteins from the endoplasmic reticulum (ER) requires co-expression of both ABC half-transporters [85,86], suggesting that formation of a heterodimer is necessary to target ABCG5 and ABCG8 to the apical plasma membrane. Recently, studies using genetically modified mice have been conducted to elucidate the role of the twinned transporters ABCG5 and ABCG8. Transgenic mice that overexpress both human proteins display markedly elevated biliary cholesterol secretion rates, increased fecal neutral sterol excretion, and markedly reduced plasma phytosterol levels [87]. It was therefore suggested that ABCG5 and ABCG8, upon their expression on the apical membrane in the liver, function as an export pump for neutral sterols (e.g. cholesterol) to the bile, whilst ABCG5 and ABCG8 in the intestine play an important role in reducing intestinal absorption. Recently, several mutations and a number of polymorphisms have been identified in ABCG5 and ABCG8 in humans. Strikingly, mutations in either ABCG5 or ABCG8 have been related to the occurrence of a rare autosomal recessive disorder, called ß-sitosterolemia [88]. The disease is characterized by enhanced trapping of cholesterol and other sterols, including plant and shellfish sterols, within the intestinal cells

and the inability to concentrate these sterols in the bile. As a consequence affected individuals have strongly increased plasma levels of plant sterols, for example, ß-sitosterol, campesterol, stigmasterol, avenosterol, and 5-saturated stanols, whereas total sterol levels remain normal or are just moderately elevated. Despite the almost normal total plasma sterol levels, the disease shares several clinical characteristics with homozygous familial hypercholesterolemia. Patients suffer from tendon and tuberous xanthomas at an early age, premature development of atherosclerosis, and coronary artery disease [89].

1.5 Regulators of genes involved in lipid metabolism

The observation that several hepatic proteins are crucial factors involved in the different pathways of hepatic lipid metabolism, and thereby total body lipid homeostasis, has inspired scientists to intensively search for regulators of these factors on a transcriptional basis. Importantly, regulation of genes on a transcriptional level is primarily controlled by transcription factors, which are nuclear receptors that, upon activation, are able to bind to specific sequences in DNA resulting in a changed expression of their target genes. In the past, medicinal chemists and pharmacologists have not ventured into the field of transcription regulation due to the fear that drugs that interfere with transcription regulation may not be selective or efficacious. However, the past 5 years have seen some exciting developments in the field of signal transduction in general, and transcription regulation in particular with the discovery of a specific group of nuclear hormone receptors, the orphan receptors. Like all nuclear receptors, the orphan receptors share a common domain structure that includes a highly conserved DNA binding domain (DBD) with two zinc fingers and a ligand binding domain (LBD) (Fig.4A) [90]. The DBD interacts with specific DNA sequences known as response elements. The LBD, located in the carboxyl terminal of the protein, serves as the docking site for ligands and also contains dimerization motifs and transcriptional activation domains. The binding of a ligand to the LBD results in a conformational change in the receptor that facilitates interaction with accessory proteins and activation of target gene expression. However, prior to DNA binding and gene activation, these so-called orphan nuclear receptors need to form a receptor-dimer complex with a common heterodimeric partner, namely the retinoic X receptor (RXR) [91], which is bound and activated by 9-cis-retinoic acid (Fig.4B). In addition, this novel subfamily of transcription factors has been proven to be very important in disease (reviewed by Gurnell and Chatterjee [92]), since its members have been identified as critical players in carbohydrate metabolism, inflammation, reproduction, and nutritional status (Table 2). Interestingly, several orphan nuclear receptors have also been suggested to play a role in the regulation of (hepatic) lipid metabolism. These include the liver X receptor, farnesoid X receptor, peroxisome proliferator-activated receptor, and pregnane X receptor.

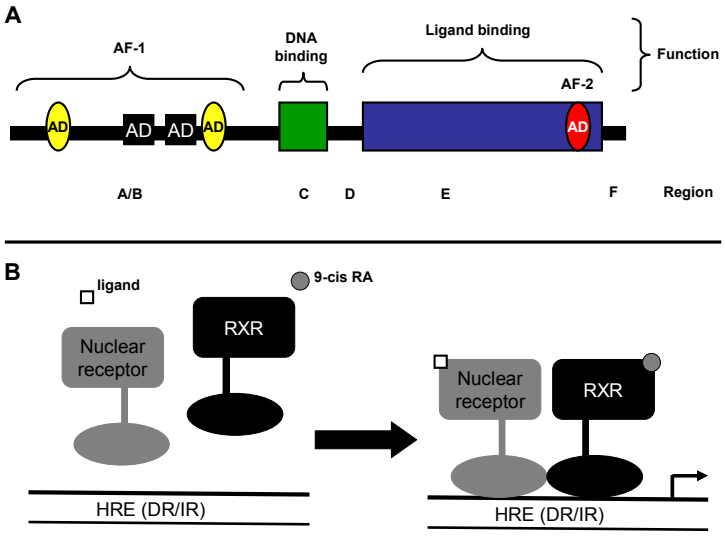

Fig.4. A) Structural and functional organisation of the nuclear receptor superfamily. Nuclear receptors consist of six domains (A–F) based on regions of conserved sequence and function. The evolutionarily conserved regions C and E are indicated as boxes, and a black bar represents the divergent A/B, D and F regions. The N-terminus (A/B region) contains one autonomous transcriptional activation function (AF-1). The highly conserved C region harbours the DNA-binding domain that confers sequence-specific DNA recognition. The ligand-binding domain (E region) is a highly structured domain comprising a ligand-dependent activation function (AF-2). The activation domains (ADs) contain transcriptional activation functions that can activate transcription when fused to a heterologous DNA-binding domain. **B) Nuclear receptors form heterodimeric complexes with RXR and affect target gene transcription**. Upon binding their specific ligands, nuclear receptors heterodimerize with 9-cis retinoic acid (9-cis RA)-activated RXR. This complex subsequently binds to hormone response elements (HREs) in the promoter of the nuclear receptor target gene leading to transactivation/transrepression.

Table 2
Nuclear receptors, their ligands, and associated functions and diseases

Receptor	HRE	Natural ligand	Synthetic ligand	Biological function	Disease
VDR	DR-3	Vitamin D3	Calcipotriene	Calcium absorption	Osteoporosis
TR	DR-4	T3	GC-1	Basal metabolic rate	Graves disease, Thyroid cancer
RARα,β,γ	DR-2 DR-5	All-trans RA	TTNPB	Vitamin A signalling, body health	Cancer, dermatology
PPARα	DR-1	Palmitic acid	Fenofibrate	Triglyceride	Hyperlipidemia, heart disease
PPARγ	DR-1	PGJ2	Rosiglitazone	Fat storage	Diabetes
PPARδ	DR-1	EPA	GW501516	Fatty acid metabolism, VLDL production	Hyperlipidemia
RXRα,β,γ	DR-1	9-cis RA	LG100268	Essential heterodimer partner	Cancer, insulin resistance
LXRα,β	DR-4	24(S),25-EPC	T1317	Cholesterol homeostasis	Heart disease
FXR	IR-1	CDCA	Fexaramine	Bile acid metabolism	Cholestasis
PXR	DR-3	LCA	Hyperforin	Drug and hormone detoxification	Drug-drug interaction
CAR	DR-5	Androstanol	TCPOBOP	Drug and hormone detoxification	Drug-drug interaction

HRE, hormone response elements; DR, direct repeat; IR, inverted repeat; T3, 3,5,3'-triiodo-L-thyronine; RA, retinoic acid; PGJ2, prostaglandin J2; EPA, eicosapentanoic acid; 24(S),25-EPC, 24(S),25-epoxycholesterol; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; TTNPB ([(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl]benzoic acid]; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

1.5.1 Liver X receptor (LXR)

The LXR subfamily consists of two members, LXRα and LXRβ. Both subtypes are expressed in the enterohepatic system, but each has a distinct pattern of expression in other tissues. Whereas LXRβ is ubiquitously expressed, LXRα expression is restricted to tissues rich in lipid metabolism, such as brown and white adipose tissue, intestine, kidney, and liver [93]. Upon activation by naturally occurring oxysterols derived from tissue-specific cholesterol metabolism (e.g. 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol), LXR forms the obligate heterodimer with RXR [94]. Subsequently, the RXR-LXR heterodimer is able to bind a DNA hormone response element (termed an LXRE) that consists of two hexanucleotide repeats separated by 4 nucleotides (DR4) in the promoter and/or distal enhancers of its target genes, resulting in a stimulation of target gene expression [95]. Due to the rapid generation of LXR deficient mice and specific (synthetic) activators of LXR, several target genes of LXR involved in liver lipid metabolism have currently been identified. In rodents, LXR has been shown to control the regulatory cascade of bile acid synthesis by activating CYP7A1 transcription through an LXRE in the CYP7A1 promoter [96]. In addition, the expression of the ABC transporters involved in cholesterol efflux to the serum (ABCA1) and bile (ABCG5/G8) has been shown to be strongly regulated by LXR activators [97,98]. Furthermore, LXR is able to induce the gene expression of the HDL receptor SR-BI in hepatocytes and preadipocytes [99]. Moreover, LXRs regulate the esterification and storage of cholesterol by an indirect means that involves the coordinate regulation of another important lipid metabolic pathway, fatty acid synthesis. Under high cholesterol conditions, LXRs stimulate transcription of sterol regulatory element-binding protein 1c (SREBP-1c), the master regulator of genes involved in fatty acid synthesis [100]. Increased SREBP-1c protein results in increased cleavage of this membrane-bound basic helix-loop-helix transcription factor and the transcription of a number of fatty acid-synthesizing enzymes, including stearoyl-CoA desaturase 1 (SCD1) [101]. SCD1 is an enzyme responsible for the 9-cis desaturation of stearoyl-CoA and palmitoyl-CoA, converting them to oleoyl-CoA and palmitoleoyl-CoA, respectively. Increased oleoyl-CoA, the preferred substrate for ACAT, enables increased esterification of cholesterol for storage. Since LXR is able to stimulate the expression of genes involved in different cholesterol and fatty acid synthesis/metabolism pathways, LXR can thus be considered a very important regulator of hepatic lipid homeostasis.

1.5.2 Farnesoid X receptor (FXR)

In contrast to the ubiquitous expression of LXR, expression of FXR is restricted to the enterohepatic system, kidneys, and adrenals [102]. Upon activation by its endogenous ligands, primary (e.g. chenodeoxycholic acid) and secondary (e.g. lithocholic acid and deoxycholic acid) bile acids, FXR forms an obligate heterodimer with RXR and binds to an inverted hexanucleotide repeat spaced by one nucleotide (IR1) in the promoter of its

target genes [103]. From multiple in vitro and in vivo studies it has become clear that FXR is the key factor that regulates bile acid homeostasis. In more detail, it maintains bile acid homeostasis by inducing the hepatic expression of genes involved in the export of bile acids such as the bile salt efflux pump (BSEP) [104], and by inhibiting the genes responsible for bile acid synthesis and uptake, CYP7A1 and sodium taurocholate cotransporter polypeptide (NTCP), respectively, through stimulation of the nuclear receptor small heterodimer partner (SHP) [105,106]. Interestingly, FXR has recently also been implicated in the regulation of serum triglyceride metabolism, since it is able to induce the hepatic expression of an activator (ApoC-II) [107] and to reduce the hepatic expression of an inhibitor (ApoC-III) of LPL activity [108]. However, FXR's main function is to serve as a bile acid sensor and regulator of bile acid synthesis, since FXR deficient mice develop cholestasis and severe liver damage [109]. In this light, recent studies have indicated that FXR is a promising therapeutic target for treating or preventing cholesterol gallstone disease.

1.5.3 Peroxisome proliferator-activated receptor (PPAR)

The PPAR subfamily consists of three members, PPARα, PPARγ, and PPARδ (also called PPARβ), which all bind fatty acids and fatty acid metabolites [110]. The different PPARs all heterodimerize with RXR and are able to bind the same DNA hormone response elements (termed PPREs) that consists of two hexanucleotide repeats separated by 1 nucleotide (DR1) in the promoter of their target genes. In the liver, PPARα is the most abundantly expressed PPAR subtype. Research has indicated that in the liver PPARα is able to regulate the expression of genes encoding peroxisomal, microsomal and some mitochondrial fatty acid metabolizing enzymes such as fatty acyl-CoA oxidase (ACO) [111] and enoyl-CoA hydratase (Ech-1)/3-hydroxyacyl-CoA dehydrogenase (HCDH) [112]. Thus PPARα is a strong activator of beta and omega fatty acid oxidation in mitochondria and peroxisomes, and microsomes, respectively. Since the PPARγ and PPARδ are not abundantly expressed in liver, the definite role of these nuclear receptors in liver lipid homeostasis has yet to be determined. However, from studies using genetically modified mice it has become evident that PPARδ activation is able to compensate for PPARα deficiency in skeletal muscle [113], which opens up the possibility that, in the liver, PPARδ might also have a role in the regulation of fatty acid oxidation. PPARγ is mainly expressed in adipose tissue where it coordinates the expression of many hundreds of genes responsible for establishment of the mature adipocyte phenotype, resulting in the development of adipose tissue [114]. Strikingly, treatment of mice with thiazolidinediones (TZDs), synthetic ligands of PPARγ, has been shown to decrease hepatic glucose production and increase glycogen synthesis in diabetic animals by increasing hepatic glucokinase expression [115], suggesting an important role for PPARγ in the regulation of glucose homeostasis.

1.5.4 Pregnane X receptor (PXR)

PXR (also called PAR/SXR) is the most recently established member of the orphan nuclear receptor subfamily. It is highly expressed in liver, and to a minor extent, in colon and small intestine. The name PXR is based on the fact that the receptor is activated by various natural and synthetic pregnanes [116]. However, progesterone, glucocorticoids, and multiple drugs (e.g. phenobarbital) are also able to activate PXR. In agreement with its orphan function PXR also heterodimerizes with RXR upon activation, resulting in DNA binding to DNA hormone response elements that consists of two hexanucleotide repeats separated by 3 nucleotides (DR3) in the promoter of its target genes. The observation that the PXR expression in tissues correlates with the CYP3A expression [117] has led to the assumption that PXR might be a regulator of CYP3A1 expression. In accordance, studies have shown that PXR activation increases the expression of CYP3A4 [118] and CYP3A7 [119] through binding to a PXR response element in their promoters. Importantly, CYP3A4 is the predominant expressed CYP expressed in human liver, constituting up to 60% of total hepatic P450 protein. CYP3A4 is involved in the metabolism of an extensive range of endogenous steroids and xenobiotics, making a significant contribution to the termination of the action of steroid hormones, elimination of foreign chemicals, and activation of several potent carcinogens [120]. It has been estimated that in excess of half of all therapeutic drugs are metabolized in full or part by this enzyme. Since PXR is a strong regulator of CYP3A4, the current vision on the role of PXR in the liver is that of xenosensor. Interestingly, recent data have also indicated an important role for PXR in the protection of the liver against bile acid toxicity, since litocholic acid (a toxic bile acid) is able to activate PXR, resulting in stimulation of the expression of several bile acid-metabolizing CYPs, bile acid transporters, and sulfotransferases that serve to detoxify bile acids such as lithocholic acid [121].

1.6 Outline of the Thesis

In the first part of the thesis the role of scavenger receptor class B type I (SR-BI) in lipid metabolism was studied. In Chapter 2, the relative importance of SR-BI in the removal of cholesterol esters from HDL was quantified in vivo. Recently, Out et al. have established a novel role for SR-BI in postprandial triglyceride metabolism [18]. In Chapter 3 additional studies are described on the role of SR-BI in chylomicron remnant metabolism in which adenoviral hepatic overexpression of SR-BI was used to determine the effect on chylomicron remnant metabolism. Finally, we also show that SR-BI, in addition to its role in lipoprotein metabolism, is important for the maintenance of adequate fasting glucose levels (Chapter 4).
The second part of the thesis focuses on the role of nuclear receptors in regulation of hepatic gene expression. In Chapter 5, the effect of a Western-type diet was studied on serum and hepatic lipid composition and the expression of genes involved in hepatic lipid metabolism in C57Bl/6

mice to gain insight into the nuclear receptor-mediated response of the liver to atherogenic diet feeding. In addition, the effect of a natural FXR agonist, taurocholic acid, on hepatic gene expression and atherosclerosis in ApoE deficient mice was explored in Chapter 6. Importantly, the liver consists of several different cell types with specific localizations and functions. Therefore, in Chapter 7 the expression of nuclear receptors and ABC transporters involved in hepatic lipid metabolism was studied in rat parenchymal, endothelial and Kupffer cells. In addition, the hepatic cell type specific regulation of genes involved in lipid metabolism by nuclear receptors on an atherogenic diet was investigated in Chapter 8. Furthermore, the initial nuclear receptor-mediated response of liver parenchymal cells to an increase in serum lipid levels by a Western type diet was studied in LDL receptor deficient mice using microarray technology (Chapter 9).

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2

Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice

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Scavenger receptor class B type I (SR-BI) has been identified as a functional HDL binding protein that can mediate the selective uptake of cholesteryl ester (CE) from HDL. To quantify the in vivo role of SR-BI in the process of selective uptake, HDL was labeled with cholesteryl ether ([^{3}H] CEt-HDL) and ^{125}I-tyramine cellobiose ([^{125}I]TC-HDL) and injected into SR-BI knockout (KO) and wild-type (WT) mice. In SR-BI KO mice, the clearance of HDL-CE from the blood circulation was greatly diminished (0.043 ± 0.004 pools/h for SR-BI KO mice vs. 0.106 ± 0.004 pools/h for WT mice), while liver and adrenal uptake were greatly reduced. Utilization of double-labeled HDL ([^{3}H]CEt and [^{125}I]TC) indicated the total absence in vivo of the selective decay and liver uptake of CE from HDL in SR-BI KO mice. Parenchymal cells isolated from SR-BI KO mice showed similar association values for [^{3}H]CEt and [^{125}I]TC in contrast to WT cells, indicating that in parenchymal liver cells SR-BI is the only molecule exerting selective CE uptake from HDL.
Thus, in vivo and in vitro, SR-BI is the sole molecule mediating the selective uptake of CE from HDL by the liver and the adrenals, making it the unique target to modulate reverse cholesterol transport.

2.1 Introduction

In both mice [1,2] and humans [3] there is a strong inverse relation between the blood levels of HDL and the development of atherosclerosis. The atheroprotective effect of HDL is ascribed to its role in reverse cholesterol transport (RCT), as first proposed by Glomset [4], in which HDL accepts cholesterol from peripheral cells, including those in the arterial wall, and delivers it to the liver for biliary secretion [reviewed in Refs. 4–10]. In addition, HDL can deliver its cholesteryl ester (CE) to the adrenals and testis or ovary for steroid hormone synthesis [11,12]. At both the liver and steroidogenic tissues, cholesterol delivery occurs via selective cellular uptake of HDL-CE without stoichiometric degradation of HDL protein [13,14]. Acton and coworkers [15] provided the first evidence that scavenger receptor class B type I (SR-BI) can mediate the selective uptake of HDL-CE in Chinese hamster ovary cells stably transfected with mouse SR-BI. Furthermore, treatment of the adrenocortical cell line Y1-BS1 with antibodies directed against mouse SR-BI resulted in a dramatic decrease in the selective uptake of HDL-CE (16).

In vivo, the expression levels of rat and mouse SR-BI mRNA and protein are highest in liver and steroidogenic tissues (adrenal gland, testis, and ovary) [17,18], all tissues that display selective uptake of HDL-CE. We showed earlier [19] that changes in SR-BI expression in rat liver, induced by estradiol treatment or a high-cholesterol diet, correlated with changes in the selective uptake of HDL-CE in vivo, supporting a function of SR-BI in mediating the selective uptake of HDL-CE. Adenovirus-mediated hepatic overexpression of SR-BI in mice on both sinusoidal and canalicular surfaces of hepatocytes resulted in the virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol [20]. A similar decrease in plasma HDL cholesterol levels was found in transgenic mice overexpressing SR-BI under the control of the apolipoprotein A-I promoter [21]. These studies indicated that SR-BI expression in the liver can regulate blood HDL metabolism and influence cholesterol secretion into bile. Studies with transgenic mice with liver-specific overexpression of SR-BI showed increased total and selective uptake of HDL-CE by the liver compared with nontransgenic controls [22,23]. In addition, in SR-BI attenuate (att) mice with an SR-BI promoter mutation resulting in decreased expression of the receptor, the hepatic uptake of HDL-CE was decreased accordingly [24]. Further evidence for the role of SR-BI in RCT was provided by studies in SR-BI knockout (KO) mice. These animals displayed impaired biliary cholesterol secretion [25,26] and increased plasma cholesterol concentration in large apolipoprotein A-I-containing particles, together with low adrenal gland cholesterol content [27,28).

Although the aforementioned studies are consistent with a regulatory role of SR-BI in HDL metabolism, there is no report that addresses the quantitative contribution of SR-BI in vivo to the selective uptake of HDL-CE by the liver and the adrenals. By using SR-BI KO mice in the present study, we made a quantitative comparison of the metabolism of HDL-CE in SR-BI KO and wild-type (WT) mice to assess the contribution of SR-BI to the serum decay and hepatic and adrenal uptake of HDL-CE. The data obtained are compared with data for holo-particle uptake, determined by analyzing the

fate of ^{125}I-tyramine-cellobiose-labeled HDL ([^{125}I]TC-HDL) in the animals, to assess whether additional HDL binding proteins perform selective CE uptake from HDL. In addition, studies with isolated parenchymal liver cells from WT and SR-BI KO mice demonstrate the quantitative importance of SR-BI for the high level of selective uptake of HDL-CE by these cells.

2.2 Experimental Procedures

2.2.1 Materials

Egg yolk phosphatidylcholine was from Fluka (Buchs, Switzerland). Cholesteryl [1,2(n)-^{3}H]oleoyl ether ([^{3}H]CEt), cholesteryl[1,2(n)-^{3}H] ester ([^{3}H]CE), and ^{125}I (carrier free in NaOH) were obtained from Amersham (Piscataway, NJ). The PL phospholipids kit, the CHOD-PAP (cholesterol oxidase-peroxidase aminophenazone) kit, and the GPO-PAP (glycerolphosphate oxidase-peroxidase aminophenazone) kit were from Roche Diagnostics (Mannheim, Germany). Hypnorm and Thalamonal were from Janssen Pharmaceutica (Titusville, NJ), and ketamine was from Bela-Pharm (Vechta, Germany). Ethylmercurithiosalicylate (thimerosal), BSA (fraction V), and collagenase type IV were from Sigma-Aldrich (St. Louis, MO). DMEM was from BioWhittaker (Walkersville, MD). All other chemicals were of analytical grade.

2.2.2 Animals

Heterozygous (+/-) SR-BI mice on a 129(agouti)/C57BL/6 background were kindly provided by Dr. Monty Krieger (Massachusetts Institute of Technology). The offspring of these mice was analyzed by polymerase chain reaction as described [27] for the presence of the targeted or WT SR-BI alleles. Experiments were carried out with homozygous mutant (SR-BI-/-) progeny. The WT (SR-BI+/+) littermates were used as controls. All animals used were between 8 and 10 week old males. Animals were maintained on a 12 h light/dark cycle and had unlimited access to regular chow diet (SRM-A; Hope Farms, Woerden, The Netherlands) and water. Animal welfare was in accordance with institutional guidelines.

2.2.3 Phospholipid liposome preparation

Unilamellar liposomes were prepared from egg yolk phosphatidylcholine and labeled with [^{3}H]CEt or [^{3}H]CE as described [29].

2.2.4 Isolation and labeling of lipoproteins

Human HDL and LDL were isolated from blood of healthy subjects by differential ultracentrifugation as described by Redgrave, Roberts, and West [30] and dialyzed against PBS with 1 mM EDTA. HDL (1.063<d<1.21) was

labeled with [^{3}H]CEt or [^{3}H]CE via exchange from donor particles as reported previously [31]. Donor particles were formed by sonication of egg yolk phosphatidylcholine supplemented with 50 µCi of either [^{3}H]CE of [^{3}H]CEt. Sonication was carried out with a MSE soniprep 150 for 40 min (amplitude, 12 µm) at 52°C under a constant stream of argon in a 0.1 M KCl, 10 mM Tris, 1 mM EDTA. 0.025% NaN3 buffer, pH 8.0. Donor particles with a density of 1.03 g/ml were isolated by density gradient centrifugation. HDL was labeled by incubating HDL with donor particles (mass ratio of HDL protein/particle phospholipid = 8:1) in the presence of human lipoprotein-deficient serum as the CE transfer protein source (1:1, v/v) for 8 h at 37°C in a shaking-water bath under argon. Ethylmercurithiosalicylate (thimerosal; 20 mM) was added to stimulate CE transfer and to inhibit phospholipid transfer and lecithin:cholesterol acyltransferase activity. Radiolabeled HDL was then isolated by density gradient ultracentrifugation.
The specific activity varied between 5 and 8 dpm/ng protein. For some experiments, HDL was doubly labeled with [^{125}I]TC. Synthesis and subsequent radioiodination of TC were performed as described earlier [32]. Coupling of [^{125}I]TC to HDL was done as described by Bijsterbosch, Ziere, and Van Berkel [33]. To 50 µl of 0.3 mM [^{125}I]TC were successively added 20 µl of 0.75 mM cyanuric chloride in acetone and 10 µl of 3.0 mM NaOH. After 20 s, 20 µl of 2.25 mM acetic acid was added. The resulting activated ligand was added to 1–2 mg of HDL in 1 ml of 20 mM sodium tetraborate buffer, pH 9.0, containing 0.12 M NaCl and 1 mM EDTA. After 30 min at room temperature, the reaction was quenched by the addition of an equal volume of 0.2 M NH_4HCO_3. Unbound label was removed by exhaustive dialysis against phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA). Less than 1% of the labeled material was trichloroacetic acid soluble. The specific activity was 20–30 cpm/ng protein. Before use, the radiolabeled HDL was checked for hydrolysis of the CE label and for its composition and physical properties [29]. Hydrolysis of the CE was <5%. Labeled HDL was used only when there were no differences in physical behavior and composition compared with the unlabeled HDL.
LDL was dialyzed against PBS with 10 µM EDTA and, where indicated, oxidized by exposure to $CuSO_4$ as described [34].

2.2.5 Plasma decay, organ association, and liver cell distribution

A dose of 200 µg (apolipoprotein) ± 1.2 x 10^6 dpm (plasma decay and organ association) or 500 µg (apolipoprotein) ± 3 x 10^6 dpm (liver cell distribution) of [^{3}H]CEt-HDL (total volume of 100 or 250 µl) was injected into the tail vein of WT and SR-BI KO mice. At 3 min after injection, a blood sample was drawn to verify the injected dose. At the indicated time points, blood samples were drawn to measure serum decay. For analysis of organ association, the liver, adrenals, and various other organs (stomach, intestines, thymus, pancreas, spleen, kidneys, thyroid, heart, aorta, veins, lungs, lymph nodes, muscles, skin, adipose tissue, bones, and reproductive organs) were isolated for radioassay at 1, 2, 4, and 24 h after tracer injection. The organs were weighed, solubilized, and counted for ^{3}H

radioactivity in a Packard liquid scintillation unit and for ^{125}I in a counter (Packard). Correction was made for the radioactivity in the blood present in the organs at the time of sampling as determined by injection of screened [125I]BSA. The values are in µl/g organ (means ± SEM; n = 4): liver, 84.7 ± 8.9; heart, 68.1 ± 2.9; lung, 125.9 ± 13.2; stomach, 22.9 ± 3.2; kidney, 135.2 ± 7.2; adrenal, 110.2 ± 9.5; spleen, 64.6 ± 9.1; small intestine, 46.3 ± 9.0; large intestine, 35.2 ± 3.0; skin, 13.1 ± 3.4; muscle, 13.7 ± 2.5; and bone, 30.1 ± 5.2. When double-labeled HDL was used, the [^{3}H]CEt radioactivity was measured in a Packard β counter with a double label program. Within the used $^{3}H/^{125}I$ activity ratios the percentage recovery for ^{3}H is 100 ± 2%. Computer analysis using an interactive curve peeling program was applied to fit a least-squares multiexponential curve to each set of serum decay data and to calculate fractional catabolic rates (FCRs) according to the model of Matthews [35]. The uptake by liver parenchymal, endothelial, and Kupffer cells was determined at 2 h after injection. After a liver lobule was tied off (total liver sample), the parenchymal liver cells were isolated as indicated under “In vitro studies with freshly isolated hepatocytes”. The endothelial and Kupffer cells were collected from the parenchymal cell supernatant by centrifugation for 10 min at 500 g and separated further via centrifugal elutriation as described [36]. The purity of each fraction was examined via a staining reaction for peroxidase activity followed by light microscopy. The purity was 80–90% and >95% for Kupffer cells and endothelial cells, respectively. The total liver sample and the cell isolations were solubilized and analyzed for protein content and radioactivity.

2.2.6 Protein determination

Protein was determined according to Lowry et al. [37] with BSA as the standard.

2.2.7 In vitro studies with freshly isolated hepatocytes

Between 10 and 11 AM, WT (SR-BI+/+) and SR-BI KO (SR-BI–/–) mice were anesthetized by subcutaneous injection with a mixture of 1.5% (w/v) ketamine, 8.5% (v/v) Thalamonal, and 1.5% (v/v) Hypnorm in PBS at a dose of 5–7.5 ml/kg. Collagenase perfusion (0.06% (w/v) collagenase) of the liver was then performed for 12 min as described [38]. Liver cells were collected by mincing the liver in ice-cold Hank's buffer containing 0.3% (w/v) BSA followed by filtration through nylon gauze to remove large debris. Three subsequent washing steps (10 min, 50 g, 4°C) with ice-cold Hank's buffer were performed to separate the parenchymal cells (pellet) from the endothelial and Kupffer cells (supernatant). The pellet consisted of pure parenchymal cells as judged by light microscopy. Cell viability was examined by trypan blue exclusion and was 80–90% in every case. After resuspension in oxygenated DMEM supplemented with 2% (w/v) BSA (pH 7.4), 1 mg of parenchymal cell protein was incubated with the indicated amounts of ligand at 37°C for 3 h in a final volume of 0.5 ml. During the incubation, the cells were shaken at 150 rpm and briefly oxygenated every hour. The viability of the cells remained greater than 80% during these

incubations. Subsequently, the cells were washed twice with wash buffer (0.15 M NaCl, 2.5 mM $CaCl_2$, and 50 mM Tris-HCl, pH 7.4) containing 0.2% (w/v) BSA and once with the same buffer without BSA, after which the cells were lysed in 0.1 N NaOH and protein content and radioactivity were measured

2.3 Results

2.3.1 Serum decay and liver and adrenal association

Upon injection of [^{3}H]CEt-HDL into WT mice, 51.7 ± 3.1% of the injected label was removed from the blood during the first 4 h. In SR-BI KO mice, a severely delayed clearance was noted (Fig.1). In WT mice, the liver uptake of CEt from HDL increased gradually up to 4 h after injection. At this time point, 31.3 ± 4.9% of the injected dose (ID) was recovered in the liver (Fig.1). This indicates that, in agreement with data obtained in rats [30], also in mice the liver is responsible for the majority of the removal of CEt from HDL. In SR-BI KO mice, the liver uptake of CEt from HDL was greatly diminished: at 4 h after injection, only 3.3 ± 0.8% of the ID accumulated in the liver, indicating an almost 90% inhibition of liver uptake in the absence of SR-BI ($P<0.05$). Serum decay kinetics and the rate at which the liver accumulated CEt tracer were determined in both WT and SR-BI KO mice. The activity of the liver was expressed as organ FCR per gram of tissue. This represents the fraction of the plasma pool of the traced HDL component cleared per hour per gram of tissue. The serum decay rate and the liver FCR of the [^{3}H]CEt-HDL were higher in WT mice compared with SR-BI KO mice. In WT mice, the serum FCR was 0.106 ± 0.004 pools/h (n=4); serum FCR was 0.043 ± 0.004 pools/h in SR-BI KO animals (n = 4; $P=0.002$). The liver FCR was 38.7 ± 6.6 x 10^{-3} serum pools/h/g in WT mice vs. 4.8 ± 0.80 x 10–3 serum pools/h/g in KO mice ($P=0.036$). These values indicate a severely delayed capacity for CE uptake in the SR-BI KO mice.
We observed earlier that SR-BI deficiency induced a 1.8-fold increase in total serum cholesterol levels [28]. Interestingly, this increase in total cholesterol levels was mainly the result of a 3.2-fold increase in free cholesterol, and CEs were only increased by 1.3-fold. Rigotti et al. [27] reported that apolipoprotein A-I levels were not different between WT and SR-BI KO mice. Correction for the HDL-CE pool size indicates that the HDL-CE mass influx into the liver was reduced from 22.0 ± 3.8 µg HDL-CE/h in WT mice to 4.1 ± 0.7 µg HDL-CE/h in SR-BI KO animals. To verify the possible presence of a selective CE decay and uptake in the absence of SR-BI, we injected double-labeled HDL, whereby the protein moiety was additionally labeled with [^{125}I]TC (Fig.2, Table 1). In WT mice, the decay rate of [^{3}H]CEt was higher than that of [^{125}I]TC, indicative of selective CE removal from serum. In contrast, the decay rates of [^{3}H]CEt-HDL and [^{125}I]TC-HDL in SR-BI KO mice were identical, reflecting the absence of a selective CE removal pathway in these animals. The liver uptake values of [^{3}H]CEt-HDL were similar to those for single CE-labeled HDL (30.3 ± 1.2% for WT mice and 4.4 ± 0.9% for KO mice), whereas the uptake values for

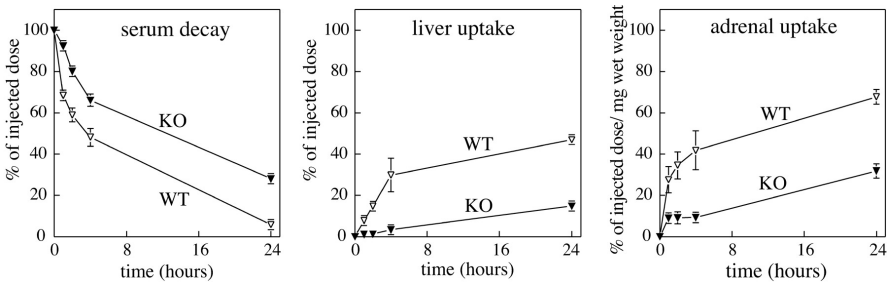

Fig.1. Serum decay, liver association, and adrenal association of HDL labeled with cholesteryl ether ([^{3}H]CEt-HDL) in wild-type (WT) and scavenger receptor class B type I (SR-BI) knockout (KO) mice. A dose of 200 µg (apolipoprotein; 1.2 x 10^6 dpm) of [^{3}H]CEt-HDL was injected into the tail vein of WT (open symbols) and SR-BI KO (closed symbols) mice. At the indicated time points, the animals were killed, serum was collected, and liver and adrenal biopsies were taken. The tissue samples were weighed, combusted in a Hewlett-Packard 306 sample oxidizer, and counted for radioactivity. Serum decay and liver and adrenal association were calculated. Correction was made for the radioactivity in the blood present in the organs at the time of sampling (see "Experimental Procedures"). For serum and liver, data are expressed as percentage of injected dose (ID), whereas for the adrenal, association values are expressed as percentage of ID/mg wet weight x 10^3. Values are means±SEM of four animals for each time point.

Fig.2. Serum decay of double-labeled [3H]CEt-HDL and HDL labeled with tyramine-cellobiose ([125I]TC-HDL) in WT and SR-BI KO mice. A dose of 200 µg (apolipoprotein) was injected into the tail vain of WT [open circles ([^{3}H]CEt-HDL) and open triangles ([^{125}I] TC-HDL)] and SR-BI KO [closed circles ([^{3}H]CEt-HDL) and closed triangles ([^{125}I]TC-HDL)] mice. At the indicated time points, blood was taken and radioactivity was determined as described in Materials and Methods. The data are expressed as percentage of ID ± SEM of four animals at each time point.

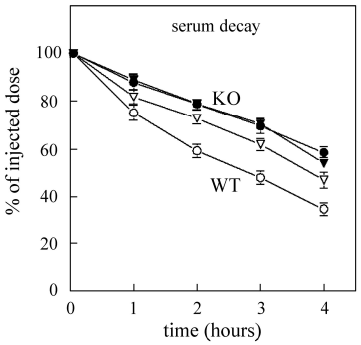

Table 1
Liver and adrenal association of [^{3}H]CEt-HDL and [^{125}I]TC-HDL at 4 h after injection into SR-BI KO mice and WT mice

	[3H]CEt-HDL		[125I]TC-HDL	
Tissue	WT mice	SR-BI KO mice	WT mice	SR-BI KO mice
Liver (% ID)	30.3 ± 1.8	4.4 ± 0.9 [a]	4.8 ± 0.6	5.4 ± 0.4
Adrenals (%ID/mg wet weight x 10^3)	45.3 ± 5.0	6.3 ± 1.7	6.1 ± 1.6	3.1 ± 0.2

HDL labeled with tyramine-cellobiose ([^{125}I]TC-HDL) and cholesteryl ether ([^{3}H]CEt-HDL) was injected into the tail vein of wild-type (WT) and SR-BI knockout (KO) mice. At 4 h after injection, the animals were killed, serum was collected, and liver and adrenals were taken. Tissue samples were weighed, solubilized, and together with the serum samples assayed for ^{3}H or ^{125}I counts. Correction was made for the contribution of serum to the measured organ-associated radioactivity (see Experimental Procedures). Data are expressed as percentage of injected dose (ID) for liver and as percentage of ID/mg wet weight x 10^3 for the adrenals. The values are means±SEM of five to eight animals.

[a] Significant difference for [^{3}H]CEt-HDL ($P<0.05$) between WT and KO mice. The WT [^{125}I]TC-HDL values did not differ significantly from the KO [^{3}H]CEt-HDL values or the KO [^{125}I]TC-HDL values.

[^{125}I]TC (4.8 ± 0.6% for WT mice and 5.4 ± 0.4% for KO mice) indicated that no residual selective uptake of CE from HDL was observed in SR-BI KO mice. Because the observed liver uptake did not account fully for the serum decay, the distribution of radioactivity over extra-hepatic tissues was also analyzed at various time points after injection; the data for 24 h are given in Table 2. It was found that the majority of the extra-hepatic label was present in skin (15–18%), bones (6–8%), muscle (3%), and intestine (4%), whereas the other tissues contained <1% for each tissue. The total recovery of label was between 80% and 90% for both WT and KO mice in every case. There was no significant difference in extra-hepatic distribution between WT and SR-BI KO animals except for the adrenals. The major differences in adrenal uptake occurred during the first 4 h after injection (Fig.1), during which the uptake in WT mice increased to 45.3 ± 5.0% of the ID/mg wet weight x 10^3, whereas uptake was only 6.3 ± 1.1% of the ID/mg wet weight x 10^3 in the SR-BI KO mice (Table 1).

Table 2
Percent radioactivity in tissues determined 24 h after injection of [^{3}H]CEt-HDL in WT and SR-BI KO mice

Organ	WT mice	SR-BI KO mice
Liver	46.9 ± 2.4	14.8 ± 1.2
Heart	0.4 ± 0.1	0.7 ± 0.2
Lung	0.7 ± 0.1	0.7 ± 0.2
Stomach	0.6 ± 0.2	0.6 ± 0.2
Kidney	0.6 ± 0.1	0.4 ± 0.2
Adrenal	0.5 ± 0.1	0.2 ± 0.1
Spleen	0.7 ± 0.2	1.0 ± 0.2
Small intestine	4.0 ± 0.3	3.3 ± 0.2
Large intestine	2.5 ± 0.3	2.0 ± 0.2
Skin	14.7 ± 2.4	18.1 ± 4.8
Muscle	4.1 ± 0.3	3.7 ± 0.4
Bone	8.2 ± 0.6	9.3 ± 1.9
Serum	4.9 ± 0.7	32.0 ± 1.0
Recovery	88.8%	86.8%

[^{3}H]CEt-HDL was injected into the tail vein of WT and SR-BI KO mice. At 24 h after injection, the animals were killed, serum was collected, and tissues were removed. Tissues were assayed for ^{3}H counts, and correction was made for the contribution of serum to the measured organ-associated radioactivity (see Experimental Proceudres). The values are means±SEM of four animals in each group

2.3.2 In vivo liver cell distribution

At 2 h after injection of [^{3}H]CEt-HDL, parenchymal, endothelial, and Kupffer cells were isolated from the livers of WT and SR-BI KO mice to examine the potential roles of SR-BI in the process of CEt uptake by these various cell types. For total liver, there was an 81.6% lower association of CEt in SR-BI KO animals compared with WT mice (13.1 ± 1.3% and 71.3 ± 3.6% of the ID/mg protein x 10^{-3} for KO and WT mice, respectively) (Fig.3). It can be calculated that in the WT liver, parenchymal cells were responsible for 88% of the [^{3}H]CEt uptake from HDL, when taking into account the contribution of these cells to total liver mass. The absence of SR-BI from the liver parenchymal cells resulted in an 87% decrease of CEt uptake (62.4 ± 1.1%

uptake of the ID/g protein x 10^{-3} in WT parenchymal cells vs. 8.0 ± 0.6% uptake of the ID/mg protein x 10^{-3} in SR-BI KO parenchymal cells). For liver endothelial cells, no significant difference was observed. In the Kupffer cell fraction from SR-BI KO mice, the uptake of CEt from HDL was 51.5% lower compared with WT mice (69.6 ± 13.9% and 143.4 ± 7.5% of the ID/mg protein x 10^{-3}, respectively). Because parenchymal liver cells are the predominant cell type in the liver (92.5%), the diminished uptake of CEt by the SR-BI KO liver is mainly the consequence of the absence of SR-BI in the parenchymal liver cells and is only to a minor extent caused by the SR-BI deficiency in the Kupffer cells.

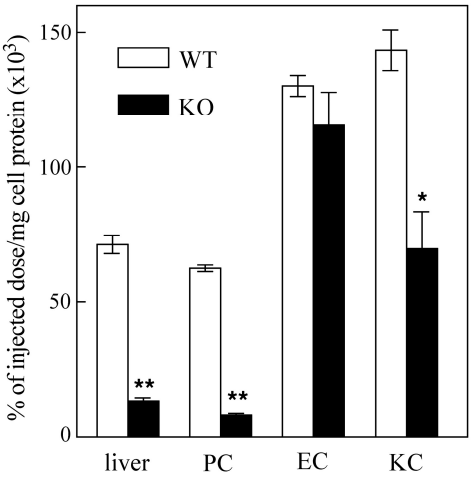

Fig.3. In vivo distribution of [3H]CEt-HDL in parenchymal, liver endothelial, and Kupffer cells in WT and SR-BI KO mice at 2 h after injection. A dose of 500 µg (apolipoprotein; 3 x 10^6 dpm) of [^{3}H] CEt-HDL was injected into the tail vein of WT (open bars) and SR-BI KO (closed bars) mice. At 2 h after injection, a liver lobule was tied off and the liver was perfused at 37°C. Parenchymal cells (PC), endothelial cells (EC), and Kupffer cells (KC) were further isolated at 4°C. The total liver sample and the cell isolations were solubilized and counted for radioactivity. Values are means±SEM of four isolations in both groups. *P<0.05 and ** P<0.005 between WT and KO mice (unpaired Student's t-test).

2.3.3 In vitro parenchymal liver cell association

Parenchymal liver cells from WT and SR-BI KO mice were isolated and incubated for 3 h at 37°C with different concentrations of HDL doubly labeled with [^{3}H]CE and [^{125}I]TC. As described by Pittman et al. [39], values for CE association are expressed in terms of apparent particle uptake (i.e., from the amount of ^{3}H-labeled tracer associated with the cells, the amount of HDL protein apparently taken up is calculated). In WT parenchymal liver cells, the CE association increased with increasing ligand concentration and exceeded the association to SR-BI KO cells by 3- to 5-fold (Fig.4). In SR-BI KO parenchymal liver cells, at every ligand concentration used, the association of HDL-derived [^{3}H]CE was similar to the [^{125}I]TC association, indicating that SR-BI KO parenchymal liver cells performed no residual selective CE uptake from HDL (Fig.4). With [^{3}H]CEt-HDL, similar data were obtained as with the [^{3}H]CE label.

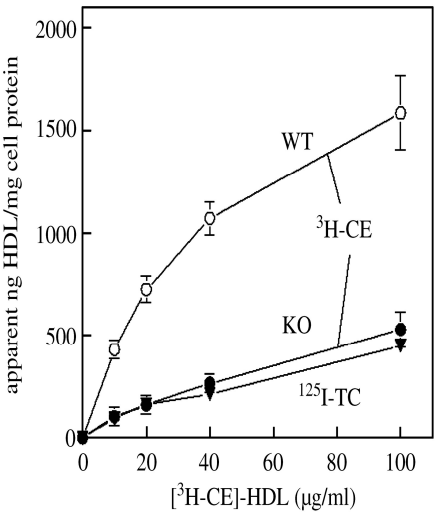

Fig.4. Concentration dependence of HDL labeled with cholesteryl ester ([^{3}H]CE-HDL) and [^{125}I]TC-HDL association to isolated parenchymal liver cells from WT and SR-BI KO mice. Parenchymal liver cells from WT (open symbols) and SR-BI KO (closed symbols) mice were incubated for 3 h at 37°C with the indicated concentrations of HDL doubly labeled with [^{3}H]CE (circles) and [^{125}I]TC (triangles) in DMEM with 2% BSA. The association of [^{3}H]CE is expressed as apparent uptake [i.e., the amount of HDL protein that would deliver the measured amounts of [^{3}H]CE (39)], whereas for [^{125}I]TC the association is expressed in ng HDL/mg cell protein. Values are means±SEM of four cell isolations from both groups. The [^{125}I]TC values for WT and SR-BI KO mice are graphically similar in the plots and do not differ significantly from the [3H]CE-HDL values in the SR-BI KO mice (Student's t-test).

2.3.4 Effect of LDL and oxidized LDL on selective uptake of [^{3}H]CE from HDL

Previously, we had shown that oxidized LDL (oxLDL) is a very potent inhibitor of HDL-CE association with rat parenchymal liver cells. Based on these inhibition characteristics, we suggested that SR-BI mediates the efficient uptake of HDL-CE by these cells [28]. In the present study, the availability of SR-BI KO mice enabled us to determine if this assumption is true. Incubation of freshly isolated WT and SR-BI KO mouse parenchymal liver cells with 10 µg/ml [^{3}H]CE-HDL for 3 h at 37°C resulted in an association of 476.3 ± 37.8 ng/mg cell protein for WT cells and a 5-fold lower association of 89.5 ± 18.9 ng/mg cell protein for SR-BI KO cells (Fig.5). The efficiency of modified LDL to compete for this [3H]CE-HDL association was examined by including the indicated amounts of unlabeled native or oxidized LDL in the incubation (Fig.5). Addition of native LDL only marginally decreased the cell association (18% in WT cells and 20% in SR-BI KO cells at the highest concentration of 100 µg/ml). Competition with oxLDL at a concentration of 100 µg/ml resulted in a 73% reduction of [^{3}H]CE-HDL cell association to WT cells. With an oxLDL concentration as low as 25 µg/ml, a 52% decrease was achieved (Fig.5). In contrast, in the SR-BI KO parenchymal liver cells, oxLDL did not efficiently decrease the association of [^{3}H]CE-HDL. This indicates that apparently oxLDL is a very efficient inhibitor of the SR-BI-mediated selective uptake of CE from HDL

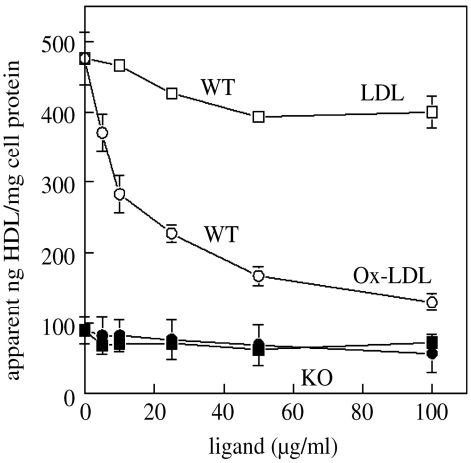

Fig.5. Effect of increasing concentrations of LDL or oxidized LDL (oxLDL) on the association of [3H]CE-HDL to WT and SR-BI KO mouse parenchymal liver cells. Parenchymal liver cells from WT (open symbols) and SR-BI KO (closed symbols) mice were incubated for 3 h at 37°C with 10 µg/ml [^{3}H]CE-HDL and the indicated concentrations of LDL (squares) or oxLDL (circles) in DMEM with 2% BSA. The association is expressed as apparent uptake [i.e., the amount of HDL protein that would deliver the measured amounts of [^{3}H]CE (39)]. Values are means ± SEM of four cell isolations. Where error bars are not visible, they fall within the symbols.

2.4 Discussion

Selective delivery of CE from HDL to the adrenals and the liver is important for the supply of substrate for steroid hormone synthesis and the maintenance of lipid homeostasis, respectively. Based on the following evidence, SR-BI is suggested to be a functional receptor for HDL: i) SR-BI is highly expressed in the steroidogenic organs and the liver in rats [17] and mice [18]; ii) SR-BI can bind HDL via interaction with its apolipoproteins [40]; and iii) SR-BI can mediate the selective uptake of HDL-CE [15]. In the present work, we used SR-BI KO mice to assess the quantitative role of SR-BI in the selective uptake of CE from HDL in vivo by comparing the uptake in these animals with that in WT mice. Our data show that, in the intact animal, the initial rate at which CE is taken up from HDL by the liver and the adrenals is greatly diminished in SR-BI KO animals compared with WT animals. Calculation of the liver FCR for WT and KO animals indicated that CE uptake from HDL by the liver occurred eight times faster in WT animals. This shows the quantitative importance of SR-BI as a rate-limiting factor in the process of selective uptake of CE from HDL by the liver. It is consistent with the finding that in SR-BI KO mice biliary cholesterol secretion is impaired [25,26] and that plasma HDL cholesterol concentrations are increased by 125% [27]. In agreement with the rate-determining role of SR-BI in the selective CE uptake from HDL, it has been reported that overexpression of SR-BI in vivo resulted in a dramatic decrease in plasma HDL cholesterol levels [20–22] and increased total and selective uptake of HDL-CE by the liver [22] compared with nontransgenic controls. Furthermore, our data are consistent with the decreased CE uptake from HDL by the liver observed in SR-BI att mice, which have an attenuated expression of the SR-BI receptor [24]. Analysis of the organ distribution of [^{3}H]CE at various times after injection indicated that in addition to the liver, only the uptake by the adrenals is impaired in the absence of SR-BI. This is in accordance with the previously reported low adrenal gland cholesterol content in homozygous SR-BI KO mice [27]. Comparison of the uptake values obtained for [3H]CE and [^{125}I]TC, a nondegradable and accumulating label located in the protein moiety of

HDL, indicates that in SR-BI KO mice the residual CE uptake can be ascribed to HDL holo-particle association and/or uptake. This implies not only that SR-BI is the determining factor for the selective uptake of CE from HDL but also that no additional routes for the selective uptake of CE from HDL are active in vivo. Our data obtained with SR-BI KO animals compared with WT mice thus form the final proof that SR-BI is solely responsible for the selective uptake of CE from HDL in the blood circulation by the liver and the adrenals.

Recently, in a preliminary report, Rinninger's group [41] came to the same conclusion by studying the kinetics of autologous HDL in SR-BI KO mice.

The liver does contain, in addition to the parenchymal cells, endothelial and Kupffer cells. We have shown previously [19] that, in rats, SR-BI protein is expressed in both the parenchymal and Kupffer cells. Treatment with estradiol or a high-cholesterol diet led to downregulation of SR-BI protein expression in the parenchymal cells and resulted in an upregulation of SR-BI protein expression in the Kupffer cells. These changes in SR-BI protein expression correlated with changes in the ability of these cells to perform selective uptake of CE from HDL [19]. In the present study, isolation of parenchymal, endothelial, and Kupffer cells at 2 h after in vivo administration of [^{3}H]CEt-HDL revealed that in the SR-BI KO mice CEt-HDL uptake is greatly impaired in parenchymal cells but in liver endothelial cells no difference in uptake of CEt from HDL was observed. Kupffer cells from SR-BI KO mice showed a 2-fold decrease in CE uptake from HDL compared with WT mice. These data obtained in mice are consistent with our earlier data for the rat. Because the parenchymal liver cells account for 92.5% of total liver protein, we conclude that the SR-BI-mediated selective uptake of CE from HDL by the liver is mainly exerted by the parenchymal cells.

In view of the major role of the parenchymal liver cells in the removal of CE from HDL, additional in vitro studies were performed with these cells from WT and SR-BI KO mice. Pioneering studies by Glass and coworkers [11,14] have shown that the uptake route of HDL-CE by liver parenchymal cells is characterized by the selective uptake of the CE without simultaneous uptake of the holo-particle. We have shown previously that the selective uptake of HDL-CE by rat parenchymal liver cells can be blocked efficiently by oxLDL [29] and also that changes in SR-BI protein expression, induced by a high-cholesterol diet or estradiol treatment, correlated with changes in the selective uptake of HDL-CE in vitro [19]. Our present data with isolated parenchymal liver cells from SR-BI KO mice indicate that the uptake of HDL-CE is greatly diminished compared with WT parenchymal liver cells and also that inhibition by oxLDL of the selective uptake process no longer occurs. This indicates that, similar to the in vivo observations, SR-BI expression is essential for the efficient uptake of CE from HDL. In addition, the present data support our earlier suggestion [29] that the inhibition of CE uptake from HDL by oxLDL indicates that SR-BI is mainly responsible for the selective uptake process. By simultaneous measurement of [^{3}H]CE uptake and [^{125}I]TC uptake (reflecting holo-particle uptake) from HDL in parenchymal cells from SR-BI KO mice, we conclude that these cells, in contrast to WT parenchymal liver cells, no longer exert selective CE uptake from HDL. These data are consistent with the in vivo

uptake values and indicate that for this major liver cell type SR-BI is essential for the selective uptake of CE from HDL. Furthermore, the data indicate that the parenchymal liver cells do not contain additional receptor systems that can facilitate this unique uptake route.
In conclusion, both our in vivo and in vitro data prove that SR-BI is mainly responsible for the efficient and selective uptake of HDL-CE by the liver and the adrenals, making it an important target for pharmaceutical interference with the RCT process.

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3

Adenovirus-mediated hepatic overexpression of scavenger receptor class B type I accelerates chylomicron metabolism in C57BL/6J mice

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The function of scavenger receptor class B type I (SR-BI) in mediating the selective uptake of HDL cholesteryl esters is well established. In SR-BI-deficient mice, we recently observed a delayed postprandial triglyceride (TG) response, suggesting an additional role for SR-BI in facilitating chylomicron (CM) metabolism. Here, we assessed the effect of adenovirus-mediated hepatic overexpression of SR-BI (Ad.SR-BI) in C57BL/6J mice on serum lipids and CM metabolism. Infection of 5×10^8 plaque-forming units per mouse of Ad.SR-BI significantly decreases serum cholesterol (>90%), phospholipids (>90%), and TG levels (50%), accompanied by a 41.4% reduction ($P<0.01$) in apolipoprotein B-100 levels. The postprandial TG response is 2-fold lower in mice treated with Ad.SR-BI compared with control mice (area under the curve = 31.4 ± 2.4 versus 17.7 ± 3.2; $P<0.05$). Hepatic mRNA expression levels of genes known to be involved in serum cholesterol and TG clearance are unchanged and thus could not account for the decreased plasma TG levels and the change in postprandial response.
We conclude that overexpression of SR-BI accelerates CM metabolism, possibly by mediating the initial capture of CM remnants by the liver, whereby the subsequent internalization can be exerted by additional receptor systems such as the LDL receptor and LDL receptor-related protein 1 (LRP1).

3.1 Introduction

Scavenger receptor class B type I (SR-BI) binds HDLs and mediates the selective uptake of cholesteryl esters (CEs) from HDL without concomitant uptake of HDL protein [1]. The major apolipoproteins from HDL (apolipoprotein A-I (ApoA-I), ApoA-II, and ApoC-III) mediate the binding of HDL to SR-BI [2]. Recently, it was shown that lipid-free ApoE also binds to SR-BI and enhances CE uptake from lipoproteins [3]. In addition to HDL, SR-BI was found to bind a broad spectrum of ligands, including maleylated BSA, anionic phospholipids (PLs), modified lipoproteins (acetylated LDL, oxidized LDL, and hypochlorite-modified LDL), and native lipoproteins (HDL, LDL, and VLDL) [4–7]. In contrast, SR-BI does not bind polyanions (e.g., fucoidin and polyinosinic acid), which are well-known ligands for scavenger class A receptors. In addition to CE, SR-BI selectively takes up a variety of other molecules, such as lipoprotein-associated PL [8,9], HDL-associated CE hydroxides [10], and triglycerides (TGs) [9,11].

The importance of SR-BI in HDL cholesterol metabolism is readily observed in genetically altered mice. SR-BI-deficient mice are characterized by an increase in serum cholesterol levels, reflected in enlarged, cholesterol-rich HDL particles and impaired HDL cholesterol clearance [12]. Conversely, adenoviral hepatic SR-BI overexpression results in decreased serum HDL cholesterol content as well as increased liver uptake and subsequent delivery of HDL cholesterol to the bile [13]. In contrast with HDL cholesterol metabolism and despite several studies in both SR-BI transgenic mice [14-17] and in mice with adenovirus-mediated overexpression of SR-BI [18–20], the role of SR-BI in the metabolism of apoB-containing lipoproteins is still under discussion.

Chylomicrons (CMs) are TG-rich lipoproteins that transport dietary lipids from the intestine to the liver [21]. In the intestines, CMs are formed by the addition of lipids to ApoB-48, the structural protein of CM, which is mediated by microsomal triglyceride transfer protein (MTP). Upon entering the circulation, CMs are converted to remnants by the TG-hydrolyzing action of LPL and the acquisition of apolipoproteins such as apoE. CM remnants are subsequently taken up by the liver by an ApoE-mediated process (reviewed in Refs. 22–24). The essential role of ApoE in remnant clearance was indicated by the accumulation of remnants in ApoE-deficient mice [25]. Several ApoE-dependent recognition sites have been suggested to contribute to the removal of remnants, including the (ApoB and ApoE) LDL receptor receptor [25–30] and the LDL receptor-related protein/2-macroglobulin receptor 1 (LRP1) [29,31,32]. However, it is generally accepted that for the initial liver recognition of remnants, the so-called "capture step," additional systems are needed. The initial sequestration step was suggested to involve heparan sulfate proteoglycans [26,33], the lipolysis-stimulated receptor [34–36], a TG-rich lipoprotein receptor [37,38], the asialoglycoprotein receptor [39], LPL [40] and/or HL [41], and a specific remnant receptor [42–44]. We recently observed a reduced recognition of 160 nm TG-rich CM-like emulsion particles to freshly isolated hepatocytes from SR-BI deficient mice [45]. Furthermore, the postprandial TG response to an intragastric fat load is 2-fold higher in SR-BI deficient mice compared with wild-type littermates. These data suggest that SR-BI facilitates CM-

remnant metabolism possibly by mediating the initial binding/capture of remnants by the liver [45]. However, it remains to be established to what extent this facilitating role is critically dependent on SR-BI protein levels.
The aim of the present study was to further substantiate the role of SR-BI in CM metabolism by assessing the effect of adenovirus-mediated hepatic overexpression of SR-BI. It appears that adenovirus-mediated hepatic overexpression of SR-BI in C57BL/6J mice results in a decrease in plasma TG, a decrease in VLDL/CM-associated TG, and a modified postprandial TG response. In addition, a tendency to an increase in MTP expression was observed, suggesting increased VLDL production. These data support our earlier suggestion and indicate that besides its role in HDL metabolism, SR-BI levels modulate the kinetics of CM (remnant) metabolism.

3.2 Experimental Procedures

3.2.1 Animals

In all experiments, 10–12 week old male C57BL/6J mice (Broekman Institute BV, Someren, The Netherlands), weighing ~25 g were used. Mice were fed a regular chow diet containing 4.7% fat and no cholesterol (SDS, Whitham, UK). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

3.2.2 Treatment with recombinant adenovirus

Construction of a recombinant replication-deficient adenoviral vector expressing mouse SR-BI (Ad.SR-BI) has been described previously [46]. A total of 5 x 10^8 plaque-forming units (pfu) of Ad.SR-BI or Ad.LacZ (control) was injected into the tail vein of the mice (n=4–5 per group) at 3 h after injection of Ad.LacZ (5 x 10^8 pfu) to saturate the uptake of viral particles by Kupffer cells [47]. Before injection and 5 days after injection, mice were fasted overnight and a blood sample for lipid determination was collected by tail bleeding. Subsequently, mice were anesthetized [subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health), fentanyl citrate, and fluanisone (1.26 and 2 mg/kg, respectively; Janssen Animal Health)] and exsanguinated by eye bleeding. A whole body perfusion was performed using phosphate-buffered saline containing 1 mM EDTA (4°C, 100 mm Hg) for 15 min. After perfusion, liver lobules were excised and either kept in 3.7% formalin overnight, embedded in OCT compound (Tissue-Tec), and frozen in liquid nitrogen for histological analysis or snap frozen in liquid nitrogen and stored at –80°C until RNA isolation, Western blotting, or hepatic lipid composition analysis.

3.2.3 Analysis of gene expression by real-time quantitative PCR

mRNA analysis was performed as described previously [48,49]. Total RNA was extracted from the liver by the acid guanidinium thiocyanate-phenol chloroform extraction method according to Chomczynski and Sacchi [50]. cDNA was synthesized from 2 µg of total RNA using RevertAid M-MuLV reverse transcriptase according to the protocols supplied by the manufacturer. Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR-green technology (Eurogentec) with the primers listed in Table 1. Hypoxanthine guanine phosphoribosyl transferase (HPRT), β-actin, and acidic ribosomal phosphoprotein PO (36B4) were used as the standard housekeeping genes. Relative gene expression was calculated by subtracting the threshold cycle number of the target gene from the average threshold cycle number of HPRT, β-actin, and 36B4 and raising 2 to the power of this difference. The average threshold cycle number of three housekeeping genes was used to exclude the possibility that changes in the relative expression were caused by variations in the separate housekeeping gene expressions.

Table 1
Primers for quantitative real-time PCR analysis

Gene	GenBank Accesion	Forward primer	Reverse primer	Amlicon size
SR-BI	U76205	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA	65
LDLR	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC	68
LRP1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA	95
MTP	L47970	AGCTTTGTCACCGCTGTGC	TCCTGCTATGGTTTGTTGGAAGT	50
ABCA1	NM013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC	96
HMGCR	M62766	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT	69
ABCG1	NM053502	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG	85
ABCG5	NM053754	CGCAGGAACCGCATTGTAA	TGTCGAAGTGGTGGAAGAGCT	67
ABCG8	NM130414	GATGCTGGCTATCATAGGGAGC	TCTCTGCCTGTGATAACGTCGA	69
CYP7A1	NM012942	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	75
CYP27	M38566	GTGTCCCGGGATCCCAGTGT	CTTCCTCAGCCATCGGTGA	66
BSEP	NM021022	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA	76
HL	NM008280	CAGCCTGGGAGCGCAC	CAATCTTGTTCTTCCCGTCCA	62
LPL	NM008509	CCAGCAACATTATCCAGTGCTAG	CAGTTGATGAATCTGGCCACA	72
HPRT	X62085	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
36B4	X1526775	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
β-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA	75

3.2.4 Western blotting

Immunoblotting on protein from total liver was performed as described previously [49].In short, after running equal amounts of total liver protein (25 µg) on a 7.5% SDS-PAGE gel, SR-BI was detected using rabbit polyclonal anti-SR-BI peptide (496–509) IgG (Abcam, Cambridge, UK) as a primary antibody and goat-anti-rabbit IgG (Jackson ImmunoResearch) as a secondary antibody. LDL receptor and LRP1 were detected using goat anti-LDL receptor (C-20) IgG and goat anti-LRP (N-20) IgG (Santa Cruz Biotechnology, Inc.) as primary antibodies, respectively. As a secondary antibody, mouse anti-goat IgG was used (Jackson ImmunoResearch). Finally, immunolabeling was detected by enhanced chemiluminescence (Biosciences). For quantitation, ImageQuant 5.2 software was used.

3.2.5 Lipid analysis

Serum concentrations of total cholesterol, free cholesterol, PL, and TG were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). Precipath L was used as an internal standard. The distribution of total cholesterol, PL, and TG over the different lipoproteins in serum was analyzed by fractionation of 30 µl of pooled serum using a Superose 6 column (3.2 x 30 mm, Smart-system; Pharmacia, Uppsula, Sweden) and determination of total cholesterol, PL, and TG as described above.

3.2.6 ApoB-100 ELISA

Determination of plasma ApoB-100 levels was carried out using an enzyme-linked immunosorbent assay with a monoclonal antibody against murine apoB-100 (LF3) essentially as described by Zlot et al. [51], who kindly provided LF3 and rabbit antisera against mouse ApoB (rabbit865).

3.2.7 Hepatic lipid composition/liver histology

Hepatic lipids were extracted according to Bligh and Dyer [52]. After dissolving the lipids in 2% Triton X-100, contents of cholesterol, CE, PL, and TG in liver tissue were determined as described above and expressed as micrograms per milligram of protein. Five micrometer cryosections were prepared on a Leica CM3050-S cryostat. Cryostat sections were routinely stained with hematoxylin (Sigma Diagnostics, St. Louis, MO) and Oil Red O (Sigma Diagnostics) for lipid visualization.

3.2.8 Intragastric fat load-induced postprandial TG response

Groups of five mice were fasted overnight. For basal TG and cholesterol levels, 50 µl blood samples were drawn just before 9:00 AM by tail bleeding into heparinized capillary tubes (time 0). At 9:00 AM, animals received an intragastric load of 400 µl of olive oil. After gavage, blood collection was performed every hour for 4 h. Plasma TG levels were measured at the various time points using enzymatic kits as described above. The distribution of TG over the different lipoproteins in plasma was analyzed by fractionation of 30 µl of pooled plasma using a Superose 6 column (3.2 x 30 mm, Smart-system; Amersham Biosciences) and determination of the TG content of the eluted fractions as described above.

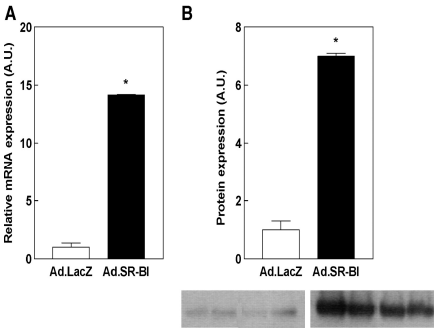

Fig.1. Hepatic SR-BI expression in Ad.SR-BI-treated mice and control mice. *A*: Analysis of SR-BI expression at 5 days after adenoviral administration by real-time quantitative PCR in C57BL/6J mice treated with Ad.LacZ [5 x 10^8 plaque-forming units (pfu)] or Ad.SR-BI (5 x 10^8 pfu) (n=5 per group). *B*: Quantitation of Western blot analysis of SR-BI expression in C57BL/6J mice treated with Ad.LacZ (5 x 108 pfu) or Ad.SR-BI (5 x 10^8 pfu). Below the quantitation histogram, a representative immunoblot of four samples per group is shown. A.U., arbitrary units. Values shown are means±SEM. *P<0.001

Table 2
Effect of SR-BI overexpression on plasma lipid levels

Day	Treatment	n	Total cholesterol	Free cholesterol	Phospholipid	Triglyceride
			mg/dl			
-3	Ad.LacZ	5	62 ± 3.7	14 ± 0.6	27 ± 0.8	113 ± 9
5			68 ± 6.0	11 ± 1.4	24 ± 1.2	95 ± 6
-3	Ad.SR-BI	5	62 ± 2.3	14 ± 1.1	27 ± 1.0	123 ± 14
5			<5.0[a]	<1.0[a]	1.8 ± 0.3[a]	63 ± 12[b]

PL, phospholipid; TG, triglyceride. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 plaque-forming units) or Ad.SR-BI (5 x 10^8 plaque-forming units). Three days before injection and at 5 days after injection, overnight-fasted plasma was collected from individual mice and assayed for total cholesterol, free cholesterol, PL, and TG. Values are means ± SEM. [a]P<0.001, [b]P<0.01 compared to Ad.LacZ.

3.3 Results

3.3.1 Adenovirus-mediated SR-BI overexpression in C57BL/6J mice results in decreased plasma VLDL/CM-associated TG levels and plasma ApoB-100 levels

SR-BI is a class B scavenger receptor that binds a broad variety of lipoprotein ligands. Recently, we showed that SR-BI is able to facilitate CM metabolism [45]. To further assess the role of SR-BI in CM metabolism, the receptor was overexpressed in livers of C57BL/6J mice by infusion with a dose of Ad.SR-BI (5 x 10^8 pfu), which resulted in 14-fold and 7-fold increases in hepatic SR-BI mRNA (Fig.1A) and protein expression (Fig.1B), respectively, at 5 days after infusion. Five days after Ad.SR-BI infusion, mice showed highly significant decreases in plasma total cholesterol and free cholesterol (>90%) and plasma PL (>90%) compared with mice treated with control adenovirus (Table 2). In addition, plasma TG levels were decreased significantly (2-fold) in Ad.SR-BI-treated mice compared with mice treated with control adenovirus (Table 2). Analysis of lipoprotein profiles revealed a depletion of both HDL and LDL-cholesterol (Fig.2A) and HDL and LDL-PL (Fig.2B) in Ad.SR-BI-treated mice compared with control virus-treated mice. Interestingly, Ad.SR-BI-treated mice also showed a significant decrease in VLDL/CM-associated TG compared with control virus-treated mice (Fig.2C).

Subsequently, ApoB-100 levels in serum of Ad.SR-BI- and Ad.LacZ-treated mice were determined using an enzyme-linked immunosorbent assay with a monoclonal antibody against murine ApoB-100 (LF3). ApoB-100 levels in serum of Ad.SR-BI-treated animals were significantly reduced compared with Ad.LacZ-treated mice (41.4% reduction; P<0.001) (Fig.3).

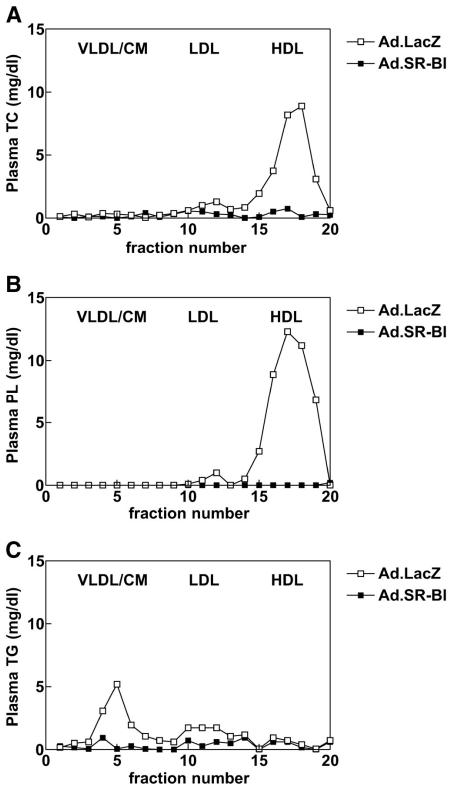

Fig.2. Lipoprotein profiles in Ad.SR-BI-treated mice and control mice. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu). After 5 days, overnight-fasted plasma was collected and total cholesterol (*A*), phospholipid (*B*), and triglyceride (*C*) levels in the lipoprotein profiles of pooled plasma of Ad.SR-BI mice (closed squares) and control mice (open squares) were determined. CM, chylomicron

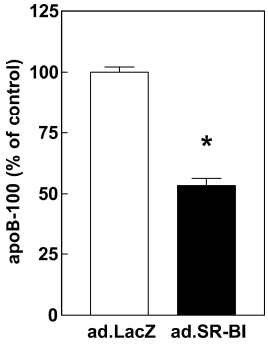

Fig.3. Plasma apolipoprotein B-100 (ApoB-100) levels. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu). After 5 days, ApoB-100 levels in serum of Ad.SR-BI- and Ad.LacZ-treated mice were determined using an enzyme-linked immunosorbent assay with a monoclonal antibody against murine ApoB-100 (LF3). Values shown are means±SEM for five mice per group. *P<0.001

3.3.2 Influence of Ad.SR-BI on hepatic lipid metabolism, hepatic lipid composition, and liver morphology

Because adenovirus-mediated hepatic overexpression of SR-BI results in a substantial decrease of plasma cholesterol, PL, and TG, we next analyzed the effect of SR-BI overexpression at 5 days after infusion on hepatic genes involved in the uptake, metabolism, and efflux of cholesterol and TG. In addition to SR-BI, the uptake of cholesterol in the liver can be mediated by receptors such as the LDL receptor and LRP1. The decrease in LDL-cholesterol and VLDL/CM-TG observed in Ad.SR-BI-treated mice was not attributable to an increase in expression of the LDL receptor or LRP1. Compared with control mice, the mRNA level of the LDL receptor was similar and that of LRP1 was decreased significantly (Fig.4A/B). In agreement with the mRNA levels, LDL receptor and LRP1 protein levels were similar and significantly decreased, respectively, in Ad.SR-BI-treated mice compared with control mice (Fig.4C/D).

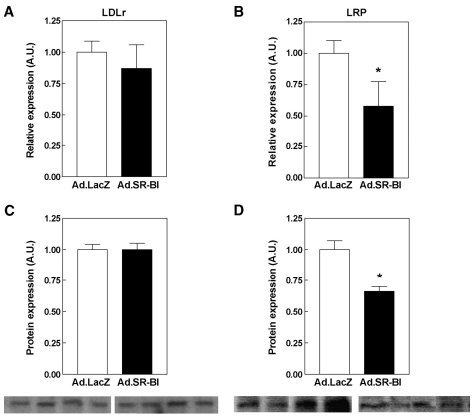

Fig.4. Effect of SR-BI overexpression on hepatic LDL receptor (LDLr) and LDL receptor-related protein 1 (LRP1) expression. At 5 days after adenoviral administration, LDL receptor (*A*) and LRP1 (*B*) expression was analyzed by real-time quantitative PCR in C57BL/6J mice treated with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu) (n=5 per group). Also shown is the quantitation of Western blot analysis of LDL receptor (*C*) and LRP1 (*D*) expression in C57BL/6J mice treated with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu). Values shown are means±SEM. *P<0.05 compared to Ad.LacZ.

The liver has three well-known routes of cholesterol disposal. Cholesterol can be used for the synthesis of VLDL and HDL. The key process in VLDL synthesis is the intracellular association of ApoB-48/ApoB-100 with lipids in which the MTP is crucially involved. MTP mRNA levels have the tendency to be increased in Ad.SR-BI-treated mice (P=0.07) (Fig.5). Recently, it was shown that ABCA1 plays an essential role in the formation of HDL [53]. ABCA1 mRNA levels are not affected by hepatic overexpression of SR-BI (Fig.5). Hepatic cholesterol levels are the consequence of lipoprotein uptake and de novo synthesis of cholesterol by the enzyme HMG-CoA reductase. HMG-CoA reductase mRNA expression was not changed (Fig.5). As a heterodimer, ABCG5 and ABCG8 mediate biliary cholesterol efflux from the liver to the bile duct [54]. Recently, ABCG1 also has been proposed to have a role in the intracellular trafficking and efflux of cholesterol in the liver [48]. Neither ABCG5 and ABCG8 nor ABCG1 mRNA expression was changed in Ad.SR-BI-treated animals (Fig.5). Finally, CYP7A1 and CYP27 are responsible for the conversion of cholesterol into

bile acids, which can be secreted from the liver into the bile via the bile salt export pump (BSEP). Whereas the level of CYP27 mRNA expression was significantly lower in mice overexpressing SR-BI, CYP7A1 and BSEP expression was not different (Fig.5). LPL and HL regulate plasma TG levels by their TG-hydrolyzing action. The decrease in VLDL/CM-TG observed in Ad.SR-BI-treated mice was not attributable to an increase in the expression of these two enzymes, because HL was significantly lower in Ad.SR-BI-treated mice and LPL was unchanged (Fig.5).

Hepatic lipid content in Ad.SR-BI-treated mice was not changed, as the hepatic levels of PL, TG (Fig.6A), free cholesterol, or CE (Fig.6B) are all similar. In accordance with the above hepatic lipid composition data, Oil Red O staining revealed no differences in lipid depots in Ad.SR-BI-treated and control mice (Fig.6C).

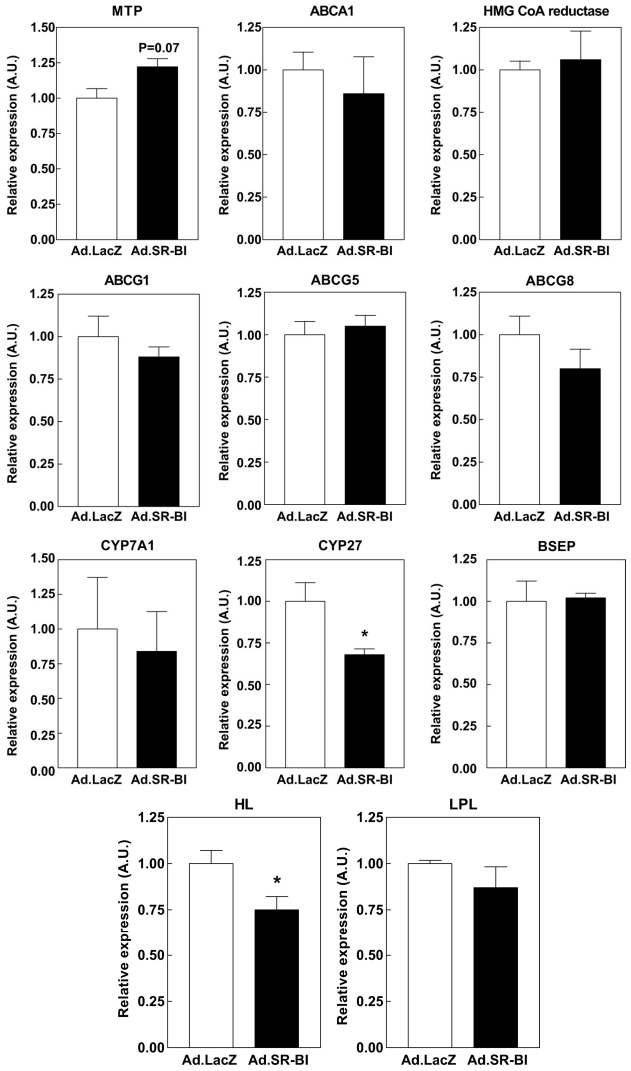

Fig.5. Effect of SR-BI overexpression on genes involved in hepatic cholesterol metabolism. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu). After 5 days, hepatic mRNA levels of the indicated genes were determined by real-time quantitative PCR. Values shown are means±SEM. *P<0.05 compared to Ad.LacZ.

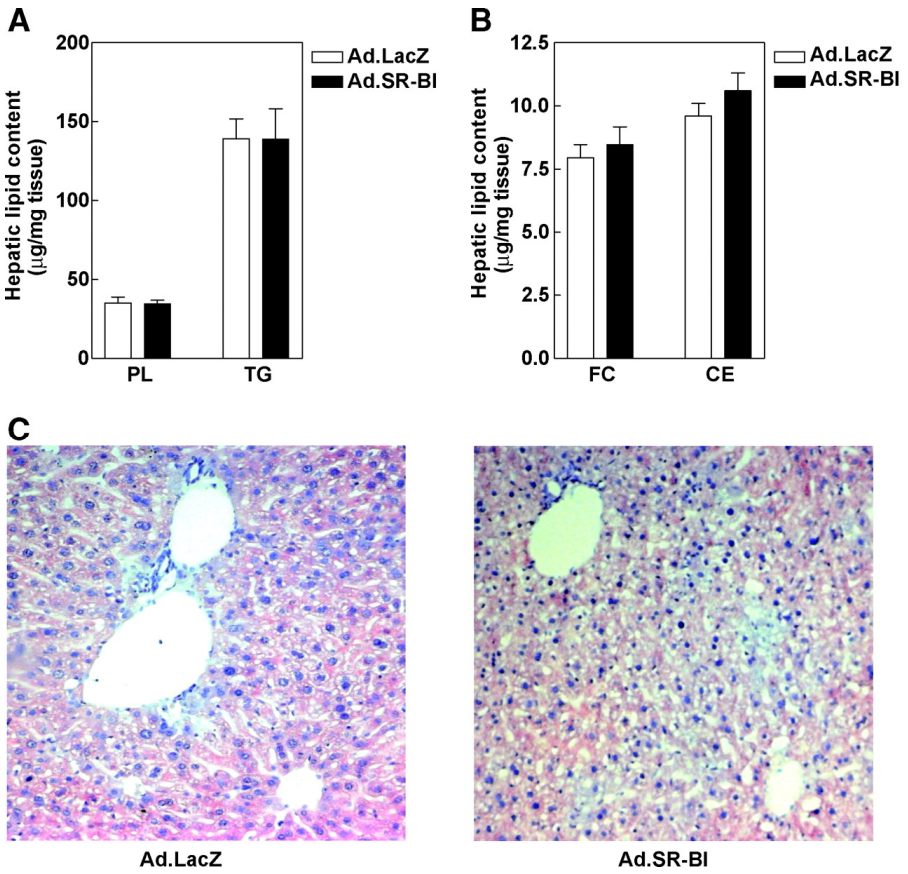

Fig.6. Hepatic lipid content in Ad.SR-BI-treated mice and control mice. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 pfu) or Ad.SR-BI (5 x 10^8 pfu). After 5 days, hepatic PL and TG (*A*), and free cholesterol (FC) and cholesteryl ester (CE; *B*) levels were analyzed. Five micrometer cryosections of livers of Ad.LacZ- and Ad.SR-BI-treated mice were stained with Oil Red O for lipid visualization and counterstained with hematoxylin. Values shown are means±SEM.

3.3.3 Effect of SR-BI overexpression on CM metabolism in vivo

We next investigated the effect of SR-BI overexpression on the postprandial TG response upon an intragastric fat load, which is an established procedure to study the kinetics of CM metabolism. After an intragastric load of olive oil, plasma TG levels were determined over a period of 4 h in Ad.SR-BI-treated and control virus-treated mice. Before gavage, Ad.SR-BI-treated mice had significantly lower basal levels of plasma TG (Table 2). At 2 h after olive oil administration, control virus-treated animals showed a postprandial increase in plasma TG (3.1-fold) (Fig.7A), which decreased again at 4 h after administration. In contrast, Ad.SR-BI-treated mice showed a 2-fold decreased TG response compared with control virus-treated mice

(area under the curve = 31.4 ± 2.4 vs. 17.7 ± 3.2; P<0.05). Specifically, the increase in plasma TG was significantly lower at 1, 2, and 3 h after gavage (Fig.7A). Analysis of lipoprotein profiles at 3 h after gavage (Fig.7B) showed that plasma TG in the VLDL/CM fraction was lower in Ad.SR-BI-treated mice compared with control virus-treated mice, and the reduction was mainly attributable to a decrease in CM-associated TG.

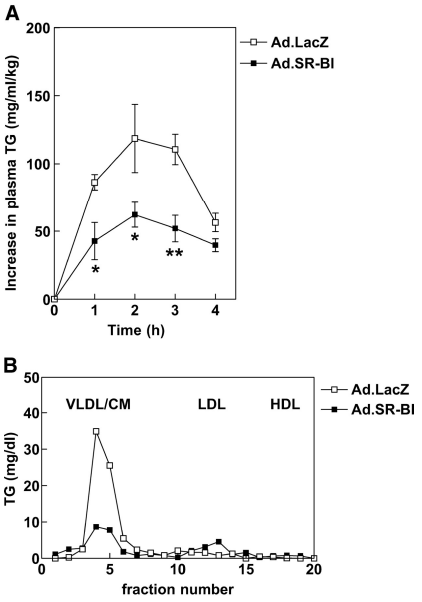

Fig.7. Effect of SR-BI overexpression on the postprandial TG response upon an intragastric fat load. C57BL/6J mice (n=5 per group) were injected with Ad.LacZ (5 x 10^8 pfu; open squares) or Ad.SR-BI (5 x 10^8 pfu; closed squares). After 5 days, overnight-fasted mice received an intragastric load of 400 µl of olive oil at time 0. *A*: Subsequently, plasma TG levels were determined at the indicated times, and the data are expressed as increases in TG levels relative to time 0. *B*: At 3 h after olive oil administration, TG levels in the lipoprotein profiles of pooled plasma were determined. Values shown are means±SEM. *P<0.05 and **P< 0.01 compared to Ad.LacZ.

3.4 Discussion

SR-BI is a multiligand cell surface receptor capable of binding HDL, LDL, VLDL, modified LDL and BSA, and liposomes containing anionic PL [4–7]. Although the function of SR-BI in the selective uptake of CE from HDL is undisputable [12], conflicting information on a potential role of SR-BI in the metabolism of ApoB-containing lipoproteins exists [14–20].

Using SR-BI-deficient mice, we recently showed that SR-BI can facilitate CM (remnant) metabolism [45]. In the present study, we investigated to what extent the role of SR-BI in VLDL/CM (remnant) metabolism is critically dependent on SR-BI protein levels by assessing the effect of adenovirus-mediated hepatic overexpression of SR-BI in C57BL/6J mice. Adenovirus-mediated overexpression of SR-BI led to a significant decrease in HDL-cholesterol and was accompanied by a decrease in the main apolipoprotein constituent of HDL (ApoA-I) (data not shown), as also observed in other studies [13,15,16,46], and a substantial increase in biliary cholesterol [13]. Strikingly, plasma TG levels, VLDL/CM-associated TG, and plasma ApoB-100 levels were all significantly reduced: findings that correlate with a potential role for SR-BI in VLDL/CM metabolism. In the present work, we analyzed the effect of SR-BI protein level on endogenous CM metabolism by giving an intragastric fat load to Ad.SR-BI-treated mice and control mice. After administration of olive oil, the maximum level of TG reached in the blood circulation was 2-fold lower, corresponding with a significant

decrease in the area under the curve (31.4 ± 2.4 vs. 17.7 ± 3.2; $P<0.05$) in the Ad.SR-BI-treated mice compared with control mice. Both the decreased plasma TG levels and the decreased postprandial response could have been attributed to other processes, such as decreased VLDL production, and/or indirect effects of overexpression of SR-BI on the expression of other hepatic genes involved in cholesterol and/or CM metabolism. For this reason, we assessed both the mRNA and protein levels of the LDL receptor and LRP1, which are believed to be responsible for the internalization of VLDL and CM remnants by the liver. The expression of the LDL receptor appeared unchanged, whereas the LRP level was actually lower by Ad.SR-BI administration. Thus, the decreased plasma TG levels and the change in postprandial response cannot be caused by an increased expression of these two receptors. Hepatic expression of proteins involved in cholesterol transport and/or metabolism, such as ABCA1, ABCG1, ABCG8, CYP7A1, CYP27, and BSEP, were not increased by Ad.SR-BI administration, indicating that the observed change in serum lipoproteins is not related to these proteins. Actually, only MTP had the tendency to be increased ($P=0.07$), which suggests a compensatory mechanism to resecrete the SR-BI-mediated hepatic uptake of TG in the form of VLDL. LPL and HL regulate plasma TG levels by their TG-hydrolyzing action. The decrease in VLDL/CM-TG observed in Ad.SR-BI-treated mice was not attributable to an increase in the expression of these two enzymes, because HL was significantly lower in Ad.SR-BI-treated mice and LPL was unchanged.

Combined with our earlier data in SR-BI-deficient mice, the present experiments using transient adenovirus-mediated overexpression of SR-BI in C57BL/6J indicate that SR-BI levels are important for the kinetics of postprandial lipemia. Previous studies have suggested that postprandial remnant particles may predict the onset of atherosclerosis. Consistent with our present findings, it was suggested by Arai et al. [55] that in heterozygous LDL receptor deficient mice, the transgene expression of SR-BI leads to decreased atherosclerosis, which correlated with decreased VLDL- and LDL-cholesterol levels [55]. Also, Wang et al. [15] and Ueda et al. [16,17] observed in SR-BI transgenic mice decreased levels of VLDL-ApoB and LDL-ApoB. Kozarsky et al. [56] have shown that adenovirus-mediated hepatic overexpression of SR-BI in fat-fed LDL receptor deficient mice leads to a marked decrease in HDL cholesterol and a modest decrease in intermediate density lipoprotein/LDL cholesterol. The modest reduction in non-HDL-cholesterol in these studies can be explained by the absent activity of the LDL receptor needed for the internalization of the remnants [30,57]. Furthermore, Fu, Kozarsky, and Borensztajn [20] recently observed that fibrate-induced hypercholesterolemia in ApoE deficient mice can be normalized by the overexpression of SR-BI. It was suggested that SR-BI can function as a remnant receptor responsible for the clearance of remnant particles from the circulation of ApoE-deficient mice. Together with our recent observation in SR-BI-deficient mice, our present experiments suggest that SR-BI can indeed function as an initial recognition site for VLDL/CM not only in ApoE-deficient mice but also under normal metabolic conditions.

The mechanism responsible for the initial liver capture of CM remnants has been a point of continuous dispute [22–24]. In mice without ApoE-

recognizing internalizing receptor (LRP1/LDL receptor double deficient mice), the initial association of lipoprotein remnants [30] and large emulsion particles (P.C.N. Rensen, J.K. Kruijt, and T.J.C. Van Berkel, unpublished results) with the liver is not affected, indicating that another molecular structure is responsible for the initial liver recognition. SR-BI fulfills the requirements as an initial recognition site in that it is a multi-ligand cell surface receptor with a limited substrate specificity, which includes not only apolipoproteins but also lipids such as phosphatidylserine, a remnant surface component. The present experiments are consistent with SR-BI serving as an initial remnant recognition site. The locally available ApoE [58] may subsequently be acquired and trigger internalization. Although ApoB-100 levels were decreased by Ad.SR-BI, our data do not necessarily implicate SR-BI as an internalizing receptor, because studies with LRP1/LDL receptor double deficient mice have clearly shown the decisive role of this combined system for the internalization and further catabolism of remnants [30,57] and large emulsion particles (P.C.N. Rensen, J.K. Kruijt, and T.J.C. Van Berkel, unpublished results) by the liver.

Very interesting and consistent with our data, Pérez-Martinez et al. [59] recently suggested a role for SR-BI in postprandial CM metabolism in humans. They observed that a polymorphism in the exon 1 variant at the SR-BI gene locus called genotype 1/2 was associated with a lower postprandial response compared with that in individuals with a 1/1 genotype [59]. The expression levels of hepatic SR-BI in both genotypes were not analyzed, but this knowledge could shed additional light on the role of SR-BI as a remnant receptor in humans.

In summary, we have further substantiated the proposed role of SR-BI in CM metabolism by assessing the effect of adenovirus-mediated hepatic overexpression of SR-BI. Adenovirus-mediated hepatic overexpression of SR-BI in C57BL/6J mice resulted in a decrease in plasma TG, a decrease in VLDL/CM-associated TG, and a changed postprandial TG response. These data support our earlier suggestion that SR-BI is involved in facilitating CM remnant metabolism, and the present study strengthens the notion that besides its role in HDL metabolism, SR-BI is crucially involved in facilitating CM (remnant) metabolism. We conclude that overexpression of SR-BI accelerates CM metabolism possibly by mediating the initial capture of CM remnants by the liver, leading to subsequent internalization by receptor systems such as the LDL receptor and LRP1.

3.5 Acknowledgements

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3.6 References

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4

Hepatic scavenger receptor class B type I is an important factor in the regulation of fasting serum glucose levels

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Type II diabetes is a disease with a heterogeneous phenotype, which is primarily characterized by an increase in serum glucose levels. However, patients suffering from diabetes usually also have lipid abnormalities, such as hypertriglyceridemia. Recent data have indicated that there appears to be a significant interaction between variation at the SR-BI locus and type II diabetes in determining the lipoprotein profile. Since SR-BI might thus play a role in the pathology of diabetes, in the current study the role of SR-BI in glucose metabolism was evaluated. SR-BI deficiency in mice resulted in a 42% decrease in fasting serum glucose levels, which could not be attributed to a change in the expression of genes involved in hepatic glucose synthesis (PEPCK, PGC-1) and metabolism (L-PK). However, the hepatic expression of the rate-limiting enzyme in ketogenesis, HMG-CoA synthase, was decreased by 54% due to a hampered hepatic uptake of lipoprotein-associated fatty acids via SR-BI. The subsequently lower production of ketone bodies leads to a reduction in fasting serum glucose levels due to a higher peripheral glucose uptake.
In conclusion, in addition to its role in lipid metabolism, our data indicate that functional SR-BI is important for the maintenance of adequate fasting serum glucose levels.

4.1 Introduction

Cardiovascular diseases are the most common cause of death in the Western world. Multiple risk factors for the development of cardiovascular disease have been identified, which include elevated serum lipid levels, hypertension, obesity, smoking, and type II diabetes [1]. Type II diabetes is a disease with a heterogenous phenotype, which is characterized by an increase in serum glucose levels combined with dyslipidemia i.e. elevated triglyceride and reduced high-density lipoprotein (HDL) cholesterol levels and postprandial lipemia (reviewed by Verges [2]). Several studies have indicated that the liver plays an important role in the development of the type II diabetic phenotype, contributing to dyslipidemia and the increase in glucose levels [3,4]. Interestingly, recent data by Osgood et al. have indicated that variation in the scavenger receptor class B type I (SR-BI) gene in humans is associated with both lipid concentrations and lipoprotein particle size. Moreover, there appears to be a significant interaction between variation at the SR-BI locus and type II diabetes in determining the lipoprotein profile [5]. In the liver, SR-BI is a key gene in reverse cholesterol transport since it is able to selectively remove cholesterol esters from HDL without the concomitant uptake of the HDL protein [6]. The importance of SR-BI in lipid metabolism was provided through studies in SR-BI deficient mice, which showed that absence of functional SR-BI resulted in 2-fold increased serum total cholesterol levels due to the accumulation of abnormally large (defective) HDL particles [7,8], leading to an increased susceptibility to atherosclerosis [9]. In addition to HDL, SR-BI is able to bind a wide variety of other ligands, including anionic phospholipids [10], apoptotic cells [11], and native and modified lipoproteins [6,12-14]. Moreover, SR-BI is also able to bind advanced glycation end products (AGE) [15], which are stable covalent adducts of glucose and proteins/nucleic acids that are formed without the aid of enzymes [16]. Importantly, under diabetic conditions prolonged exposure of proteins to high serum glucose levels leads to an excessive formation of AGE, which can covalently trap extravasated serum proteins to the extravascular matrix, and thus may contribute to capillary closure in the retina and glomerulus, and to arterial narrowing in the coronary, cerebral, and peripheral circulation [17]. Since hepatic SR-BI is able to bind and remove AGE from the circulation, SR-BI might be considered beneficial in the treatment of diabetes. To further investigate this potential beneficial role of SR-BI in diabetes, in the current study the role of SR-BI in the hepatic regulation of fasting serum glucose levels was studied in normal mice by determining the effect of absence of functional SR-BI on the expression of hepatic genes involved in the regulation of fasting serum glucose levels.
Here we show that, under fasting conditions, SR-BI deficiency due to a hampered hepatic uptake of lipoprotein-associated fatty acids results in a decreased hepatic expression of the rate-limiting ketogenesis enzyme HMG-CoA synthase and a subsequent fall in fasting serum glucose levels, consistent with a decreased hepatic activation of PPARalpha [18]. In conclusion, we propose an important novel role for SR-BI in the control of fasting serum glucose levels.

4.2 Experimental Procedures

4.2.1 Animals

SR-BI deficient mice were kindly provided by Dr. M. Krieger [8]. Heterozygous SR-BI deficient mice were crossbred to generate wild-type and homozygous progeny. The offspring of the mice were analysed for the presence of targeted or wild-type SR-BI alleles by PCR, as described by Van Eck et al. [9]. Male homozygous SR-BI deficient mice and wild-type littermates as controls were maintained on a sterilized regular chow (RM3, Special Diet Services, Witham, UK). At 12-16 weeks of age, overnight fasted mice were anesthetized, and blood was collected for lipid and glucose analyses. Subsequently, a whole-body perfusion was performed using phosphate-buffered saline (4°C, 100 mm Hg) for 10 min. After perfusion, the liver was excised, weighed, and frozen in liquid nitrogen and stored at -80°C. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

4.2.2 Lipid analyses

Fasting serum concentrations of free and total cholesterol, phospholipids, free fatty acids, and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 µl serum of each mouse using a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics), taking the efficiency of recovery from the column into account.

4.2.3 Glucose analysis

Fasting serum glucose levels were determined essentially as described [19]. In short, 200 µl coloring reagent, containing 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate), glucose-oxidase, and peroxidase was added to 5 µl 25-times diluted serum of SR-BI deficient and wild-type mice. After a 30-minute incubation at 37°C, the absorbance was measured at 405 nm and the glucose concentration was determined.

4.2.4 Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on livers of SR-BI deficient mice and their wild-type littermates was performed as described [20]. In short, total RNA was isolated according to Chomczynski and Sacchi [21] and reverse

transcribed using RevertAid^{TM} reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in Table 1, which were validated for identical efficiencies (slope = -3.3 for a plot of Ct versus log ng cDNA). Hypoxanthine guanine phosphoribosyl transferase (HPRT), 18S ribosomal RNA (18SrRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of HPRT, 18SrRNA, GAPDH, and 36B4 ($Ct_{housekeeping}$) and raising 2 to the power of this difference. The average Ct of four housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes.

Table 1
Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse primer	Amplicon size
18SrRNA	X00686	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG	69
36B4	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ACO	NM015729	AATTGGCACCTACGCCCAG	GTGGTTTCCAAGCCTCGAAG	63
CPT-I	NM013495	CACTACGGAGTCCTGCAACTTTG	AGCTTGAACCTCTGCTCTGCC	72
L-FABP	NM017399	TCACCATCACCTATGGACCCA	TCCAGTTCGCACTCCTCCC	67
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
HMGCS2	NM008256	TTTCATTCCGAGTGTCCAAGG	CTGACACACTAGACACCAGTTTCTCC	69
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
L-PK	NM013631	AAGACAGTGTGGGTGGACTACCA	CGTCAATGTAGATGCGGCC	70
PEPCK	NM011044	TTGAACTGACAGACTCGCCCT	GATATGCCCATCCGAGTCATG	64
PGC-1	NM008904	CCCGATCACCATATTCCAGG	GTAGTGGCTTGATTCATAGTAGTAACAGGA	89

4.2.5 Data analysis

The significance of differences in relative gene expression numbers between livers of SR-BI deficient and wild-type mice, measured by real time quantitative PCR, was calculated using a two-tailed unpaired Student's t-test on the differences in Ct ($Ct_{housekeeping} - Ct_{target\ gene}$). Probability values less than 0.05 were considered significant.

3.3 Results

In the current study, the potential beneficial role of SR-BI in the treatment of diabetes was investigated by determining whether SR-BI plays a role in the regulation of serum glucose levels in normolipidemic mice. In agreement with earlier studies, overnight (~16h) fasted SR-BI deficient mice have significantly increased serum (free) cholesterol and triglyceride levels as compared to wild-type controls, which is due to increased levels of HDL and VLDL, respectively (Table 2; Fig.1). However, no change in serum free fatty acid levels was seen upon SR-BI deficiency (Table 2). In contrast, the fasting serum glucose levels were 42% decreased in SR-BI deficient mice

as compared to wild-type littermate controls (P<0.01; Fig.2). This suggests that indeed SR-BI plays a role in the regulation of fasting serum glucose levels. Blood glucose levels are maintained by the balance between glucose uptake by peripheral tissues and glucose secretion by the liver. During fasting, blood glucose levels decrease rapidly as a result of hampered glucose intake. In response, the liver starts breaking down glycogen to glucose-6-phosphate, which is subsequently converted to free glucose that can be excreted into serum (glycogenolysis pathway). However, after a longer fasting period (>8h) the breakdown of glycogen is replaced by gluconeogenesis, a process in which hepatic pyruvate is transformed into free glucose, to preserve glycogen stores [22].

Table 2
Effect of SR-BI deficiency on fasting serum lipid levels

	n	Free cholesterol	Total cholesterol	Phospholipid	Triglyceride	FFA
		mg/dl				mM
SR-BI +/+	6	20.0 ± 0.8	62.2 ± 6.4	172 ± 4.4	81 ± 4.1	0.69 ± 0.05
SR-BI -/-	6	84.8 ± 2.9 ***	181.9 ± 8.0 ***	183 ± 3.5	108 ± 13.2 *	0.66 ± 0.09

Serum lipid levels were measured in SR-BI deficient (-/-) and wild-type (+/+) mice on a chow diet after an overnight fasting period (means+SEM). *P<0.05, ***P<0.001 compared to wild-type mice.

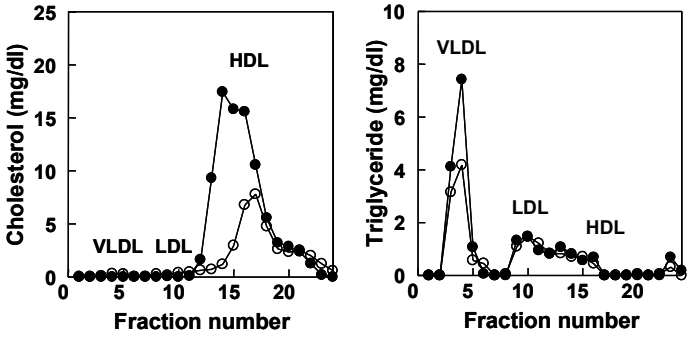

Fig.1. Effect of SR-BI deficiency on serum lipoprotein cholesterol and triglyceride distribution. Blood samples were drawn after an overnight fast from SR-BI deficient (●) and wild-type (○) mice. Sera from 6 individual mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3-6 represent VLDL; fractions 8-12, LDL; and fractions 12-20, HDL, respectively

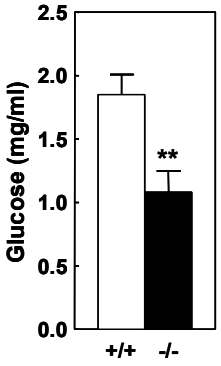

Fig.2. Effect of SR-BI deficiency on fasting serum glucose levels. Blood samples were drawn after an overnight fast from SR-BI deficient (closed bar) and wild-type (open bar) mice (means+SEM; n=6); **P<0.01 compared to wild-type mice.

Phosphoenolpyruvate carboxykinase (PEPCK) is generally considered to be the first rate-limiting step in gluconeogenesis in hepatic cells [23]. Furthermore, recent data from Yoon et al. suggested that nuclear coactivator PGC-1 is a key modulator of hepatic gluconeogenesis [24]. Interestingly, SR-BI deficiency did not have an effect on the hepatic expression of PEPCK or PGC-1 (Fig.3), which indicates that absence of functional SR-BI does affect hepatic gluconeogenesis. In contrast to gluconeogenesis, the glycolytic pathway mediates the breakdown of glucose to pyruvate. To determine whether SR-BI deficiency affected glycolysis, the expression of a key enzyme of the glycolytic pathway in the liver, L-type pyruvate kinase (L-PK) [25], was determined in SR-BI deficient and wild-type mice. Pyruvate kinase expression is highly regulated upon a demanded change in the glycolysis rate [26,27]. No significant change in the expression of pyruvate kinase was observed in SR-BI deficient mice as compared to wild-type littermate controls (Fig.3), which suggests that SR-BI deficiency did not influence the glycolysis rate.

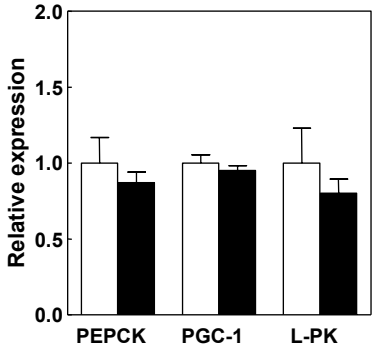

Figure 3: Effect of SR-BI deficiency on hepatic mRNA expression of genes involved in gluconeogenesis and glycolysis. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to wild-type mice (means+SEM; n=6).

Importantly, particularly under fasting “low glucose” conditions, extrahepatic tissues (e.g. brain, muscle) are also able to use an alternative source of energy generated by the liver, namely ketone bodies. These ketone bodies are produced in the liver from fatty acids by fatty acid oxidation and subsequent ketogenesis [28]. Because no change was observed in the hepatic expression of genes involved in the generation and breakdown of glucose, the effect of SR-BI deficiency on hepatic fatty acid oxidation and ketogenesis was studied by determining the effect on the expression of genes involved in these processes. The expression of the rate-limiting enzyme in the peroxisomal beta-oxidation, acyl-CoA oxidase (ACO) [29], was not different between SR-BI deficient and wild-type mice (Fig.4). Moreover, the expression of carnitine palmitoyltransferase I (CPT-I), an essential enzyme in the beta-oxidation of long-chain fatty acids in the mitochondria [30], was also unchanged (Fig.4). Furthermore, no significant change (Fig.4) was observed in the expression of liver fatty acid binding protein (L-FABP), which specifically enhances the uptake and intracellular targeting of long and medium chain fatty acids to the nucleus [31]. In contrast, the hepatic expression of HMG-CoA synthase (HMGCS), a key enzyme in ketogenesis [32], was significantly reduced by 54% (P=0.004) in SR-BI deficient mice as compared to wild-type mice (Fig.5).

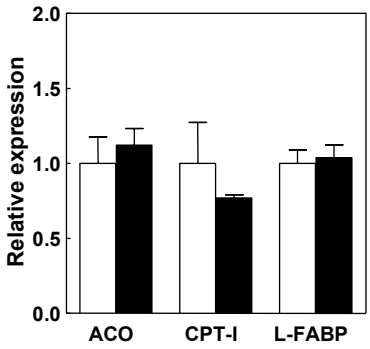

Fig.4. Effect of SR-BI deficiency on hepatic mRNA expression of genes involved in fatty acid transport and metabolism. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to wild-type mice (means+SEM; n=6).

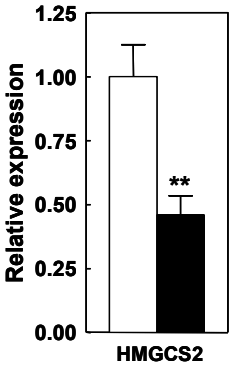

Fig.5. Effect of SR-BI deficiency on hepatic mRNA expression of the rate-limiting ketogenesis enzyme HMG-CoA synthase (HMGCS2). Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to wild-type mice (means+SEM; n=6).
**P<0.01 compared to wild-type mice.

4.4 Discussion

Recently, it has been suggested that SR-BI might play a role in diabetes. In the current study, the effect of the absence of functional SR-BI on hepatic genes involved in the regulation of fasting glucose levels was determined.
Strikingly, fasting serum glucose levels were 42% decreased in SR-BI deficient mice as compared to wild-type littermates without a change in the expression of key genes in gluconeogenesis (PEPCK and PGC-1) and glycolysis (pyruvate kinase), the major pathways involved in the regulation of hepatic glucose metabolism. Hepatic PEPCK and PGC-1 expression is stimulated by many different factors, including glucocorticoids, cAMP, and thyroid hormone [33-35], whilst the key regulator of serum glucose levels, insulin, is able to inhibit PEPCK and PGC-1 expression [24,33]. In contrast to inhibition of PEPCK expression, insulin is able to stimulate the expression of pyruvate kinase [36,37]. However, the unchanged hepatic expression of PEPCK, PGC-1, and pyruvate kinase in SR-BI deficient mice suggests that changes in insulin signalling and the associated gluconeogenesis and glycolysis pathways were not responsible for the decreased serum glucose levels observed in these mice.
It became clear from human patients carrying mutations in the HMGCS gene, which suffer from severe hypoglycaemia [38] and hypoglycemic hypoketotic coma during fasting periods [39,40] that hepatic fatty acid oxidation and subsequent ketogenesis are also important pathways involved in the regulation of fasting glucose levels, since ketone bodies can act as an alternative source of energy for peripheral tissues. The expression of key enzymes in the peroxisomal (ACO) and mitochondrial

fatty acid oxidation (L-FABP and CPT-I) was not changed, which suggests that fatty acid oxidation was not affected by SR-BI deficiency. In contrast, in fasted SR-BI deficient mice a 54% decreased hepatic HMGCS mRNA expression was observed. HMGCS catalyzes the rate-limiting step in liver ketone body formation and its activity correlates with the supply of ketone bodies which serve as an alternative extra-hepatic substrate by which extra-hepatic glucose utilization can be spared. The lack of adequate hepatic ketone body formation will lead to increased peripheral glucose utilization with consequently a net decrease in the fasting serum glucose level.
The promoter of HMGCS contains (putative) recognition sequences for several transcription factors [41], such as the forkhead transcription factor FKHRL1 [42], hepatocyte nuclear factor 4 (HNF4) [43], and peroxisome proliferator-activated receptor (PPAR) [44,45]. However, data provided by Rodriquez et al. and Hegardt have indicated that PPARalpha mediates regulation of HMGCS under fasting conditions [43,46]. This suggests that the hepatic activity of PPARalpha is decreased in SR-BI deficient mice as compared to their wild-type littermates. In agreement, 24-hours fasted PPARalpha deficient mice also have a decreased hepatic HMGCS expression and a 60% lowered serum glucose level as compared to their wild-type littermates, with an unchanged hepatic CPT-I expression [47]. In addition, the hepatic expression of a novel PPARalpha target gene, microsomal triglyceride transfer protein (MTP) [48], trended to a decrease ($P=0.09$) in SR-BI deficient mice (data not shown). Furthermore, PPARalpha activation has been shown to result in increased hepatic secretion of ApoB-containing lipoproteins [49] since MTP is the rate-limiting enzyme in VLDL ApoB secretion [50]. In accordance, the VLDL secretion rate also shows a trend to a decrease in SR-BI deficient mice as compared to wild-type mice (unpublished data, M. Van Eck, R. Out, M. Hoekstra, I.S.T. Bos, J.K. Kruijt, and Th.J.C. van Berkel). Moreover, Mardones et al. have shown that the hepatic expression of cholesterol 7α-hydroxylase (CYP7A1), another murine PPAR target gene [51], was decreased in SR-BI deficient mice [52].
Out et al. have recently shown that SR-BI deficient mice have a hampered hepatic uptake of triglyceride-rich lipoproteins [53], which are a source for intra-hepatic fatty acids, since their triglycerides upon entry in the liver are rapidly lipolysed to fatty acids by lysosomal acid lipase [54]. In addition, multiple studies have shown that the selective uptake of HDL cholesterol esters, which are also a source for intra-hepatic fatty acids via breakdown by lysosomal acid lipase, is greatly reduced in SR-BI deficient mice [8,9], since SR-BI is solely responsible for the selective uptake of cholesterol esters from HDL by the liver [55,56]. The hampered hepatic association/uptake of triglycerides and cholesterol due to absence of functional SR-BI can thus explain the increase in serum VLDL-triglyceride and HDL-cholesterol levels compared to controls observed in the current study. Moreover, the decreased uptake of lipoprotein-associated fatty acids might have caused the lower PPARalpha activity (fatty acids are potent naturally occurring activators of PPARalpha [57,58]) and decrease in hepatic HMGCS expression which will lead to lower serum glucose levels.
In conclusion, we have shown that, under fasting conditions, SR-BI deficiency due to a hampered hepatic uptake of lipoprotein-associated fatty

acids results in a decreased hepatic expression of the rate-limiting ketogenesis enzyme HMG-CoA synthase and a subsequent fall in fasting serum glucose levels. In this light, we propose an important novel role for SR-BI in the control of fasting serum glucose levels.

4.5 Acknowledgements

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5

Gene regulation in the liver by nuclear receptors plays an essential role in the prevention of diet induced atherosclerotic lesion development in C57BL/6 mice

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Since high dietary lipid levels are a common risk factor for atherosclerosis, many studies have been conducted to gain more insight into the mechanism behind atherogenesis. Wild-type mice do develop atherosclerotic lesion development on an atherogenic diet containing a combination of increased cholesterol and fat levels and cholic acid. However, no atherosclerotic lesion development is observed in wild-type mice after 20 weeks of feeding a diet containing 0.25% cholesterol and 15% fat without cholic acid. The liver is an essential organ in the modulation of total body cholesterol homeostasis, and thus atherosclerotic lesion formation, since it is able to take up lipid from the circulation for biliary efflux and subsequent excretion via the feces. To determine whether the liver plays a role in the inhibition of diet induced lesion formation, in the current study the effect of a diet containing 0.25% cholesterol, 15% fat (without cholic acid) on lipid metabolism was studied in C57BL/6 mice. Feeding C57BL/6 mice the high cholesterol/high fat diet induces an increased hepatic activation of the nuclear receptors LXR and PPARalpha resulting in significant changes in the hepatic expression of genes involved in the maintenance of hepatic and total body lipid homeostasis, such as ABCA1 (HDL production), BSEP, CYP3A11, CYP7A1, SHP (bile acid synthesis and metabolism), HMG-CoA reductase and SREBP-1 (lipid synthesis), CD36, PPARgamma (adipogenesis), and SCD1 (VLDL production). The net result of these adaptive changes leads to a non-atherogenic serum lipoprotein profile, thus preventing the development of atherosclerotic lesions in the vessel wall.

5.1 Introduction

Atherosclerosis is a common cause of death in the Western world. In the past, several risk factors for the development of atherosclerotic lesions have been identified. These include smoking, diabetes, obesity, and changed serum lipid levels. Increased plasma very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol levels are a well-established risk factor for the development of atherosclerotic lesions [1,2], while increased levels of high-density lipoprotein (HDL) have been shown to inhibit atherogenesis [3,4]. To elucidate the pathways by which the different lipoproteins mediate their pro- or anti-atherogenic effect, several murine models of dyslipidemia have been generated. Mice that lack ApoE (ApoE deficient mice) spontaneously develop atherosclerotic lesions on a regular chow diet due to an accumulation of long circulating VLDL remnants [5]. In addition, LDL receptor deficient mice develop lesions on a diet containing increased levels of cholesterol and fat, due to an accumulation of VLDL remnants and LDL [6-8]. Moreover, scavenger receptor class B type I (SR-BI) deficient mice fed a high cholesterol/high fat diet are susceptible to atherosclerotic lesion development [9]. Wild-type mice also do develop atherosclerotic lesion development on an atherogenic diet containing a combination of increased cholesterol and fat levels and cholic acid (Paigen diet) [10]. However, no atherosclerotic lesion development is observed in wild-type mice after 20 weeks of feeding a diet containing 0.25% cholesterol and 15% fat without cholic acid (Western type diet) [9], which suggests that wild-type mice are resistant to the induction of atherosclerotic lesion formation by this diet.

The liver is an essential organ in the modulation of total body cholesterol homeostasis, and thus atherosclerotic lesion formation, since it is able to take up lipid from the circulation for biliary efflux and subsequent excretion via the feces [11,12]. Furthermore, the liver is the key organ involved in the production of HDL, which is an essential component of the reverse cholesterol transport pathway. To determine whether the liver plays a role in the inhibition of diet induced lesion formation, in the current study the effect of an atherogenic high cholesterol/high fat Western type diet containing 0.25% cholesterol, 15% fat (without cholic acid) on lipid metabolism was studied in C57BL/6 mice.

Here we report that, as compared to a regular chow diet, feeding a high cholesterol/high fat diet leads to increased serum levels of the pro-atherogenic lipoprotein VLDL (4.2-fold) and the anti-atherogenic lipoprotein HDL (1.7-fold) in C57BL/6 mice, with a minor increase in hepatic lipid levels. The changes in the serum lipoprotein profile are due to increased hepatic activation of the nuclear regulators LXR and PPARalpha resulting in significant changes in the hepatic expression of genes involved in the maintenance of hepatic and total body lipid homeostasis, such as ABCA1 (HDL production), BSEP, CYP3A11, CYP7A1, SHP (bile acid synthesis and metabolism), HMG-CoA reductase and SREBP-1 (lipid synthesis), CD36, PPARgamma (adipogenesis), and SCD1 (VLDL production). The net result of these adaptive changes is a non-atherosclerotic serum lipoprotein profile, thus preventing the development of atherosclerotic lesions in the vascular wall.

5.2 Experimental Procedures

5.2.1 Animals

Male C57BL/6 mice (10-12 weeks old) were obtained from Charles River Laboratories (Maastricht, The Netherlands). Mice were maintained on sterilized regular chow containing 4.3% (w/w) fat with no added cholesterol (RM3; Special Diet Services, Witham, U.K.) or were fed a semi-synthetic diet containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W; Hope Farms, Woerden, The Netherlands) for two weeks. Subsequently, after an overnight (~16 h) fasting period, serum was drawn for lipid analyses, which was followed by a whole-body perfusion using phosphate-buffered saline (4°C, 100 mm Hg) for 10 min. After perfusion, the liver was excised, weighed, and frozen in liquid nitrogen and stored at -80°C. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

5.2.2 Lipid analyses

Fasting serum concentrations of free and total cholesterol, phospholipids, and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 μl serum of each mouse using a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics), taking the efficiency of recovery from the column into account. Hepatic lipids were extracted according to Bligh and Dyer [13]. After dissolving the lipids in 2% Triton X-100, contents of total cholesterol and triglycerides in liver tissue were determined as described above and expressed as μg lipid/mg of protein.

5.2.3 VLDL triglyceride production

Mice fed a regular chow or high cholesterol/high fat diet were injected intravenously after an overnight fast with 500 mg of Triton WR 1339 (Sigma) per kg body weight as a 15 g/dl solution in 0.9% NaCl. Previous studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions [14]. Blood samples (50 μl) were taken at 0, 1, 2, 3, and 4 hours after Triton WR-1339 injection. Serum triglycerides were determined enzymatically as described above. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as mg/dl/h.

5.2.4 Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on isolated liver was performed as described [15]. In short, total RNA was isolated according to Chomczynski and Sacchi [16] and reverse transcribed using RevertAid^{TM} reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in Table 1. The primers used were validated for identical efficiencies (slope = -3.3 for a plot of threshold cycle number (Ct) versus log ng cDNA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18SrRNA), β-actin, and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of GAPDH, 18SrRNA, β-actin, and 36B4 (Ct housekeeping) and raising 2 to the power of this difference. The average Ct of four housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions.

Table 1
Primers for quantitative real-time PCR analysis

Gene	GenBank Accesion	Forward primer	Reverse primer	Amplicon size
18SrRNA	X00686	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG	69
36B4	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ABCA1	NM013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC	96
β-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA	75
BSEP	NM021022	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA	62
CD36	NM007643	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGT T	110
CYP3A11	NM007818	GGATGAGATCGATGAGGCTCTG	CCAGGTATTCCATCTCCATCACA	74
CYP7A1	NM007824	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	95
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
HMGCR	M62766	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT	69
LDLR	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC	68
LRP1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA	95
PPAR(g)	NM011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT	131
SCD1	NM009127	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA	65
SHP	L76567	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC	72
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG	63
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC	74

5.2.5 Data analysis

The significance of differences in relative gene expression numbers between livers isolated from mice fed a regular chow or a high cholesterol/high fat diet, measured by real time quantitative PCR, was calculated using a two-tailed Student's t-test on the differences in Ct ($Ct_{housekeeping} - Ct_{target\ gene}$). The difference in Ct values was tested for normality using Graphpad Instat 3 software (Graphpad Software, Inc.). Probability values less than 0.05 were considered significant.

5.3 Results

Serum lipid levels in C57BL/6 mice are greatly affected upon feeding a high cholesterol/high fat diet containing 0.25% cholesterol and 15% fat for two weeks. Free cholesterol and cholesterol ester levels are increased 81% (P=0.0012) and 92% (P<0.001), respectively, with a 78% (P=0.002) increase in the serum phospholipid level as compared to the chow diet (Table 2). In addition, the serum triglyceride level trends to an increase (30%; P=0.14) as compared to the chow diet. On the chow diet, cholesterol in the serum of C57BL/6 mice is mainly present in HDL (72.6%), and to a minor extent in LDL (26.0%) and VLDL (1.4%) (Fig.1). However, the high cholesterol/high fat diet induced a marked 4.2-fold (P=0.032) increase in the level of VLDL-associated cholesterol, without changing the VLDL lipid composition (Fig.1).
The liver plays an essential role in the regulation of serum VLDL (cholesterol) levels, since it is able 1) to decrease serum levels through receptor-mediated uptake of VLDL, and 2) to increase circulating VLDL levels by stimulating the synthesis and subsequent secretion of VLDL. Importantly, the hepatic mRNA expression of the LDL receptor and the LDL receptor-related protein (LRP1), the two receptors that are predominantly involved in the serum clearance of VLDL (remnant) particles by the liver [17,18], were unchanged by the high cholesterol/high fat diet (Fig.2). This suggests that the uptake of VLDL was not changed by high cholesterol/high fat diet feeding. In contrast, the VLDL secretion rate was significantly increased by 20% (P=0.014) upon feeding the high cholesterol/high fat diet (Fig.3). These combined data thus suggest that the rise in the serum VLDL cholesterol level by the high cholesterol/high fat diet was the result of an increased VLDL secretion rate.

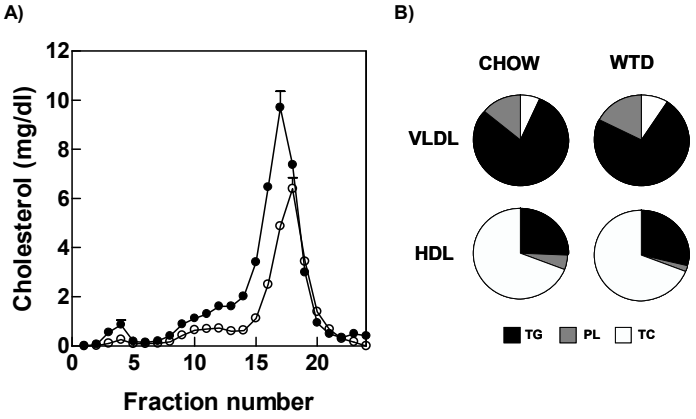

Fig.1. Effect of a high cholesterol/high fat diet on the serum cholesterol distribution and lipoprotein lipid composition. Blood samples were drawn after an overnight fast while feeding a regular chow (○) or a high cholesterol/high fat diet (●) for two weeks. *A*) Sera from 5 individual C57BL/6 mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3-7 represent VLDL; fractions 8 to 15, LDL; and fractions 15 to 19, HDL, respectively (means+SEM; n=5). *B*) VLDL and HDL were isolated by ultracentrifugation from pooled serum of 5 overnight fasted C57BL/6 mice on a high cholesterol/high fat or chow diet for two weeks and the lipid composition was determined (in w/w% of total lipids).

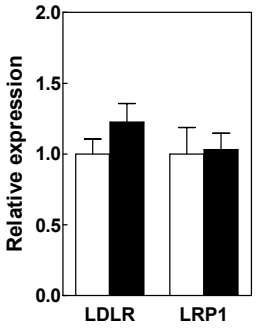

Fig.2. Effect of a high cholesterol/high fat diet on mRNA expression of genes involved in VLDL and LDL uptake in livers of C57BL/6 mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=4/5).

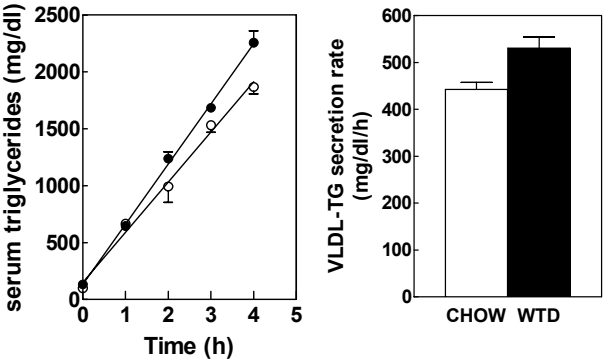

Fig.3. Effect of a high cholesterol/high fat diet on the VLDL production rate. C57BL/6 mice were fed a regular chow (○) or a high cholesterol/high fat diet (●) for two weeks and fasted overnight before Trion WR1339 injection. Subsequently, triglyceride accumulation was measured over time and the VLDL production rate was determined (means+SEM; n=5). *P<0.05 as compared to chow diet.

Strikingly, next to a 4.2-fold increase in the pro-atherogenic lipoprotein VLDL, the high cholesterol/high fat diet also significantly stimulated the serum level, but not lipid composition, of the anti-atherogenic lipoprotein HDL by 69% (P<0.001; Fig.1). As for VLDL, serum HDL levels are highly controlled by the liver. Hepatic expression of the ATP-binding cassette transporter A1 (ABCA1) is important for generation of nascent HDL [19,20], whilst functional expression of scavenger receptor class B type I (SR-BI) is necessary for the selective uptake of cholesterol esters from mature HDL into the liver [21,22]. The mRNA expression of SR-BI was not significantly changed by the high cholesterol/high fat diet (Fig.4). In contrast, the expression of ABCA1 was increased by 43% upon feeding the high cholesterol/high fat diet. However, the increase failed to reach statistical significance (p=0.16) due to a large variation in the expression of ABCA1 in the chow-fed mice (Fig.4). ABCA1 mRNA expression can be stimulated by the nuclear receptor liver X receptor (LXR), which upon its activation by oxysterols and subsequent heterodimerization with the retinoic X receptor (RXR) is able to bind a LXR/RXR response element in the promoter of ABCA1 [23].

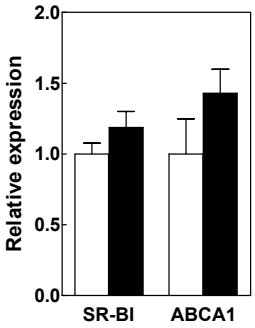

Fig.4. Effect of a high cholesterol/high fat diet on mRNA expression of genes involved in HDL synthesis/metabolism in livers of mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=4/5).

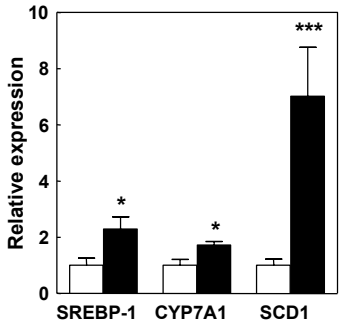

Fig.5. Effect of a high cholesterol/high fat diet on mRNA expression of LXR target genes in livers of mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=4/5). *P<0.05, ***P<0.001 compared to chow diet.

To determine the effect of the high cholesterol/high fat diet on hepatic LXR activation, the effect on additional established LXR target genes was investigated. The expression of sterol regulatory element-binding protein 1 (SREBP-1), cholesterol 7α-hydroxylase (CYP7A1) and stearoyl-CoA desaturase-1 (SCD1) was significantly induced 2.3-, 1.7-, and 7.0-fold, respectively, by the high cholesterol/high fat diet (Fig.5), which suggests that indeed LXR activation was stimulated on the diet.

Increased expression of CYP7A1 has been shown to result in an increased formation of bile acids [24]. To determine whether the high cholesterol/high fat diet did increase the formation of bile acids, the effect of the diet on the hepatic expression of bile acid-regulated genes was investigated. In agreement with an increased bile acid formation, a significant increase in the expression of the bile acid receptor farnesoid X receptor (FXR) target genes small heterodimer partner (SHP; 1.4-fold) and bile salt efflux pump (BSEP; 1.5-fold) was observed (Fig.6). In contrast, the expression of 6α-testosterone hydroxylase (CYP3A11) was 6.8-fold (P<0.001) decreased by the high cholesterol/high fat diet (Fig.6).

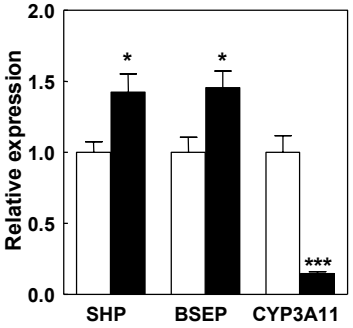

Fig.6. Effect of a high cholesterol/high fat diet on the mRNA expression of genes involved in bile acid metabolism in livers of mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=4/5). *P<0.05, ***P<0.001 compared to chow diet.

Interestingly, recent data provided by Seo et al. [25] suggest that LXR also plays a role in the execution of adipocyte differentiation through an induction of the expression of peroxisome proliferators-activated receptor (PPAR) gamma, a central regulator of adipocyte gene expression and differentiation [26]. In accordance, the expression of PPARgamma as well as the expression of fatty acid translocase CD36/FAT, which is strongly induced during the adipocyte differentiation [27] and regulated by PPARgamma [28], was increased by the high cholesterol/high fat diet (Fig.7). Since increased hepatic adipogenesis is associated with an increased storage of fat in the liver, the effect of the high cholesterol/high fat diet on hepatic lipid levels was determined. The diet did affect hepatic lipid content, but to a relatively small extent. The hepatic total cholesterol level was increased 38% (P=0.005), while the hepatic concentration of triglycerides, the main lipid constituent in liver, remained at the control level upon feeding the high cholesterol/high fat diet (Fig.7). Multiple studies have indicated that an increase in hepatic lipid levels results in a repressed expression of HMG-CoA reductase, the essential enzyme in de novo cholesterol synthesis [29-31]. Accordingly, the hepatic HMG-CoA reductase expression was reduced by the high cholesterol/high fat diet to 63% (P=0.022) of that on the chow diet (Fig.7).

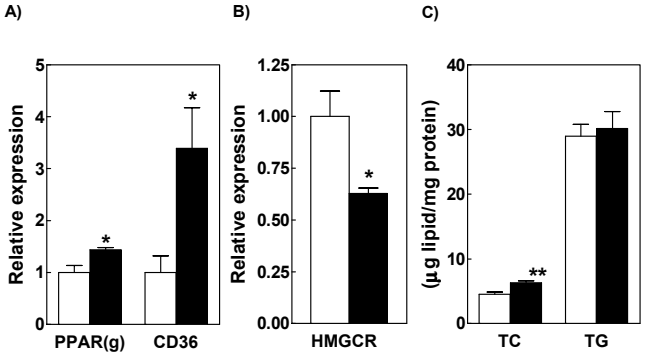

Fig.7. Effect of a high cholesterol/high fat diet on the mRNA expression of genes involved in *A*) adipogenesis and *B*) de novo cholesterol synthesis in livers of mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks. ***C*) Effect of a high cholesterol/high fat diet on hepatic lipid levels of mice fed a regular chow (white bars) or a high cholesterol/high fat diet (black bars) for two weeks**. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=4/5). *P<0.05, **P<0.01 compared to chow diet.

5.4 Discussion

Clinical data provided by Castelli have indicated that in humans a 2-fold rise in LDL levels results in a ~2-fold increased risk for cardiovascular diseases (CVD), whilst a 2-fold rise in HDL levels results in a ~4-fold decreased risk for CVD [32]. The observed rise in the anti-atherogenic HDL level of C57BL/6 mice on the high cholesterol/high fat diet in the current study might

therefore abrogate the increased atherogenic risk caused by the rise in pro-atherogenic VLDL levels, resulting in the absence of atherosclerotic lesion formation in wild-type mice on this diet as observed by Van Eck et al. [9].
The increase in the serum VLDL level correlates with an increased VLDL secretion rate, while the expression of receptors involved in the hepatic uptake of VLDL, the LDL receptor and LRP1 [17,18], is unchanged. The rise in serum HDL levels can be explained the increased hepatic expression of ABCA1. The essential role for ABCA1 in the production/lipidation of HDL is apparent from mice with a targeted disruption of ABCA1, which leads to a virtual absence of HDL cholesterol [33]. The increase in ABCA1 expression can be the consequence of an increased hepatic LXR activation as judged from the concomitant increases in the expression of several other LXR target genes such as SREBP-1 [34], CYP7A1 [35], and SCD1 [36] on the high cholesterol/high fat diet. The high cholesterol/high fat diet does also stimulate the expression of SHP and BSEP, which are target genes of FXR. Makishima et al. [37] have shown that bile acids are physiological ligands for nuclear receptor FXR and the increased expression of SHP/BSEP therefore suggests that the high cholesterol/high fat diet induced the formation of bile acids. This is in agreement with in vitro and in vivo data provided by Pandak et al. [38] and Miyake et al. [24], respectively, that an increase in the expression of CYP7A1, as observed on the high cholesterol/high fat diet, results in a marked activation of the classic pathway of bile acid biosynthesis. Since FXR activation through stimulation of SHP leads to an inhibition of CYP7A1 expression, the increased expression of CYP7A1 observed on the diet suggests that the stimulatory effect of LXR overrides the inhibitory effect of FXR, as already proposed by Gupta et al. [39]. Strikingly, the expression of another gene product stimulated by the bile acid lithocholic acid through activation of the nuclear receptor pregnane X receptor (PXR), CYP3A11 [40], was decreased upon feeding the high cholesterol/high fat diet. A recent study has indicated an important role for PPARalpha in the downregulation of hepatic CYP3A11 expression by peroxisome proliferators [41]. Combined with the fact that the high cholesterol/high fat diet contains a high level of fatty acids, potent activators of PPARalpha [42], these findings suggest that feeding the mice the high cholesterol/high fat diet resulted in a significant induction of the hepatic PPARalpha activity. In agreement, the expression of an established PPARalpha target gene, carnitine palmitoyltransferase-I (CPT-I) [43], was stimulated by the high cholesterol/high fat diet (data not shown). Furthermore, the promoter of SCD1 contains a cis-linked PPAR response element [44], which suggests that the striking 7-fold increase in SCD1 expression might, next to the increase in LXR activation, also be caused by an increased activation of PPARalpha. In earlier studies, the hepatic SCD1 activity has been shown to correlate with plasma triglyceride concentrations [45,46]. In parallel, the serum triglyceride concentration did indeed increase on the high cholesterol/high fat diet, but the increase failed to statistical significance. The absence of a striking increase might be due to the enhanced PPARalpha activation, since Staels et al. have suggested that activation of PPARalpha by fibrates results in a decreased VLDL production [47]. However, the VLDL secretion rate was increased. This corresponds to the observations of Grefhorst et al. that increased hepatic LXR activation

coincides with an increased VLDL-triglyceride secretion rate [48]. Although an increased PPARalpha activity was observed on the high cholesterol/high fat diet, no change in the mRNA expression of PPARalpha or PPARdelta was seen (data not shown), which indicates that increased PPARalpha activity was not caused by an increased transcription of the PPARalpha gene, but was likely due to an enhanced activation of the PPARalpha/RXR complex. In contrast, the expression of PPARgamma and its target gene CD36/FAT was increased on the diet, due to the increased LXR activation. Yu et al. have indicated that overexpression of PPARgamma in the liver leads to an induction of adipogenic transformation of hepatocytes with adipose tissue-specific gene expression and lipid accumulation, resulting in a novel type of adipogenic hepatic steatosis [49]. These findings correspond to our observation that the high cholesterol/high fat diet induced an increase in the hepatic lipid content. However, the rise (38%) in the intra-hepatic cholesterol level was relatively small. This might be the combined result of 1) an increased VLDL secretion, 2) an increased lipidation of HDL via ABCA1, 3) an increased metabolism of cholesterol to bile acids by CYP7A1 for subsequent excretion into bile via BSEP, and 4) a decreased cholesterol synthesis due to inhibition of HMG-CoA reductase expression.
In conclusion, we propose that the liver by maintaining its intra-hepatic lipid balance, through increased activation of both LXR and PPARalpha, plays an essential role in the resistance of wild-type mice to high cholesterol/high fat diet induced atherosclerotic lesion development. The net result of these adaptive changes is a non-atherosclerotic serum lipoprotein profile, thus preventing the development of atherosclerotic lesions in the vascular wall (Fig.8).

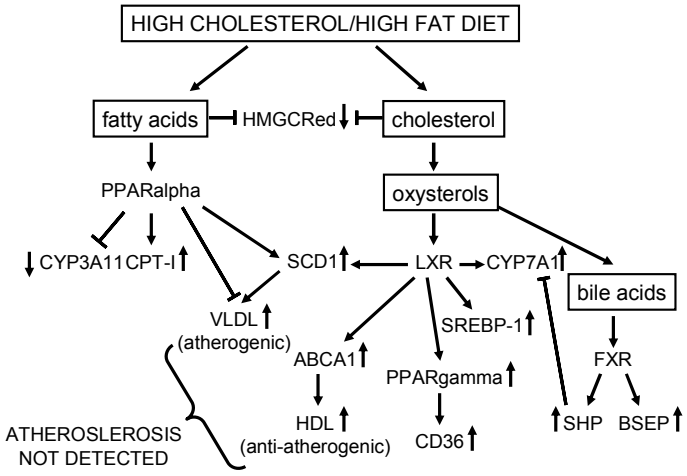

Fig.8. Overview of the effects of the high cholesterol/high fat diet on hepatic genes involved in lipid metabolism and serum lipoprotein levels. Depicted are the pathways involved in the regulation of gene expression. Dietary fatty acids activate the nuclear receptor PPARalpha, whilst dietary cholesterol-derived oxysterols activate the nuclear receptor LXR leading to marked alterations in the hepatic expression of genes involved in bile acid formation, adipogenesis, and VLDL and HDL synthesis. However, the net result of these adaptive changes is a non-atherosclerotic serum lipoprotein profile.

5.5 Acknowledgements

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6

FXR treatment induces atherosclerotic lesion formation in ApoE deficient mice

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Progressive familial intrahepatic cholestasis represent a group of inherited, autosomal recessive disorders, which are characterized by progressive liver disease with impairment of bile flow, resulting in complete liver failure and early death. Recently, several studies have suggested that ligands for the nuclear receptor farnesoid X receptor (FXR) may have therapeutic use in treating cholestatic liver disease. However, until now no conclusive evidence for a role of bile acids or FXR in atherosclerotic lesion formation has been described. In the current study the effect of FXR activation by bile acids on atherogenesis was studied in ApoE deficient mice, a well-established atherosclerotic mouse model. Taurocholic acid, a naturally occurring bile acid that is able to activate FXR, does indeed inhibit hepatic bile acid synthesis and cholesterol synthesis. However, due to a 50% downregulation of the LDL receptor in the liver, serum VLDL cholesterol levels are increased upon FXR activation, resulting in a marked 3.9-fold increase in atherosclerotic lesion formation.
We conclude that the administration of statins, which lead to upregulation of LDL receptors, can be beneficial in order to maintain the attractivity of FXR as a promising therapeutic target for treating or preventing gallstone disease.

6.1 Introduction

Progressive familial intrahepatic cholestasis (PFIC) represents a group of inherited, autosomal recessive disorders with various causes, including defects of hepatocanalicular transport of bile salts or phospholipids and defects of bile acid synthesis. Cholestasis may occur due to impairment of a specific molecular process, or as a secondary consequence of structural damage of the hepatocyte or bile duct. Clinically, PFIC is characterized by progressive liver disease with impairment of bile flow, leading to elevated plasma concentrations of biliary constituents, resulting in jaundice and liver toxicity [1-3]. Recently, Alvarez et al. have shown that the hepatic expression of the nuclear receptor farnesoid X receptor (FXR; NR1H4) was dramatically reduced in patients with cholestatic liver disease [4]. In addition, Liu et al. have recently shown that administration of a synthetic agonist for FXR is able to inhibit liver toxicity in a rat model for ANIT induced intrahepatic cholestasis [5]. Furthermore, Moschetta et al. have shown that treatment with a synthetic FXR agonist prevented sequelae of cholesterol gallstone disease in a mouse model for human cholesterol gallstone disease [6]. These combined findings suggest that FXR agonists may have therapeutic use in treating cholestatic liver disease.
Importantly, FXR is activated by naturally occuring bile acids, such as chenodeoxycholic acid, deoxycholic acid, and cholic acid. Furthermore, high expression of FXR has been detected in intestine, liver, adrenal, and kidney, where the receptor functions in the regulation of the bile acid metabolism through several mechanisms: 1) it decreases the bile acid synthesis via inhibition of cholesterol 7α-hydroxylase (CYP7A1) expression [7], 2) it increases the bile acid excretion rate via stimulation of the bile salt efflux pump (BSEP; ABCB11) expression [8], and 3) it stimulates bile acid glucuronidation through increasing UDP-glucuronosyltransferase 2B4 (UGT2B4) expression, leading to increased urinary excretion [9].
Interestingly, in addition to their function in regulating bile acid homeostasis via FXR, multiple animal studies have indicated a role for bile acids in the initiation and progression of atherosclerosis, an important cause of death in the Western world. Lichtman et al. have shown that adding cholic acid to a high fat diet leads to a more pronounced atherosclerotic lesion formation in LDL receptor deficient mice [10]. Cholic acid feeding of C57Bl/6 mice induces a reduction of plasma HDL levels [11], which has been associated with an increased risk for cardiovascular disease [12]. In contrast, fatty acid bile acid conjugates inhibit atherosclerosis formation in C57BL/6 mice [13], whilst hyodeoxycholic acid also efficiently suppresses atherosclerosis formation and plasma cholesterol levels in chow- and cholesterol-fed LDL receptor deficient mice [14]. In summary, the role of bile acids in atherogenesis remains unclear. Furthermore, the role of the bile acid-activated receptor FXR in atherosclerosis has not been clarified. Therefore, in the present study the effect of FXR activation by bile acids on atherogenesis was studied in mice that lack ApoE (ApoE deficient mice). These mice spontaneously develop atherosclerosis on a regular chow diet [15], which makes them a good model to study atherosclerotic plaque formation.

Here we show that taurocholic acid, a naturally occurring bile acid that is able to activate FXR, inhibits hepatic bile acid synthesis and cholesterol synthesis. Furthermore it appears to downregulate the LDL receptor, leading to a rise in serum VLDL, resulting in a marked increase in atherosclerotic lesion formation. We conclude that the administration of statins, which leads to upregulation of LDL receptors, can be beneficial in order to maintain the attractivity of FXR as a promising therapeutic target for treating or preventing gallstone disease.

6.2 Experimental Procedures

6.2.1 Animals

Ten-week-old male ApoE deficient (ApoE-/-) mice were obtained from Jackson Labs (Bar Harbor, Mass). The mice were kept on a regular dark/light cycle, and received water and a regular chow diet (chow) or a regular chow diet with 0.5% taurocholic acid (TCA) added for 2 or 8 weeks, respectively. After an overnight fasting period (~16h), mice were anesthetized and blood was collected for analysis of serum lipids. Subsequently, a whole-body perfusion was performed using phosphate-buffered saline (4°C, 100 mm Hg) for 10 min. After perfusion, the liver and heart plus aortic root were excised. The liver was frozen in liquid N2 and stored at –80°C. The heart plus aortic root were stored in 3.7% formalin. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

6.2.2 Lipid analyses

Fasting serum concentrations of free cholesterol, cholesterol esters, and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The total cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 μl serum of each mouse using a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics), taking the efficiency of recovery from the column into account. Hepatic lipids were extracted according to Bligh and Dyer [16]. After dissolving the lipids in 1% Triton X-100, contents of free cholesterol, cholesterol esters, and triglycerides in liver tissue were determined as described above and expressed as μg/mg of protein.

6.2.3 VLDL triglyceride production

ApoE-/- mice fed a regular chow or a regular chow with 0.5% taurocholic acid added for 2 weeks were injected intravenously after an overnight fast with 500 mg of Triton WR1339 (Sigma) per kg body weight as a 15 g/dl solution in 0.9% NaCl. Previous studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions [17]. Blood samples were taken at 0, 1, 2, 3, and 4 hours after Triton WR1339 injection. Serum triglycerides were analysed enzymatically as described above. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as mg/ml/h. At 4 hours after Triton WR1339 injection, the mice were anesthetized and an additional large blood sample was taken via the orbital plexus. From the large blood sample 1.25 ml pooled serum of 5 mice was brought to 1.063 g/ml with potassium bromide in a volume of 4 ml, transferred to SW41 centrifuge tubes, and layered with 1.063 g/ml, 1.019 g/ml, and 1.006 g/ml salt solutions. After 22 hours at 40.000 rpm and 4°C, the VLDL fraction was carefully removed by pipetting off 1.2 ml. In this VLDL, triglycerides, phospholipids, and cholesterol were measured enzymatically. This composition of the VLDL is a mixture of VLDL that circulated before administration of Triton WR1339 and nascent VLDL produced during the 4-h period after Triton WR1339 administration. To obtain the composition of nascent VLDL, the contribution of circulating VLDL was determined and corrected for, as described previously [18].

6.2.4 Assessment of Lesion Formation in the Aortic Root

Serial sections of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion area was quantified in oil red O-stained cryostat sections using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, England). Mean lesion area (in μm^2) was calculated from 10 consecutive oil red O-stained sections, starting at the appearance of the tricuspid valves.

6.2.5 Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on livers of chow- or TCA-fed ApoE deficient mice was performed as described [19]. In short, total RNA was isolated according to Chomczynski and Sacchi [20] and reverse transcribed using RevertAid^{TM} reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec). Primers (Eurogentec; Table 1) were designed using Primer Express Software and validated for identical efficiencies (slope = -3.3 for a plot of Ct versus log ng cDNA). Hypoxanthine guanine phosphoribosyl transferase (HPRT), 18S ribosomal RNA (18SrRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of

the target gene from the average Ct of HPRT, 18SrRNA, GAPDH, β-actin, and 36B4 ($Ct_{housekeeping}$) and raising 2 to the power of this difference. The average Ct of five housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes.

6.2.6 Data analysis

The significance of differences in atherosclerotic lesion size or relative gene expression numbers in the liver of chow- or TCA-fed mice was calculated using a two-tailed unpaired Student's t-test. Probability values less than 0.05 were considered significant

Table 1
Primers for quantitative real-time PCR

Gene	Forward primer	Reverse primer
18SrRNA	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCA1	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG5	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT
ABCG8	CCGTCGTCAGATTTCCAATGA	GGCTTCCGACCCATGAATG
ACAT-1	TGTTTCTCTGGGCCATCCA	CTTCAGGGCATGAGCCATATG
ACAT-2	CAGAGGGCCAAGGTGGC	CAACCTGCCGTCAAGACATG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
BSEP	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAG
CYP7A1	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
GADPH	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HMGCR	TCTGGCAGTCAGTGGGAACTATT	CCTCGTCCTTCGATCCAATTT
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
LDLR	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC
LRP1	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA
SHP	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC
SR-BI	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG

6.3 Results

Earlier studies by Srivastava et al. have indicated that in C57BL mice feeding cholic acid decreases serum HDL levels, whilst inducing serum VLDL/LDL levels [11,21]. In the current study we determined whether FXR activation by taurocholic acid (TCA), the taurine conjugate of cholic acid, also resulted in changed serum lipid levels in the ApoE-/- mice, a well-established atherosclerosis mouse model. ApoE-/- mice were fed a regular chow diet or a regular chow diet with 0.5% TCA for 8 weeks and subsequently serum free cholesterol (FC) and cholesterol ester (CE) levels were measured. After 8 weeks on a regular chow diet, free cholesterol and cholesterol ester levels were significantly increased by 78% ($P<0.001$) and 84% ($P<0.001$), respectively, as compared to 0 weeks of diet feeding (Table 2). Strikingly, compared to the animals fed the regular chow diet alone for 8 weeks, the TCA-fed animals showed a remarkable 1.9-fold ($P<0.001$) and 3.5-fold ($P<0.001$) further increase in free cholesterol and cholesterol esters (Table 2).

Table 2
Primers for quantitative real-time PCR

Diet	Time (weeks)	Free cholesterol (mg/dl)	Cholesterol ester (mg/dl)
Chow	Baseline	147 ± 8.3	490 ± 46
	8	264 ± 27	900 ± 61
TCA	Baseline	156 ± 7	531 ± 34
	8	528 ± 50 ***	3406 ± 395 ***

Serum free cholesterol (FC) and cholesterol ester (CE) levels were measured in ApoE-/- mice before and after 8 weeks of 0.5% TCA (TCA) or regular chow (Chow) diet feeding. Data represent means±SEM of 8-10 mice. *** $P<0.001$ compared to chow diet at 8 weeks.

Multiple studies have indicated that a persistently high level of serum cholesterol ultimately leads to a series of responses that promote atherosclerosis (22,23). Since the level of cholesterol was increased more rapidly in TCA-fed ApoE deficient mice, the effect of TCA on plaque formation was studied in these mice. In agreement with an important role for a rise in serum lipids in atherogenesis, TCA feeding of ApoE deficient mice induced a significant 3.9-fold increase in atherosclerotic plaque development as compared to chow-fed mice ($P<0.001$; Fig.1). This suggests an important role for bile acid activation of FXR in the development of atherosclerosis in ApoE-/- mice.
The increase in plaque formation (3.9-fold) seems to be the direct result of the increase in serum total cholesterol level (3.4-fold). Importantly, the liver is an essential organ in the removal of excess cholesterol from the body by mediating metabolism to bile acids and excretion to the bile. To determine whether the liver plays a role in the TCA-mediated increase in VLDL, in a separate experiment ApoE-/- mice were fed TCA or chow for only 2 weeks to increase the serum cholesterol level, but to minimize the possibility of a secondary response to the increase in serum cholesterol.

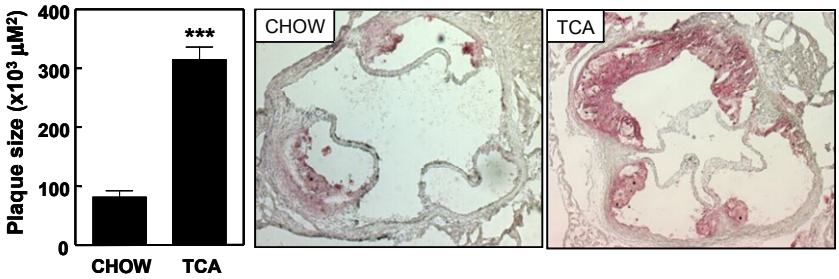

Fig.1. Taurocholic acid treatment induces atherosclerotic lesion development. Formation of atherosclerotic lesions was determined at the aortic root in ApoE-/- mice after 8 weeks of feeding a regular chow diet (chow) or a regular chow diet with 0.5% taurocholic acid (TCA) added. *Left*, quantification of the mean lesion area (means±SEM; n=8-10). *Right*, representative photomicrographs of oil red O-stained cross-sections of the aortic root at the level of the tricuspid valves. ***P<0.001 compared to the chow diet.

Table 3
Effect of 2-week TCA feeding on serum and hepatic lipid levels in ApoE-/- mice

		Free cholesterol	Cholesterol ester	Triglyceride
Serum (mg/dl)	Chow	164 ± 7.1	630 ± 43	160 ± 21
	TCA	242 ± 9.7 ***	1239 ± 27 ***	101 ± 8.7 *
Liver (µg/mg protein)	Chow	7.0 ± 1.0	15.4 ± 2.8	33.5 ± 3.3
	TCA	9.7 ± 0.9	12.0 ± 0.8	26.5 ± 1.2

Serum and hepatic lipid levels were measured in ApoE-/- mice after 2 weeks of 0.5% TCA (TCA) or regular chow (Chow) diet feeding. Data represent means±SEM of 5 mice. *P<0.05, ***P<0.001 compared to chow diet.

As anticipated, feeding mice a 0.5% TCA for 2 weeks significantly induced serum free cholesterol and cholesterol ester levels by 48% (P<0.001) and 97% (P<0.001), respectively, as compared to mice on a regular chow diet (Table 3). Analysis of the lipoprotein fractions separated by FPLC revealed that ApoE-/- mice on the chow diet primarily transport their serum cholesterol in VLDL particles (Fig.2a). The increase in the serum total cholesterol level induced by 2-week TCA feeding could be attributed to an increase in the VLDL cholesterol level (Fig.2a). However, TCA feeding significantly reduced the serum triglyceride level by 37% (P=0.032; Table 3). As a result, the circulating VLDL of TCA-fed mice contained a 23% higher cholesterol concentration and a 56% lower triglyceride concentration as compared to circulating VLDL from mice fed the regular chow diet (Fig.2b). In contrast, the secretion rate of nascent VLDL was 0.80±0.11 mg/ml/h for TCA-fed and 1.02±0.09 mg/ml/h for chow-fed animals indicating that the VLDL secretion rate was not significantly affected by TCA treatment. Moreover, no change in the secreted VLDL composition was seen after TCA treatment (Fig.2b), indicating that the changes in circulating VLDL composition were not the result of differences in the VLDL production.

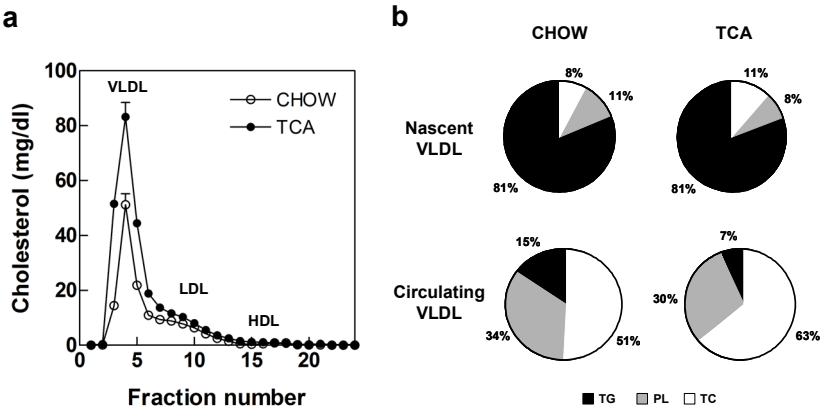

Fig.2. Taurocholic acid treatment induces serum VLDL levels and changes the circulating VLDL composition. (a) Blood samples of ApoE-/- mice were drawn after an overnight fast while feeding a regular chow diet (○) or a regular chow diet with 0.5% taurocholic acid (●) added for 2 weeks. Sera from 5 individual mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3-7 represent VLDL; fractions 8-15, LDL; and fractions 15-19 HDL, respectively. (b) VLDL was isolated by ultracentrifugation from pooled plasma of 5 ApoE-/- mice on a regular chow diet (chow) or a regular chow diet with 0.5% taurocholic acid added for 2 weeks after an overnight fast (circulating VLDL) or 4 h after Triton WR1339 administration (nascent VLDL), and lipid composition was determined (in w/w% of total lipids).

In addition to a change in the serum lipid levels, TCA feeding also affected the lipid levels in the livers of ApoE-/- mice. The hepatic free cholesterol level had a tendency to increase 38% (P=0.083), whilst the cholesterol ester and triglyceride levels were respectively 22% and 21% lowered by TCA as compared to regular chow controls (Table 3).

To evaluate the cause of the change in serum and hepatic lipid levels, gene expression analysis using real-time PCR was performed on genes involved in cholesterol and bile acid metabolism. The biological function of FXR in the liver is to regulate bile acid metabolism through repression of bile acid and induction of bile acid excretion (24,25). In accordance, the hepatic mRNA expression of the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1), was decreased by TCA to only 5.2±0.5% of the control value (P<0.001; Fig.3). In agreement with data provided by Goodwin et al. (26) that ligands for FXR repress CYP7A1 expression through a direct increase in the nuclear receptor small heterodimer partner (SHP), TCA treatment significantly stimulated SHP expression (P=0.007; Fig.3). As observed for SHP, TCA feeding also increased the expression of the bile salt efflux pump (BSEP; Fig.3), which is another established gene product regulated by bile acids and synthetic FXR ligands. However, the increase failed to reach statistical significance due to a large variation in the expression of BSEP in the TCA-fed mice.

The expression of HMG-CoA reductase (HMGCR), the rate-limiting enzyme in de novo cholesterol synthesis was decreased for 24% (P=0.013) by TCA (Fig.3). In addition, TCA similarly decreased the expression of the

cholesterol ester synthesis enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT) type 1 by 33% (P=0.005; Fig.3). In contrast, hepatic ACAT-2 expression, which under standard feeding conditions is ~3-fold lower than the ACAT-1 expression (relative expression 0.14±0.015 vs 0.43±0.028), was not changed by TCA (Fig.3), suggesting an ACAT type-specific inhibition by TCA.

Interestingly, data provided by Rudling have indicated that the mRNA levels for the LDL receptor and HMGCR are regulated in parallel in the liver in vivo during various metabolic perturbations as well as at normal physiologic conditions [27]. We therefore also investigated the effect of TCA on the LDL receptor and other receptors involved in hepatic cholesterol uptake. Strikingly, the LDL receptor expression was 50% decreased (P=0.002) upon TCA feeding, with minor changes in the SR-BI, and LRP1 expression (Fig.3).

In contrast to the strong decrease in the CYP7A1 and LDL receptor expression, no change in the expression of the ABC transporters ABCA1, ABCG5, or ABCG8 was observed as a result of TCA feeding (Fig.3). This suggests that TCA feeding 1) decreased the synthesis of bile acids from cholesterol by CYP7A1, 2) decreased the uptake of cholesterol via the LDL receptor, but that it 3) had no effect on the efflux of cholesterol to the serum or the bile.

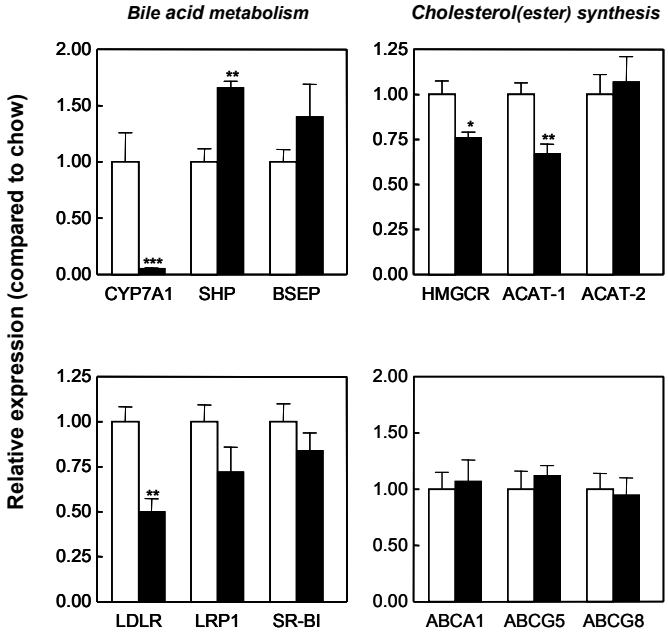

Fig.3. Taurocholic acid treatment affects the hepatic expression of genes involved bile acid metabolism, cholesterol(ester) synthesis, and cholesterol uptake, but not efflux. Gene expression analysis was performed by means of real-time quantitative PCR on the livers of ApoE-/- mice fed a regular chow diet (white bars) or a regular chow diet with 0.5% taurocholic acid added (black bars) for 2 weeks. Values are expressed as fold induction compared to the chow diet (means±SEM; n=5). *P<0.05, **P<0.01, ***P<0.001 compared to the chow diet.

6.4 Discussion

Recent studies have indicated that FXR agonists may have therapeutic use in treating cholestatic liver disease [5,28-30]. Importantly, bile acids are the natural occurring ligands for FXR and some contradictory data have been published on the role of bile acids, and thus FXR, in atherosclerosis, a major cause of death in the Western world. Therefore, in the present study the effect of FXR activation by bile acids on atherogenesis was studied in ApoE deficient mice. These mice spontaneously develop atherosclerotic plaques on a regular chow diet, and they are therefore considered to be an established animal model to study atherogenesis.
Strikingly, feeding ApoE-/- mice TCA, a natural FXR ligand, for 8 weeks resulted in a marked increase in atherosclerotic lesion development. Hakamata et al. have shown that VLDL/LDL from ApoE-/- mice is able to transform macrophages to foam cells [31]. The effect of TCA on lesion development in ApoE-/- mice, therefore, seems to be a direct result of the increased circulating VLDL cholesterol level observed after TCA treatment. The secretion rate and composition of nascent VLDL was not changed by two weeks TCA feeding, which indicates that the circulating VLDL level was induced through another mechanism. Importantly, without changing the VLDL secretion rate, TCA feeding did decrease the serum triglyceride concentration in ApoE-/- mice resulting in a changed circulating VLDL composition (relatively more cholesterol and less triglycerides). This is in agreement with data provided by Kast et al. [32], which showed that FXR activation induced hepatic expression of ApoC-II, an activator of lipoprotein lipase (LPL) [33], and data from Claudel et al. [34], which showed that FXR agonists suppress the hepatic expression of ApoC-III, an inhibitor of LPL [35], leading to a subsequent decrease in serum triglycerides in vivo. Importantly, in the current study also a significant increase in hepatic ApoC-II expression was observed (data not shown). These combined findings indicate that in ApoE-/- mice, TCA treatment stimulated LPL activity, resulting in cholesterol-rich/triglyceride poor circulating VLDL particles. However, albeit an unchanged VLDL secretion rate and an increase in LPL activity, TCA treatment still led to an increase in the serum VLDL level. Since the uptake VLDL is also an important factor in the control of the serum VLDL level, the effect of TCA on hepatic VLDL catabolism was studied. It is generally accepted that cholesterol ester enriched VLDL clearance by the liver is mediated by the LDL receptor, but also by an LDL receptor-independent recognition site on parenchymal cells, possibly LRP1 [36,37]. However, since LRP1 binds ApoE, which is absent in ApoE-/- mice, an essential role for LRP1 in VLDL clearance is unlikely. Murayama et al. showed that overexpression of the LDL receptor in ApoE-/- mice resulted in a marked lowering of plasma ApoB-containing lipoproteins and a drastic suppression of early atherogenesis pointing to the LDL receptor as an important mediator of liver uptake of cholesterol ester-rich VLDL [38]. Strikingly, TCA feeding resulted in a 50% decrease in LDL receptor expression in ApoE-/- mice, which might explain the increase in VLDL cholesterol levels and the subsequent increase in atherogenesis in these mice. The co-ordinate decreases in the expression of the LDL receptor and HMGCR by TCA are in agreement with the observations of Rudling that the

mRNA levels for the LDL receptor and HMGCR are regulated in parallel in the liver in vivo [27]. Since activators of the nuclear receptor LXR have been shown to induce the hepatic expression of ABCG5/G8 [39] and ABCA1 [40], the absence of an effect of TCA on ABCG5/G8 and ABCA1 indicates that TCA treatment did not affect the activation status of LXR. In contrast, the nuclear receptor FXR was activated by TCA, as expected, as can be concluded from the observed rise in SHP and BSEP expression and the drastic decrease in CYP7A1 expression.

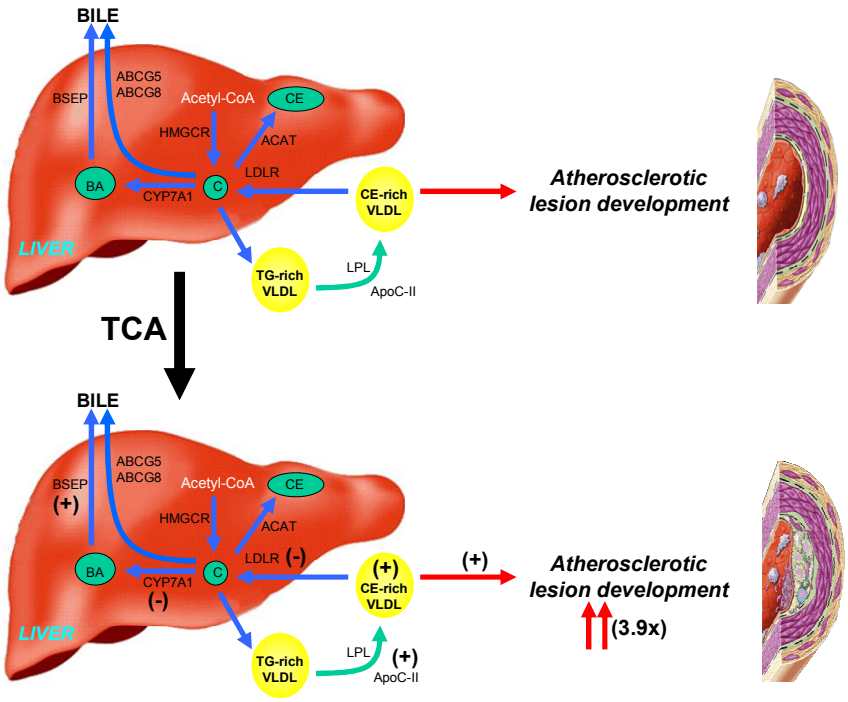

Fig.4. Proposed mechanism of the pro-atherogenic effect of taurocholic acid (TCA). TCA, by activating FXR, stimulates hepatic BSEP expression and inhibits CYPA1 expression, resulting in a subsequent decrease in LDL receptor (LDLR) expression. The combination of hepatic LDL receptor inhibition and increased serum LPL activity, due to increased ApoC-II levels, leads to an accumulation of cholesterol ester-rich VLDL in the circulation, which consequently results in a 3.9-fold increase in atherosclerotic lesion formation.

In conclusion, we have provided data that taurocholic acid, a naturally occurring bile acid that is able to activate FXR, inhibits hepatic bile acid synthesis and cholesterol synthesis and downregulates the LDL receptor. This latter phenomenon leads to a rise in serum VLDL, resulting in a marked increase in atherosclerotic lesion formation (Fig.4). In the light that FXR agonists are promising therapeutic agents for treating or preventing gallstone disease, the observed increase in atherosclerotic lesion formation can be considered as contraindication. However, the observed downregulation of LDL receptors might be reversed by statin treatment, a

frequently used drug to treat or prevent cardiovascular disease in humans. Statins inhibit HMG-CoA reductase, leading to cholesterol depletion in the liver and subsequent upregulation of LDL receptors. Upregulation of LDL receptors in the liver induces LDL-cholesterol removal from blood and consequently leads to a lowering in the risk for atherosclerosis. Therefore we suggest that the treatment of patients at risk for cholesterol gallstone disease and acute pancreatitis with FXR agonists have to be combined with statin treatment in order to prevent FXR's pro-atherogenic action.

6.5 Acknowledgements

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7

Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells

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Hepatic cholesterol(ester) uptake from serum coupled to intracellular processing and biliary excretion are important features in the removal of excess cholesterol from the body. ATP-binding cassette (ABC) transporters play an important role in hepatic cholesterol transport. The liver consists of different cell types and ABC transporters may exert different physiological functions dependent on the individual cell type. Therefore, in the current study, we compared using real-time PCR the mRNA expression of ABC transporters and genes involved in the regulation of cholesterol metabolism in liver parenchymal, endothelial, and Kupffer cells.

It appears that liver parenchymal cells contain high expression levels compared to endothelial and Kupffer cells of SR-BI (~3 fold), PPARα and PPARγ (8-20 fold), CYP7A1 (>100 fold) and ABCG5/G8 (~5 fold). Liver endothelial cells show a high expression of CYP27, LXRβ, PPARδ and ABCG1, suggesting a novel specific role for these genes in endothelial cells. In Kupffer cells, the expression level of LXRα, ABCA1 and in particular ABCG1 is high leading to an ABCG1 mRNA expression level which is 70 fold higher than for parenchymal cells. It can be calculated that 51% of the total liver ABCG1 expression resides in Kupffer cells and 24% in endothelial cells, suggesting an intrahepatic specific role for ABCG1 in Kupffer and endothelial cells. Due to a specific stimulation of ABCG1 in parenchymal cells by a high cholesterol diet, the contribution of praenchymal cells to the total liver increased from 25 to 60%.

Our data indicate that for studies on the role of ABC transporters and their regulation in liver, their cellular localization should be taken into account, allowing proper interpretation of metabolic changes, which are directly related to their (intra)cellular expression level.

7.1 Introduction

Transport of cholesterol through the body is an important process in the maintenance of total body cholesterol homeostasis. One specific component is the transport of excessive cholesterol from the periphery to the liver by the high-density lipoprotein (HDL). This process is called reverse cholesterol transport (RCT) and consists of three different stages: the efflux of peripheral cellular cholesterol to HDL, the transport of cholesterol esters through the blood to the liver, and the uptake of cholesterol esters by the liver [1]. Cholesterol esters are taken up into the liver through selective uptake by the HDL receptor, scavenger receptor class BI (SR-BI) [2], where they are primarily (~50%) catabolised to bile acids, through conversion by cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27) [3] for biliary excretion via the bile salt export protein (BSEP) [4]. A second major catabolic route (~40%) is the direct efflux of cholesterol from the liver into the bile via the ATP-binding cassette half transporters ABCG5 and ABCG8, which together function as a biliary sterol efflux regulator [5]. Additionally, cholesterol also effluxes from the liver to produce very low-density lipoproteins (VLDL), which are converted to remnants that function as precursors for low-density lipoproteins. Native HDL might also be formed by the ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux [6]. Recently, another member of the ABC transporter family, ABCG1, has been proposed to play a role in the intracellular trafficking and efflux of cholesterol [7]. However, the exact role of ABCG1 in the liver, especially in relation to ABCG5 and ABCG8, remains to be clarified.

The identification of SR-BI and novel members of the ATP-binding cassette transporter family, including ABCA1, ABCG1, ABCG5, and ABCG8, has allowed the molecular characterization of the individual transporters responsible for the intracellular trafficking and excretion of cholesterol (derivatives). In the liver, it has been shown that SR-BI is primarily responsible for the selective uptake of cholesterol esters from HDL [8], while ABCG5/G8 and ABCG1, and ABCA1, are proposed mediators of efflux to the bile and to HDL, respectively [6,9]. However, the liver is a complex tissue and contains, in addition to the parenchymal cells, which are localized around the bile canaliculi, endothelial cells, and tissue macrophages (Kupffer cells). To assess the individual function of the ABC transporters and their regulation by nuclear hormone receptors it is therefore essential to establish their cellular localization in the liver.

Here we report that key mediators in liver cholesterol homeostasis, in particular PPARα, PPARγ and ABCG1, are differentially expressed in specific cell types of the rat liver. Our data stress that it is necessary to focus on the regulation of genes involved in cholesterol homeostasis in the different cell types of the liver, to get molecular insight in their mechanism of regulation and the consequences for liver cholesterol transport.

7.2 Experimental Procedures

7.2.1 Animals

In the study, three male Wistar rats (200-250 g) were fed a chow diet containing 4.3% (w/w) fat and no cholesterol, and three rats were fed a high-cholesterol diet containing 2% (w/w) cholesterol, 5% olive oil (w/w), and 0.5% (w/w) cholic acid for two weeks. Rats were anaesthetized and the vena porta was cannulated. Subsequently, the liver was perfused for 10 min with oxygenated Hanks' buffer pH 7.4, containing HEPES (1.6 g/l). The perfusion was continued for 10 minutes with Hanks'/HEPES buffer containing 0.05% (w/v) collagenase (type IV, Sigma) and 1 mM $CaCl_2$. Parenchymal cells were isolated after mincing the liver in Hanks' buffer containing 0.3% BSA, filtering through nylon gauze and centrifugation for three times 10 min at 50 g. The pellets consisted of pure (>99%) parenchymal cells (PC) as judged by light microscopy. The supernatants were centrifuged for 10 min at 500 g in order to harvest the non-parenchymal cells. By means of centrifugal elutritation the endothelial cells (EC) and Kupffer cells (KC) were separated [10]. The purity of each cell fraction (>95% for both) was checked by light microscopy, after staining for peroxidase activity with 3,3-diaminobenzidin (Sigma).

7.2.2 Analysis of gene expression by real-time quantitative PCR

Total RNA was isolated from rat liver parenchymal, endothelial, and Kupffer cells using TriZol reagent (Life Technologies) according to the manufacturer's instructions. Purified RNA was DNAse treated (DNAse I, 10 U/μg total RNA) and reverse transcribed (RevertAid M-MuLV Reverse Transcriptase) according to the protocols supplied by the manufacturer.
Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Table 1) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and validated for identical efficiencies (slope = -3.3 for a plot of Ct versus log ng cDNA). In 96-wells optical plates, 12.5 μl SYBR Green master mix was added to 12.5 μl cDNA (corresponding to 50 ng of total RNA input) and 300 nM of forward and reverse primers in water. Plates were heated for 2 min at 50°C and 10 min at 95°C. Subsequently 40 PCR cycles consisting of 15 sec at 95°C and 60 sec at 60°C were applied. At the end of the run, samples were heated to 95°C with a ramp time of 20 min to construct dissociation curves to check that single PCR products were obtained. The absence of genomic DNA contamination in the RNA preparations was confirmed by using total RNA samples that had not been subjected to reverse transcription. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the standard housekeeping gene. Ratios of target gene and HPRT expression levels (relative gene expression numbers) were calculated by subtracting the threshold cycle number (Ct) of the target gene from Ct of HPRT and raising 2 to the power of this difference. Ct values are

defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed treshold. Target gene mRNA expressions are thus expressed relative to HPRT expression.

7.2.3 Data analysis

The significance of differences in relative gene expression numbers between the different liver cell types, from three different cell isolations, measured by real-time quantitative PCR was calculated using a two-tailed Student's t-test on the differences in Ct ($Ct_{HPRT} - Ct_{target\ gene}$). Probability values less than 0.05 were considered significant.

Table 1
Primers for quantitative real-time PCR analysis

Gene	GenBank accession	Forward primer	Reverse primer	Amplicon size
ABCA1	**	ATCTCATAGTATGGAAGAATGTGAAGCT	CGTACAACTATTGTATAACCATCTCCAAA	132
ABCG1	NM053502	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG	85
ABCG5	NM053754	CGCAGGAACCGCATTGTAA	TGTCGAAGTGGTGGAAGAGCT	67
ABCG8	NM130414	GATGCTGGCTATCATAGGGAGC	TCTCTGCCTGTGATAACGTCGA	69
CYP27	M38566	GTGTCCCGGGATCCCAGTGT	CTTCCTCAGCCATCGGTGA	66
CYP7A1	NM012942	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	75
HPRT	X62085	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
LXRα	NM031627	TCAGCATCTTCTCTGCAGACCGG	TCATTAGCATCCGTGGGAACA	144
LXRβ	NM031626	AAGCTGGTGAGCCTGCGC	CGGCAGCTTCTTGTCCTG	81
PPARα	NM013196	TGAACAAAGACGGGATG	TCAAACTTGGGTTCCATGAT	106
PPARγ	NM013124	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT	131
PPARδ	NM012141	GAGGGGTGCAAGGGCTTCTT	CACTTGTTGCGGTTCTTCTTCTG	97
SR-BI	U76205	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA	65

** Primer sequences based on an alignment of human (NM005502) and murine (XM109397) ABCA1

7.3 Results

The last step in reverse cholesterol transport is the hepatic uptake and cellular processing of cholesterol esters from HDL by SR-BI [1]. The mRNA expression of SR-BI in the different cell types of the liver was investigated with quantitative real-time PCR to determine which cell type is mainly expressing SR-BI. A significantly higher SR-BI expression was observed in parenchymal cells compared to endothelial and Kupffer cells (Fig.1).
In the liver, amongst others, cholesterol is used for bile acid synthesis. Therefore, the mRNA expression patterns of two key enzymes in bile acid synthesis, CYP7A1 and CYP27, in the different hepatic cell types were examined. A relatively high level of CYP7A1 expression was observed in parenchymal cells, which was over 200 fold higher than the expression levels found in endothelial and Kupffer cells (Fig.1). Accordingly, CYP27 expression (Fig.1) was observed in parenchymal cells, but also in endothelial cells.

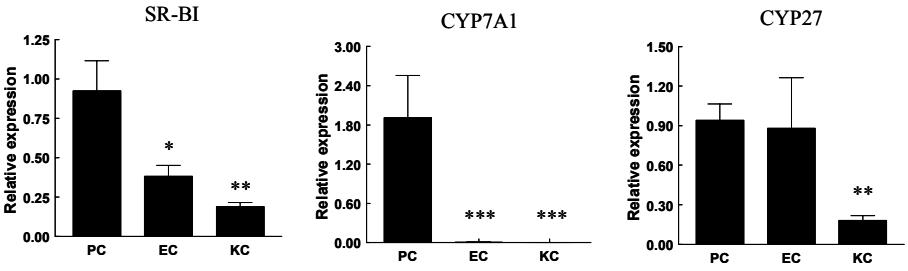


Fig.1. Relative SR-BI, CYP7A1, and CYP27 mRNA expression levels as determined by real-time quantitative PCR in rat liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells on a chow diet. Values are expressed relative to HPRT expression (means+SEM). *P<0.05, **P<0.01 and ***P<0.001 compared to PC expression (t-test, as described under "Experimental procedures").

Because a second route of cholesterol disposal from the liver is through direct excretion of cholesterol into the bile via the half transporters ABCG5 and ABCG8, we investigated whether the expression of ABCG5/8 is also higher in parenchymal cells than in non-parenchymal cells. Figure 2 clearly indicates that ABCG5 and ABCG8 expression was indeed 5- to 10-fold higher in parenchymal cells compared to endothelial and Kupffer cells.
In the liver, ABCA1 was recently suggested to be involved in the efflux of cholesterol for production of HDL [6]. Although no conclusive evidence has been shown, in the liver ABCG1, like ABCG5/G8 is proposed to play a role in biliary efflux [11]. In macrophages, ABCA1 and ABCG1 expression is induced in response to cholesterol loading and both proteins are potentially involved in cholesterol efflux to ApoA-I [12]. Since both ABCA1 and ABCG1 are implicated in the same physiological functions, we determined whether both genes also have a comparable expression distribution profile over the different cell types of the liver. Contrarily to the assumption, ABCA1 was mainly expressed in parenchymal and Kupffer cells (Fig.2), while ABCG1 was 76-fold and 27-fold higher expressed in Kupffer and endothelial cells

than in parenchymal cells, respectively (Fig.2). In contrast to ABCA1, ABCG1 is thus mainly expressed in non-parenchymal cells, which suggests a limited role of ABCG1 in the excretion of cholesterol directly into the bile under the standard feeding conditions. In addition, ABCG1 expression was analysed in the different hepatic cells isolated from rats on a high cholesterol diet. Interestingly, hepatic parenchymal cell ABCG1 expression increased ~4-fold in response to a high cholesterol diet, whilst no significant effect on endothelial and Kupffer cell ABCG1 expression was observed (Fig.3). Although ABCG1 levels were significantly increased in parenchymal cells in response to a high cholesterol diet, ABCG1 expression levels were still respectively 10- and 12-folds higher in endothelial and Kupffer cells as compared to parenchymal cells.

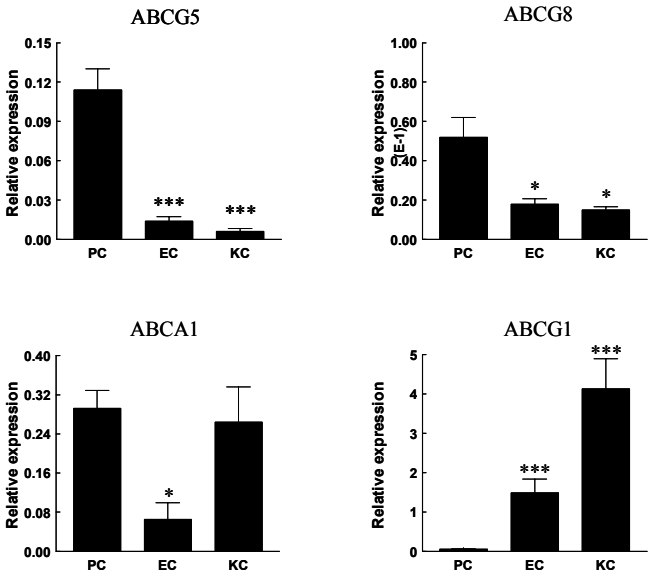
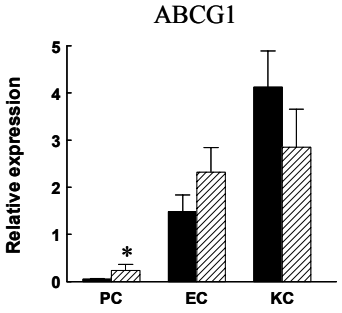


Fig.2. Relative ABCG5, ABCG8, ABCA1, and ABCG1 mRNA expression levels as determined by real-time quantitative PCR in rat liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells on a chow diet. Values are expressed relative to HPRT expression (means+SEM). *P<0.05, and ***P<0.001 compared to PC expression (t-test, as described under "Experimental Procedures").

Fig.3. The effect of a high cholesterol diet on the relative ABCG1 mRNA expression levels as determined by real-time quantitative PCR in rat liver parenchymal (PC), endothelial (EC), and Kupffer (KC). Values from rats on a chow (black bars) and on a high cholesterol diet (hached bars) are expressed relative to HPRT expression (means+SEM.). *P<0.05 compared to the ABCG1 expression on a chow diet (t-test, as described under "Experimental Procedures).



Analysis of LXR expression in the different cell types was performed to investigate a potential relation with expression patterns of the ABC transporters. LXRα had a comparable distribution pattern as found for ABCA1, with a relatively high expression in parenchymal and Kupffer cells as compared to endothelial cells (fig.4). A significantly higher expression of LXRβ was found in endothelial liver cells compared to parenchymal and Kupffer cells, respectively (Fig.4), which suggests that LXRβ may be a more important mediator in endothelial cells.
It has been shown that PPARγ activators are able to regulate LXR expression, and thereby indirectly influence ABCA1 mRNA levels [13], and that ligands for PPARδ directly regulate the expression of ABCA1 via an unknown mechanism [14]. Therefore, we also investigated the PPAR gene expression in the different cell types. Figure 4 clearly indicates that PPARα expression is found mostly in parenchymal cells, with an 82 and 23 fold higher expression in these cells than in endothelial cells and Kupffer cells, respectively. The PPARγ distribution pattern is comparable to that found for PPARα (Fig.4), suggesting a major function of these genes in parenchymal cells. PPARδ mRNA levels were almost equal in the different cells, although endothelial cell PPARδ expression was somewhat higher as compared to parenchymal and Kupffer cells (Fig.4). These data indicate that within the various liver cell types PPARδ will have a more general function.

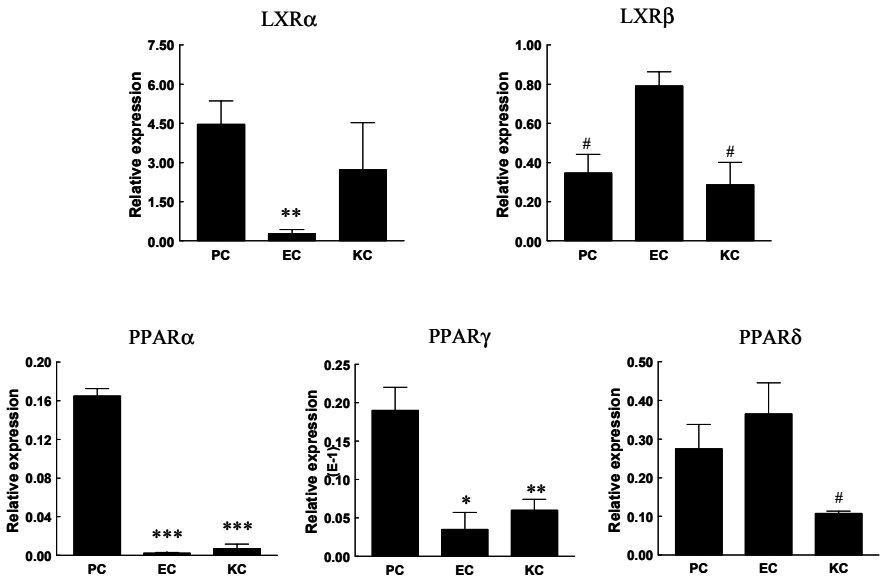


Fig.4. Relative expression levels of nuclear receptors LXR and PPAR as determined by real-time quantitative PCR in rat liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells on a chow diet. Values are expressed relative to HPRT expression (means+SEM). **P<0.01 compared to PC expression; #P<0.05 compared to EC expression (t-test, as described under "Experimental Procedures").

7.4 Discussion

Hepatic cholesterol uptake from serum coupled to intracellular processing and bile excretion are important features in the last step of reverse cholesterol transport. It has been shown that disruption of cholesterol homeostasis plays an essential role in the pathology of many diseases such as cholestasis [15] and atherosclerosis [16]. In the liver, SR-BI plays a crucial role in the selective uptake of cholesterol esters from HDL [2]. Additionally, studies on ATP-binding cassette transporters suggested that hepatic ABCA1 is involved in HDL production [6], while ABCG5/G8 and ABCG1 were indicated to mediate biliary efflux of cholesterol from the liver [7,9]. Repa et al. showed that treatment of mice with synthetic ligands of LXR markedly increased liver ABCG5/G8 expression [17]. In vitro observations by Malerod et al. also indicate that LXR is able to regulate hepatic SR-BI expression through a direct interaction with a newly discovered LXR/RXR response element in the SR-BI promoter [18]. It is however still unclear how hepatic ABCA1 and ABCG1 expression is regulated and what the precise consequence of their regulation is on hepatic cholesterol levels and transport. The liver consists of several different cell types, including parenchymal, endothelial and Kupffer cells. It is therefore important to study the expression levels of SR-BI and the ATP-binding cassette transporters in the individual hepatic cell types, to get a more detailed view on their specific functions and regulatory mechanisms in the liver. Earlier studies performed by Pieters et al. showed that uptake of HDL cholesterol esters into liver parenchymal cells is efficiently coupled to a rapid synthesis of bile acids [19]. Accordingly, Fluiter et al. observed that the receptor responsible for the selective uptake of cholesterol esters into the liver, SR-BI, has a relatively high expression in parenchymal cells as compared to endothelial and Kupffer cells [20]. These combined observations stressed an important role for parenchymal cells in the last step of the reverse cholesterol transport process.
In the current study, we investigated, using real-time quantitative PCR, the mRNA expression of genes involved in hepatic cholesterol transport and metabolism in liver parenchymal, endothelial and Kupffer cells. Real-time PCR is a highly sensitive method to quantify mRNA expression levels in vitro and in vivo. mRNA levels have been shown to strongly correlate with protein expression levels, indicating that a substantial portion of changes in protein levels are a consequence of altered mRNA levels, rather than post-transcriptional modifications [21]. Importantly, Wellington et al. showed a high concordance of ABCA1 mRNA and protein levels in the liver [22]. Additionally, we observed in the present study the highest SR-BI mRNA expression in the parenchymal cells as compared to endothelial and Kupffer cells, which is in accordance with the high Western blot protein expression data for SR-BI as reported by Fluiter et al. [20]. We thus suggest that our quantitative mRNA data for the various cell types are indicative for the activity of the particular genes of interest and their metabolic function. For SR-BI, the mRNA and protein expression data in parenchymal cells are consistent with our data that the parenchymal cholesterol ester uptake is dramatically reduced in SR-BI deficient mice as compared to non-transgenic littermates (unpublished data). Cholesterol 7α-hydroxylase

(CYP7A1) and sterol 27-hydroxylase (CYP27) are the key enzymes in classical and alternative bile acid synthesis pathways [23]. The relatively high expression of these two bile acid synthesis enzymes observed in parenchymal cells is in agreement with the data provided by Pieters et al., as it was shown that uptake of HDL cholesterol esters by the parenchymal cells is efficiently coupled to bile acid synthesis [19]. Interestingly, an equally high expression of CYP27 compared to parenchymal cells was observed in liver endothelial cells. In agreement, Reiss et al. have detected the same high levels of CYP27 activity in cultured vascular endothelial cells [24]. The observed difference between the CYP7A1 and CYP27 expression patterns suggests differential functions for these bile acid synthesizing enzymes. Interestingly, Babiker et al. suggested that CYP27 mediated elimination of cholesterol from macrophages and endothelial cells may be an alternative or complement to HDL-mediated reverse cholesterol transport under low-HDL conditions. They observed a high secretion of 3β-hydroxy-cholestenoic acid, an intermediate of the CYP27 mediated alternative bile acid formation pathway, from endothelial cells and macrophages to albumin containing medium [25]. Efflux of hepatic cholesterol to the serum compartment by ABCA1 for the production of native HDL is a second important route in maintaining cholesterol homeostasis [6]. An equally high relative expression of ABCA1 was observed in parenchymal and Kupffer cells, while a ~4 fold lower expression of ABCA1 was seen in liver endothelial cells. In macrophages, ABCA1 is a critical regulator of the specific ATP-dependent cholesterol efflux to ApoA-I, leading to an inhibition of foam cell formation [26]. Kupffer cells are liver macrophages, which play an important role in the uptake of (modified) lipoproteins [19,27]. The high uptake and an accordingly high efflux of cholesterol from Kupffer cells might be the metabolic mechanism for the relatively high ABCA1 expression level observed in these cells. Haghpassand et al. [28], and Van Eck et al. [29], have shown that monocyte/macrophage ABCA1 only minimally contributes to the overall plasma HDL levels. The observed high expression levels of ABCA1 in parenchymal cells, combined with the observation that ABCA1 functions on the basolateral surface of hepatocytes [30], suggest that the liver *does* contribute to HDL production by the efflux of cholesterol from parenchymal cells via ABCA1. A third catabolic route for hepatic cholesterol is the direct excretion into the bile, which accounts for ~40% of the total liver catabolism. Recently, two members of the ATP-binding cassette transporters, ABCG5 and ABCG8, have been shown to coordinately participate in the hepatic sterol secretion into bile [31]. Mutations in either ABCG5 or ABCG8 are sufficient to cause sitosterolemia, a disorder that is characterized by elevated plasma levels of sterols [32]. Because parenchymal cells are responsible for bile acid formation, a relatively high expression of the biliary transporters such as ABCG5/G8 in these cells compared to endothelial and Kupffer cells is consistent with their suggested function. The expression pattern of ABCG5 resembled ABCG8, which is in agreement with the statements that these transporters operate as heterodimers to regulate biliary cholesterol efflux [9,33].

Interestingly, a novel member of the ABC transporter family, ABCG1, has also been proposed to have a function in the intracellular trafficking and

biliary efflux of cholesterol in the liver [11]. Contrarily to the expectations, ABCG1 expression was mainly observed in non-parenchymal cells of the rat liver. A 76 fold and 27 fold higher ABCG1 expression was observed in Kupffer and endothelial cells than in parenchymal cell under standard feeding conditions. Although Kupffer and endothelial cells only contribute for 2.5% and 3.3% to the total liver protein, they do contain 51% and 24% of total liver ABCG1 expression, respectively. Such a high specific ABCG1 expression in Kupffer cells was not expected, although ABCG1 has also been proposed to play a role in the cholesterol efflux from peripheral macrophages [7]. Importantly, after putting rats for two weeks on a high cholesterol diet, ABCG1 expression increased 4 fold in parenchymal cells, while no significant change in ABCG1 expression in endothelial and Kupffer cells was observed. The absence of a similar induction of ABCG1 in endothelial and Kupffer cells in response to diet feeding may well be caused by an already maximal activity of ABCG1 in these cells even on a chow diet. Also, the differences in expression and intracellular localization of direct activators (e.g. LXRα) and repressors (e.g. ZNF202) of ABCG1 might contribute to the difference in its transcriptional regulation between different cell types as earlier mentioned by Schmitz et al. [11]. Although endothelial and Kupffer cell ABCG1 expressions were still 10 and 12 fold higher than that in parenchymal cells, the relative contribution of ABCG1 in the parenchymal cells to total liver increased from 25 to 60%. This suggests that under high cholesterol conditions ABCG1 might indeed contribute to the transport of cholesterol in the parenchymal cells.

Recent pharmacological interest is focussed upon the regulation of SR-BI and the ABC transporters by newly discovered nuclear hormone receptors, the liver X receptors (LXRs), and peroxisome proliferator-activated receptors (PPARs), respectively [34,35]. Therefore, we also studied their cellular localization in the different cell types of the liver. Two different types of the liver X receptor have been discovered so far, LXRα and LXRβ, respectively. A relatively high expression of LXRα was observed in parenchymal cells. In the liver, LXRα plays an essential role in the regulation of CYP7A1 and thus the formation of bile acids [36]. CYP7A1 was found almost exclusively in the parenchymal cells, which coincides with the high expression of LXRα in these cells. Contrarily, equally high LXRα expression levels were observed in Kupffer cells, where CYP7A1 expression was almost absent. In macrophages however, LXRα plays a crucial role in the regulation of lipid efflux via ABCA1 [37]. Kupffer cells also contain high expression levels of ABCA1, which is consistent with a role for LXRα in the regulation of ABCA1 in these cells. Between the different cell types of the liver, LXRβ was ubiquitously expressed, with a somewhat higher expression in endothelial cells versus parenchymal and Kupffer cells. The expression distribution of LXRβ thus does not resemble the one found for LXRα, which suggests that, in the liver, LXRβ may have a different function than LXRα.

In the liver, PPARα is suggested to play a role in the formation of bile acids, because it is able to bind a PPAR response element (PPRE) in the sterol 12α-hydroxylase promoter, leading to increased levels of cholic acid [38]. This might explain the extremely high expression of PPARα found in

parenchymal cells as compared to endothelial and Kupffer cells. The high expression of PPARγ observed in parenchymal cells suggests that PPARγ, like PPARα, also has a major function in these cells. Contrarily, PPARδ is ubiquitously expressed between the different cell types of the liver, suggesting a more general function for PPARδ in all cell types of the liver.
In conclusion, we have provided data that several ABC transporters and nuclear hormone receptors involved in liver cholesterol homeostasis are differentially expressed in the specific cell types of the liver. In order to study their intracellular transport function inside the liver it appears to be essential to take into account their cellular localization, as especially evident for ABCG1. This appears specifically true for studies on the regulation of the transporters by nuclear receptors, as metabolic changes are directly coupled to the specific (intra)cellular expression level of the cholesterol transporters.

7.5 Acknowledgements

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8

Diet induced regulation of genes involved in cholesterol metabolism in rat liver parenchymal and Kupffer cells

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Background/Aims: Feeding rodents atherogenic diets enriched in cholesterol or cholic acid changes hepatic cholesterol metabolism. In the present study, the effect of an atherogenic diet enriched in cholesterol and cholic acid on cellular hepatic cholesterol metabolism was studied.
Methods: Gene and protein expression analysis was performed on parenchymal, endothelial, and Kupffer cells isolated from rats fed a chow or atherogenic diet using quantitative real-time PCR and immunoblotting, respectively.
Results: The atherogenic diet raised the serum cholesterol concentration 11-fold, mostly in the VLDL fraction, and led to heavy lipid loading of rat liver parenchymal and Kupffer cells. Only moderate changes in the expression of genes involved in cholesterol metabolism were observed in parenchymal cells on the diet, while PPARδ expression was 6.8-fold decreased. Kupffer cells, however, showed a highly adaptive response with a 2- to 9-fold induction of SR-BI, ABCA1, and ABCG5/G8, and an 82-fold induction in CYP7A1 mRNA expression, respectively.
Conclusion: Heavy lipid loading of parenchymal cells leads to moderate gene expression changes, while Kupffer cells respond highly adaptive by stimulating the expression of genes involved in cholesterol metabolism and transport.

8.1 Introduction

The liver is an essential organ in the removal of excess cholesterol from the blood circulation for excretion into the bile [1,2]. The liver regulates its intrahepatic cholesterol homeostasis, by maintaining an appropriate balance between the regulatory free cholesterol and the more inert cholesterol ester pool. The liver consists of different cell types: parenchymal, endothelial, and Kupffer cells that may exert different functions in the hepatic flux of cholesterol. Parenchymal liver cells are located between bile canaliculi and blood sinuses, and mediate chylomicron- and very low-density lipoprotein (VLDL)-remnant and high-density lipoprotein (HDL) cholesterol ester removal from the blood circulation [3,4]. Additionally, parenchymal liver cells perform the biliary efflux of cholesterol and its conversion products, bile acids [5]. Hepatic endothelial cells line the blood sinuses, where they function in the removal of modified lipoproteins from the blood circulation and mediate their natural barrier function. Kupffer cells are tissue macrophages located within the blood sinuses of the liver. They mainly function in the removal of bacteria and in the clearance of modified lipoproteins from the circulation [6,7].
Recently, we have shown that the relative expression of genes involved in hepatic cholesterol metabolism is markedly different for rat parenchymal, endothelial and Kupffer cells under standard feeding conditions [8]. In the current study, the effect of diet-induced lipid loading on the expression of genes involved in cholesterol uptake, metabolism, and efflux were studied in rat liver parenchymal, endothelial, and Kupffer cells to gain insight in the cell specific regulation of cholesterol metabolism.
Feeding an atherogenic diet enriched in cholesterol and cholic acid disrupts liver cholesterol homeostasis leading to heavy lipid loading of rat liver parenchymal, Kupffer, and to a lesser extent of endothelial cells. The regulatory response of genes responsible for the uptake of LDL (LDL-receptor) and HDL cholesterol esters (scavenger receptor B type 1; SR-BI) and ATP-binding cassette transporter (ABC) genes involved in cholesterol trafficking (ABCA1, ABCG5, and ABCG8) were analysed on mRNA level. In addition, the genes important for the conversion of cholesterol to bile acids (cholesterol 7α-hydroxylase; CYP7A1) and the biliary secretion of bile acids (bile salt export pump; BSEP) were studied. In order to correlate the observed changes with regulation by nuclear hormone receptors, we also determined the effect of the atherogenic diet enriched in cholesterol and cholic acid on the gene expression of the nuclear receptors liver X receptor (LXR), farnesoid X receptor (FXR), and peroxisome proliferator-activated receptors (PPAR) α, γ, and δ.

8.2 Experimental Procedures

8.2.1 Animals

Male Wistar rats were obtained from Charles River Laboratories. Rats were fed a regular chow diet, containing 4.3% (w/w) fat and no cholesterol (Special Diet Services, Witham, UK), or a cholesterol/cholic acid enriched diet, containing 2% (w/w) cholesterol, 5% (w/w) olive oil, and 0.5% (w/w) cholic acid (Hope Farms, Woerden, The Netherlands) for two weeks. Subsequently, hepatic parenchymal (PC), endothelial (EC), and Kupffer cells (KC) were isolated essentially according to the method of Nagelkerke et al. [9]. The purity and identification of the cells was analysed after 3,3'-diaminobenzediene peroxidase staining and phase contrast microscopy. KC's are peroxidase postitive and stain brown under these conditions. Diet feeding had no significant effect on the viability or purity of the different cell fractions. The parenchymal fractions consisted for >99% of parenchymal cells, the endothelial fractions contained ~95% endothelial cells and ~5% Kupffer cells, whilst the Kupffer cell fractions contained ~90% Kupffer cells and 10% endothelial cells under both standard and high cholesterol/cholic acid feeding conditions. Animal experiments were performed at the Gorlaeus laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

8.2.2 Serum lipid analyses

Serum concentrations of total and free cholesterol, and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 μl serum of each rat using a Superose 6 column (3.2x30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics).

8.2.3 Assessment of lipid accumulation in liver parenchymal, endothelial, and Kupffer cells

Isolated liver parenchymal, endothelial, and Kupffer cells were cyto-spinned on gelatin-coated slides. Lipid accumulation was visualised using oil red O (Sigma). A Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd.) was used to take photographs of the stained cells (1000x magnification).

8.2.4 Immunoblotting

Immunoblotting on protein from isolated liver parenchymal, endothelial, and Kupffer cells was performed as described previously [10]. In short, after running equal amounts of total cell protein on a 7.5% SDS-PAGE gel, ABCA1 was detected using murine monoclonal ABCA1 primary antibody (AC-10; Dr. M. Hayden, Vancouver, Canada) [11] and a goat-anti-mouse IgG (Jackson ImmunoResearch) secondary antibody.

8.2.5 Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on isolated liver parenchymal, endothelial, and Kupffer cells was performed as described [8]. In short, total RNA was isolated according to Chomczynski and Sacchi [12] and reverse transcribed using RevertAidTM reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in Table 1. Hypoxanthine guanine phosphoribosyl transferase (HPRT) and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression (R.E.) numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of HPRT and 36B4 (Ct housekeeping) and raising 2 to the power of this difference. The average Ct of two housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The Ct values for 36B4 (means±SEM; n=6) were 24.6±1.0 and 24.8±1.6 for parenchymal cells, 24.8±1.1 and 25.5±0.6 for endothelial cells, and 24.0±0.8 and 24.5±1.1 for Kupffer cells on the chow or the cholesterol/cholic acid enriched diet, respectively. The Ct values for HPRT (means±SEM; n=6) were 26.3±1.1 and 26.0±1.6 for parenchymal cells, 26.8±0.7 and 25.5±0.6 for endothelial cells, and 25.3±1.4 and 25.0±1.4 for Kupffer cells on the chow or the cholesterol/cholic acid enriched diet, respectively.

8.2.6 Data analysis

The significance of differences in relative gene expression numbers between hepatic cells isolated from rats fed a chow or a cholesterol/cholic acid enriched diet, measured by real time quantitative PCR, was calculated using a two-tailed Student's t-test on the differences in Ct ($Ct_{housekeeping} - Ct_{target\ gene}$). The difference in Ct values was tested for normality using Graphpad Instat 3 software (Graphpad Software, Inc.). Probability values less than 0.05 were considered significant.

Table 1
Primers for quantitative real-time PCR analysis

Gene	GenBank accession	Forward primer	Reverse primer	Amplicon size
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ABCA1	**	ATCTCATAGTATGGAAGAATGTGAAGCT	CGTACAACTATTGTATAACCATCTCCAAA	132
ABCG5	NM053754	CGCAGGAACCGCATTGTAA	TGTCGAAGTGGTGGAAGAGCT	67
ABCG8	NM130414	GATGCTGGCTATCATAGGGAGC	TCTCTGCCTGTGATAACGTCGA	69
BSEP	NM031760	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA	77
CYP7A1	NM012942	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	95
HPRT	X62085	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
LDL receptor	NM175762	AGCCGATGCATTCCTGACTC	AGTTCATCCGAGCCATTTTCAC	68
LXRα	NM031627	TCAGCATCTTCTCTGCAGACCGG	TCATTAGCATCCGTGGGAACA	144
LXRβ	NM031626	AAGCTGGTGAGCCTGCGC	CGGCAGCTTCTTGTCCTG	81
PPARα	NM013196	TGAACAAAGACGGGATG	TCAAACTTGGGTTCCATGAT	106
PPARγ	NM013124	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT	131
PPARδ	NM012141	GAGGGGTGCAAGGGCTTCTT	CACTTGTTGCGGTTCTTCTTCTG	97
SHP	L76567	CTATTCTGTATGCACTTCTGAGCCC	GGCAGTGGCTGTGAGATGC	72
SR-BI	U76205	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA	65

** Primer sequences based on an alignment of human (NM005502) and murine (XM109397) ABCA1

8.3 Results

8.3.1 The effect of an atherogenic diet on serum and hepatic cellular lipid levels

Male Wistar rats (six per group) were fed either regular chow (4.3% (w/w) fat and no cholesterol) or a diet enriched in cholesterol and cholic acid (2% (w/w) cholesterol, 5% (w/w) olive oil, and 0.5% (w/w) cholic acid) for two weeks and fasted overnight before sacrifice. Feeding the diet enriched in cholesterol and cholic acid resulted in 4-fold increased serum free cholesterol, and 11-fold increased serum cholesterol ester levels, with unchanged serum triglyceride levels as compared to rats on chow diet (Table 2). The observed changes in serum cholesterol levels were caused by a major increase in cholesterol ester rich beta-migrating VLDL (Fig.1; [13]). The liver of rats fed the cholesterol/cholic acid enriched diet showed visible whitening as compared to control liver, indicative for heavy lipid loading (Fig.2A). Oil red O staining of isolated parenchymal, endothelial, and Kupffer cells indicated heavy lipid loading of parenchymal, Kupffer cells, and to a lesser extent of endothelial cells after two weeks of feeding the cholesterol/cholic acid enriched diet (Fig.2B). Earlier data provided by Fluiter et al. showed that the total cellular cholesterol concentration increased 12- and 23-fold in parenchymal and Kupffer cells, respectively, upon feeding this cholesterol/cholic acid enriched diet [14].

Table 2
Effect of a cholesterol/cholic acid enriched diet on serum lipid levels

Diet	*n*	Total cholesterol (mg/ml)	Free cholesterol (mg/ml)	Triglycerides (mg/ml)
Chow	6	0.39 ± 0.04	0.15 ± 0.02	0.69 ± 0.053
Cholesterol/cholic acid enriched	5	3.40 ± 0.5***	0.64 ± 0.14 **	0.56 ± 0.098

Serum lipid levels were measured in overnight fasted rats after feeding a chow or a cholesterol/cholic acid enriched diet for two weeks. Data represent means+SEM. **P<0.01 and ***P<0.001 compared to chow diet values.

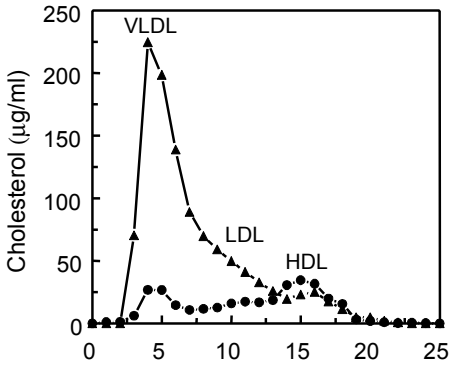


Fig.1. Effect of a cholesterol/cholic acid enriched diet on the rat serum cholesterol distribution. Blood samples were drawn after an overnight fast while feeding a regular chow (circles) or a cholesterol/cholic acid enriched diet (triangles) for two weeks. Sera from 5-6 individual rats were loaded onto a Superose 6 column and fractions were collected. Fractions 3-7 represent VLDL; fractions 8 to 15, LDL; and fractions 15 to 19, HDL, respectively.

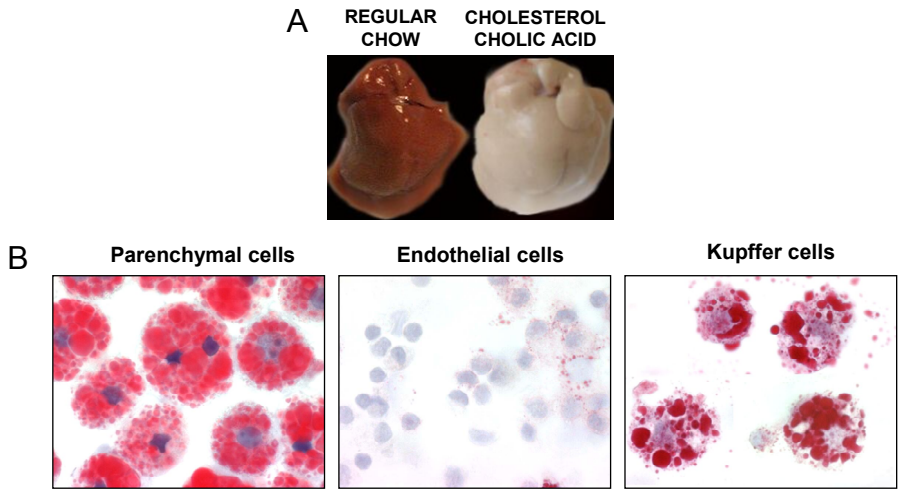


Fig.2. A cholesterol/cholic acid enriched diet induces lipid loading of rat liver parenchymal and Kupffer and to a minor extent endothelial cells leading to whitening of the liver. A) Photographs were taken of the liver during perfusion to visualize the colour difference between livers on the cholesterol/cholic acid enriched diet (*right*) as compared to the regular chow diet (*left*). B) Parenchymal, endothelial, and Kupffer cells of rats fed a cholesterol/cholic acid enriched diet for two weeks were isolated and stained for lipid content with oil red O.

8.3.2 The effect of an atherogenic diet on genes involved in the uptake of cholesterol

The effect of the atherogenic diet on the mRNA expression level of genes involved in the hepatic uptake of cholesterol from the serum was determined in parenchymal, endothelial, and Kupffer cells using real-time quantitative PCR. Parenchymal LDL receptor expression was significantly 50% decreased (R.E. 0.030 vs 0.059) by the atherogenic diet as compared to the chow diet (Fig.3). No significant effect of the diet on the LDL receptor expression was observed in endothelial and Kupffer cells (R.E. 0.0096 vs 0.0084, and 0.014 vs 0.010, respectively). The mRNA expression of the receptor involved in the selective uptake of HDL cholesterol esters, SR-BI, was not significantly changed by the diet in parenchymal and endothelial cells (R.E. 0.48 vs 0.66, and 0.17 vs 0.21, respectively). In contrast, SR-BI mRNA levels were 3-fold (Fig.3) increased in Kupffer cells, from 0.12 to 0.35 (R.E.), respectively.

8.3.3 The effect of an atherogenic diet on genes involved in cholesterol metabolism and efflux

In addition to SR-BI, several other gene products are involved in the efflux of cholesterol from the different hepatic cell types. The direct biliary efflux of cholesterol from parenchymal cells is mediated by the ABC half transporters ABCG5 and ABCG8 [15]. Recently, it has been shown that hepatic ABCA1 plays an essential role in the formation of HDL [16].

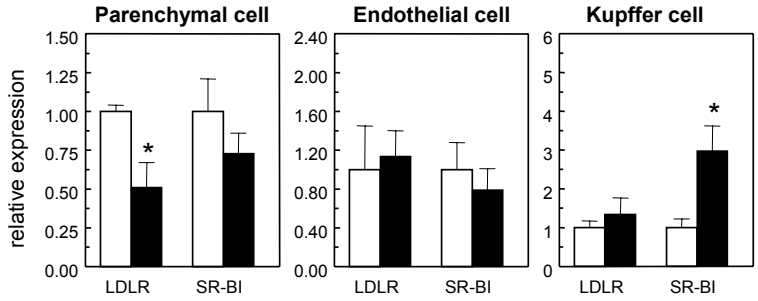


Fig.3. Effect of a cholesterol/cholic acid enriched diet on LDL receptor (LDLR) and SR-BI mRNA expression in parenchymal, endothelial, and Kupffer cells of rats fed a regular chow (white bars) or a cholesterol/cholic acid enriched diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=5-6). *P<0.05 compared to the chow diet (t-test, as described under "Experimental Procedures").

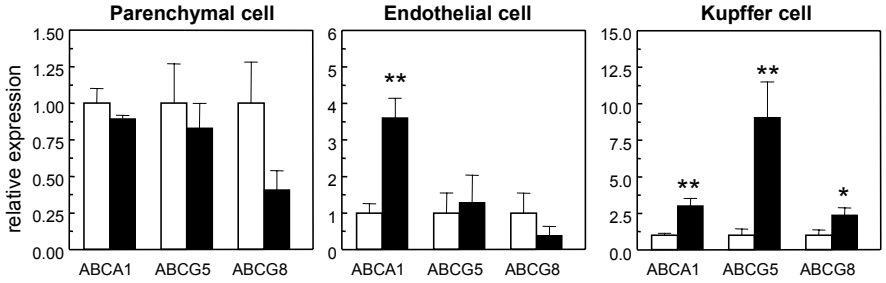
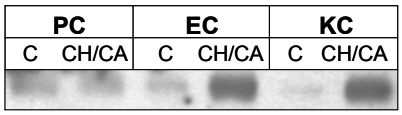


Fig.4. Effect of a cholesterol/cholic acid enriched diet on ABC transporter mRNA expression in parenchymal, endothelial, and Kupffer cells of rats fed a regular chow (white bars) or a cholesterol/cholic acid enriched diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=5-6). *P<0.05 and **P<0.01 compared to the chow diet (t-test, as described under "Experimental Procedures").

Fig.5. Effect of a cholesterol/cholic acid enriched diet on ABCA1 protein expression in parenchymal (PC), endothelial (EC), and Kupffer cells (KC) of rats fed a regular chow (C) or cholesterol/cholic acid enriched diet (CH/CA) for two weeks.



Accordingly, ABCA1 deficient mice have virtually no HDL cholesterol levels [17]. On the cholesterol/cholic acid enriched diet, ABCG5 and ABCG8 expression in parenchymal and endothelial cells is not significantly influenced. Interestingly, in Kupffer cells ABCG5 and ABCG8 expression is stimulated 9-fold (R.E. 0.049 vs 0.005) and 2.4-fold (R.E. 0.015 vs 0.006), respectively, by the cholesterol/cholic acid enriched diet (Fig.4). Furthermore, ABCA1 expression is stimulated 3.5- and 3-fold in endothelial (R.E. 0.17 vs 0.05) and Kupffer cells (R.E. 0.41 vs 0.14), but not in

parenchymal cells (R.E. 0.11 vs 0.13; Fig.4). The observed induction of ABCA1 mRNA levels in endothelial and Kupffer cells on a high cholesterol diet was also established on the protein level by western blotting using an ABCA1-specific antibody (Fig.5).
Another important route for cholesterol removal from the liver is the metabolism of cholesterol to bile acids by CYP7A1 and subsequent excretion into the bile via the BSEP. No significant change in CYP7A1 expression was detected in parenchymal and endothelial cells upon cholesterol/cholic acid diet feeding. Strikingly, the cholesterol/cholic acid enriched diet induced a highly significant 82-fold increase (R.E. 0.29 vs 0.0035) in Kupffer cell CYP7A1 expression (Fig.6). In addition, a ~17-fold increase (R.E. 0.85 vs 0.05) in Kupffer cell BSEP expression was observed, which failed to reach statistical significance due to a wide variation of expression in these cells (p=0.07). In contrast, BSEP expression was unchanged in parenchymal (R.E. 1.43 vs 1.57) and endothelial cells (R.E. 0.048 vs 0.035) in response to the cholesterol/cholic acid enriched diet (Fig.6). Surprisingly, the expression of an important repressor of CYP7A1 mRNA expression, SHP [18], was also increased 3.2-fold in Kupffer cells (R.E. 0.0094 vs 0.0028) and to a lesser extent (66%; R.E. 0.012 vs 0.007) in parenchymal cells (Fig.6).

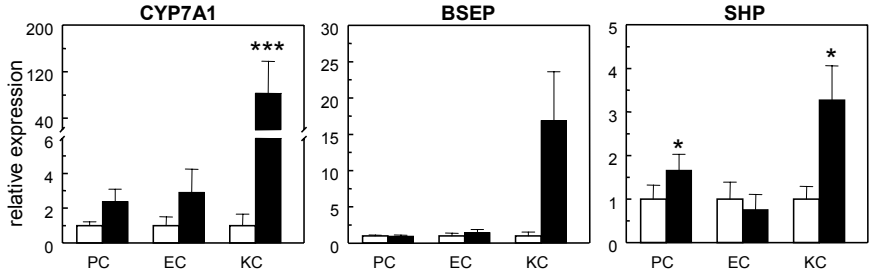


Fig.6. Effect of a cholesterol/cholic acid enriched diet on cholesterol 7α-hydroxylase (CYP7A1), bile salt export pump (BSEP), and small heterodimer partner (SHP) mRNA expression in parenchymal (PC), endothelial (EC), and Kupffer cells (KC) of rats fed a regular chow (white bars) or a cholesterol/cholic acid enriched diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=5-6). *P<0.05, ***P<0.001 compared to the chow diet (t-test, as described under "Experimental Procedures").

8.3.4 The effect of an atherogenic diet on the expression of nuclear hormone receptors

Recently it was shown that several members of the nuclear hormone receptor family are involved in the transcriptional control of genes involved in cholesterol metabolism. No significant effect on the expression of LXRα/β was detected in any cell type of the liver upon high cholesterol/cholic acid feeding, whilst also FXRα/β expression was unchanged (data not shown). Cheema et al. have shown that CYP7A1 expression can also be positively

regulated by fatty acids via binding of the nuclear hormone receptor PPARα to an additional PPARα/RXRα binding site [19]. Therefore, the regulation of PPARα expression by the cholesterol/cholic acid enriched diet was also studied in parenchymal, endothelial, and Kupffer cells. PPARα expression was significantly stimulated 7.5-fold in Kupffer cells (R.E. 0.104 vs 0.014) by the cholesterol/cholic acid enriched diet (Fig.7). In addition, the expression of PPARγ, a key regulator of intracellular fatty acid metabolism gene expression [20], was significantly increased 3.6-fold (R.E. 0.012 vs 0.032) in Kupffer cells. Finally, the expression of PPARδ, whose function in hepatic lipid metabolism remains to be clarified, was decreased 6.8-fold in parenchymal cells (R.E. 0.014 vs 0.092) on the cholesterol/cholic acid enriched diet.

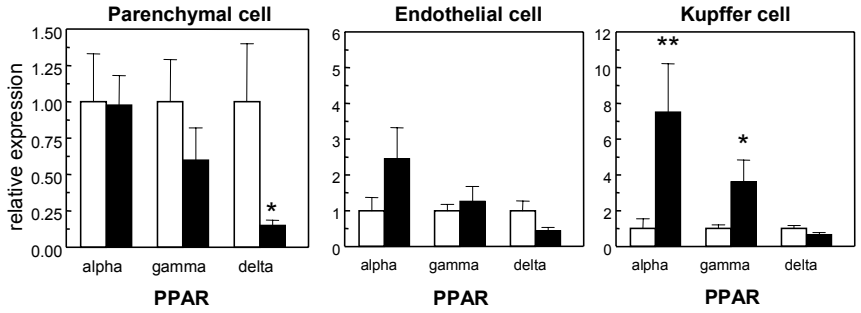


Fig.7. Effect of a cholesterol/cholic acid enriched diet on the different PPAR mRNA expression in parenchymal, endothelial, and Kupffer cells of rats fed a regular chow (white bars) or a cholesterol/cholic acid enriched diet (black bars) for two weeks. Gene expression analysis was performed by means of real-time quantitative PCR. Values are expressed as fold induction compared to the chow diet (means+SEM; n=5-6). *P<0.05 and **P<0.01 compared to the chow diet (t-test, as described under "Experimental Procedures").

8.4 Discussion

We have recently shown that the expression of genes involved in cholesterol uptake, metabolism, and efflux is markedly different between parenchymal, endothelial, and Kupffer cells in the rat liver [8]. It remains to be established whether differences in cellular localization also lead to a difference in regulation of genes involved in hepatic cholesterol metabolism. In the current study, the effect of a high cholesterol/cholic acid diet-induced lipid loading on the expression of genes involved in cholesterol uptake, metabolism, and efflux were studied in rat liver parenchymal, endothelial, and Kupffer cells to gain insight in the cell specific regulation of cholesterol metabolism. A diet, enriched in cholesterol (2%) and cholic acid (0.5%) was used, because combination of these two components in a diet is commonly used to induce aortic atherosclerotic lesion formation in rats and mice [21-23]. In addition, Chiang et al. [24] have shown that addition of cholic acid to a diet containing cholesterol induces higher rises in plasma and hepatic total cholesterol levels than the cholesterol enriched diet alone. In the current study, the cholesterol/cholic acid enriched diet drastically increased

serum levels of VLDL cholesterol (β-VLDL), leading to heavy lipid loading of parenchymal and Kupffer cells, and to a lesser extent of endothelial cells.
The expression of SR-BI, a crucial determinant in the selective uptake of HDL cholesterol esters into the liver, was not significantly affected by the diet in parenchymal and endothelial cells. Interestingly, SR-BI mRNA expression was increased in Kupffer cells in response to lipid loading by the cholesterol/cholic acid enriched diet. This observation is in agreement with data provided by Fluiter et al. [14], who showed that SR-BI protein expression in Kupffer cells is 4-fold stimulated by a high cholesterol diet. Recent studies have proposed a novel role for SR-BI in the efflux from macrophages of free cholesterol to HDL [25,26]. Since SR-BI expression is induced upon lipid loading of Kupffer cells, it is suggested that Kupffer cells, upon lipid loading, induce SR-BI mediated cholesterol efflux in order to eliminate excess cholesterol.
Recent data have indicated that ABCA1 is also a major determinant in the efflux of cholesterol from macrophages. Bortnick et al. have directly correlated ABCA1 mRNA expression with cholesterol efflux to apoAI in various cell lines [27], whilst Van Eck et al. have shown that leukocyte selective deficiency of ABCA1 results in increased susceptibility to atherosclerosis [28]. The cholesterol/cholic acid enriched diet had no effect on parenchymal ABCA1 expression, but it did significantly stimulate ABCA1 expression in endothelial and Kupffer cells. The unchanged ABCA1 expression observed in parenchymal cells is in agreement with data provided by Denis et al. [29] who indicated that ABCA1 expression is constitutive in hepatic cells, whilst sterols are able to stimulate ABCA1 expression in macrophages. Venkateswaran et al. have suggested that the increase in macrophage ABCA1 expression in response to cellular cholesterol loading is the consequence of increased activation of the nuclear hormone receptor LXRα [30]. Recently, LXRα was also shown to be a positive regulator of the hepatic expression of ABCG5 and ABCG8 [31], two half transporters, which together function as a full transporter involved in the hepatic biliary cholesterol efflux [32]. Interestingly, the expression of ABCG5 and ABCG8 was significantly increased in lipid laden Kupffer cells. The relative expression level of Kupffer cell ABCG5/G8 is rather low as compared to the expression in parenchymal cells under standard feeding conditions [8], which indicates that ABCG5/G8 normally does not play a crucial role in Kupffer cells. Since Kupffer cells do not directly efflux cholesterol into bile, the observed increase in ABCG5/G8 expression suggests a novel role for these transporters in the efflux of cholesterol from lipid laden Kupffer cells. Strikingly, no significant effect of the cholesterol/cholic acid enriched diet was observed on parenchymal and endothelial ABCG5/G8 expression. Berge et al. have shown that cholesterol feeding up-regulates the hepatic expression of ABCG5/G8 [15]. The absence of an induction of ABCG5/G8 in parenchymal cells might be the result of diet induced stimulation of SHP (66%) in these cells, since it has been shown that the nuclear receptor SHP is able to interact with LXR to modulate its transcriptional activity [33].
In the liver, CYP7A1 is a key enzyme in the conversion of cholesterol to bile acids for excretion into the bile [38]. Importantly, both LXRα and FXR control the expression of CYP7A1. In rodents, LXRα induces the

expression of CYP7A1 via binding as a heterodimer with the RXRα to AGGTCA-like response elements in its promoter [35]. In contrast, FXR inhibits CYP7A1 expression via induction of SHP. In agreement with our data, Gupta et al. [36] have shown that feeding a diet enriched in cholesterol and cholic acid stimulates hepatic SHP expression. She also suggested that under conditions of both SHP and LXR alpha activation, the stimulatory effect of LXR alpha overrides the inhibitory effect of FXR and results in an induction of rat CYP7A1 mRNA levels [36]. This is in agreement with our data that CYP7A1 was slightly increased in parenchymal and endothelial cells by the cholesterol/cholic acid enriched diet. The major induction (~80 fold) in Kupffer cell CYP7A1 expression by the diet also suggests a major role for LXRα compared to FXR in the regulation in these cell types. Interestingly, a role for CYP7A1 in the metabolism and efflux of cholesterol in macrophages has already been proposed by Moore and Davis [37] and our data provide the first evidence that in vivo loading of macrophages markedly induces the CYP7A1 efflux pathway.

Feeding the cholesterol/cholic acid enriched diet increased serum β-VLDL levels and led to an 8-fold increase in PPARα expression in Kupffer cells. Although the expression level of PPARα was still very low as compared to the expression in parenchymal cells, these data do suggest that PPARα possibly was involved in the huge stimulation of CYP7A1 expression observed in Kupffer cells. In addition, the observed increase in Kupffer cell SR-BI expression might be caused by the induction of PPARα expression as well, since Chinetti et al. [38] have suggested that PPARα is a direct regulator of SR-BI expression in macrophages.

Multiple studies have suggested a major role for PPARγ in the regulation of macrophage ABCA1 expression and cholesterol efflux to apoAI [39,40]. A small but significant induction in PPAR□ expression was observed solely in Kupffer cells, which suggests that, in addition to LXRα, PPARγ might also contribute to the observed induction in ABCA1 expression. In contrast, a strong decrease in PPARδ expression was found in parenchymal cells, but not Kupffer and endothelial cells. Although several studies have suggested that PPARδ is involved in muscle and intestinal fatty acid homeostasis [41,42], the exact function of PPARδ in hepatic cholesterol homeostasis remains to be clarified. Oliver et al. have shown that PPARδ activation does decrease serum VLDL levels and lead to an increase in serum HDL levels, thereby stimulating reverse cholesterol transport [43]. It will be interesting to study the effect of low parenchymal PPARδ expression on the VLDL secretion by these cells.

In conclusion, we have shown that heavy lipid loading of parenchymal cells leads to moderate gene expression changes, while Kupffer cells respond highly adaptive by stimulating the expression of genes involved in cholesterol metabolism and transport. The highly adaptive response of endothelial and in particular Kupffer cells will enable efficient transport of cholesterol to extracellular acceptors (ApoA-I and HDL), thereby preventing malfunctioning of the cells under atherogenic conditions.

8.5 Acknowledgements

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9

Microarray analysis on early gene expression changes in liver parenchymal cells of LDL receptor deficient mice on a Western type diet

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The liver is an essential organ in the clearance of excess cholesterol from the blood and subsequent excretion into the bile. The liver contains several different cell types, which all have their specific localization and function. However, the majority of the liver cells consist of parenchymal cells, which are the major player in cholesterol and glucose metabolism. To gain insight in the pathways involved in the response of liver parenchymal cells to increased dietary lipid levels, in current study the time-dependent changes in gene expression in this specific cell type upon feeding a Western type diet were determined using microarray analysis. Western type diet feeding resulted in a drastic increase in both free (5.8-fold) and total (5.2-fold) serum cholesterol levels in the first two weeks, with only small additional increases after 4 and 6 weeks. During the first two weeks of Western type diet feeding 244 genes displayed a change in gene expression of at least 2-fold; 158 genes were upregulated, and 86 were downregulated. The largest number of genes (total 41; upregulated 30, downregulated 11) was associated with biological processes involving lipid, fatty acid and steroid metabolism. Dietary (time-dependent) upregulation of genes involved in glycolysis and fatty acid synthesis could be confirmed using quantitative real-time PCR. The data indicate that liver parenchymal cells stimulate glycolysis and lipogenesis to maintain (intracellular) homeostasis in response to an increased dietary lipid level. Furthermore, our studies indicate a very important role for the transcription factors SREBP and LXR in the (initial) response of the parenchymal liver cells to the increased levels of dietary lipid.

9.1 Introduction

Cholesterol is an important constituent of membranes in all different organs and cells in the body. In steroidogenic tissues (e.g. testis, ovaries) and the liver cholesterol serves also as a precursor in the formation of steroid hormones and bile acids, respectively. However, due to the increased consumption of diets containing relatively high levels of cholesterol and/or fat in the Western world, it is expected that an excess of circulating plasma cholesterol in the general population will rise dramatically. Increased levels of circulating cholesterol are considered to be a major risk factor for atherosclerosis and subsequent cardiovascular diseases (e.g. myocardial infarction, stroke) [1], which are the leading cause of death in the Western world. In addition to the dietary risk for high plasma cholesterol levels, several mutations in the LDL receptor gene are associated with familial hypercholesterolemia. Familial hypercholesterolemia is a dominantly inherited error of metabolism characterised by raised plasma low-density lipoprotein (LDL) levels, xanthomas of skin and tendons, and premature heart disease due to atherosclerosis of the coronary arteries [2].
The liver is an essential organ in the clearance of excess cholesterol from the blood and subsequent excretion into the bile [3,4]. Due to the important role of the liver in metabolism, several studies have recently been conducted using microarray technology to determine the molecular mechanisms underlying long-term high fat diet-induced alterations in total mouse liver [5-8]. The liver however contains several different cell types, which all have their specific localization and function. Kupffer cells are tissue macrophages strategically located within the liver sinusoids, and their function is the removal of bacteria and the clearance of modified lipoproteins. In addition, hepatic endothelial cells line the sinusoids, where they function in the removal of modified lipoproteins and mediate their natural barrier function. However, the majority of the liver cells consist of parenchymal cells (~60%), which occupy >90% of the total liver protein mass. Parenchymal liver cells are located between bile canaliculi and sinusoids, where they mediate the uptake and metabolism of cholesterol for biliary excretion. Since the parenchymal liver cell is the major player in cholesterol and glucose metabolism, we have quantified in the current study the time-dependent changes in gene expression in this specific cell type upon feeding a Western type diet containing 0.25% cholesterol and 15% fat to gain insight in the pathways involved in the response of liver parenchymal cells to increased dietary lipid levels. For this purpose we have subjected RNA from isolated parenchymal cells of atherosclerosis prone mice (LDL receptor deficient mice) to microarray analysis.
Western type diet feeding resulted in a drastic increase in both free (5.8-fold) and total (5.2-fold) cholesterol levels in the first two weeks, with only small additional increases after 4 and 6 weeks. Since the serum lipid levels already stabilized at a higher level after 2 weeks of Western type diet feeding, we specifically focused on the early changes in gene expression in parenchymal liver cells. During the first two weeks of Western type diet feeding 244 genes displayed a change in gene expression of at least 2-fold; 158 genes were upregulated, and 86 were downregulated. The largest number of genes (total 41; upregulated 30, downregulated 11) were

associated with biological processes involving lipid, fatty acid and steroid metabolism. Dietary (time-dependent) upregulation of genes involved in glycolysis and fatty acid synthesis could be confirmed using quantitative real-time PCR. The data indicate that liver parenchymal cells stimulate glycolysis and lipogenesis to maintain (intracellular) homeostasis in response to an increased dietary lipid level. In addition, our studies indicate a very important role for the transcription factors SREBP and LXR in the (initial) response of the parenchymal liver cells to the increased levels of dietary lipid.

9.2 Experimental Procedures

9.2.1 Animals

Homozygous LDL receptor deficient mice [9,10] were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Female mice were maintained on sterilized regular chow containing 5.7% (wt/wt) fat and no cholesterol (Hope Farms, Woerden, The Netherlands), or were fed a semi-synthetic Western type diet containing 15% (wt/wt) cacao butter and 0.25% (wt/wt) cholesterol for 2, 4, or 6 weeks. Subsequently, parenchymal liver cells were isolated essentially according to the method of Nagelkerke et al. [11]. The purity and viability of the cells was analysed using trypan blue staining and phase contrast microscopy. Western type diet feeding had no effect on the viability or purity of the isolated cells; the liver parenchymal cell fractions consisted for >99% of parenchymal cells with a viability >95% under both standard and Western type diet feeding conditions.

9.2.2 Serum lipid analyses

Serum concentrations of free and total cholesterol were determined using enzymatic colorimetric assays (Roche Diagnostics). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 µl serum of each mouse using a Superose 6 column (3.2×30 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics).

9.2.3 Microarray analysis

The Mouse Genome Survey Arrays used in the study contained 32.996 different spots, which included transcripts from the public domain as well as from the Celera library.
Total RNA from liver parenchymal cells was isolated according to Chomczynski and Sacchi [12]. Double stranded cDNA was prepared from 2 µg of total RNA. An in vitro transcription (IVT) reaction was used to

synthesize 50-100 μg of UTP-digoxigenin-labeled cRNA. Equal amounts of cRNA (10 μg) from 2 pooled RNA samples of 2 mice (total of 4 mice) per time point was hybridized to Mouse Genome Survey Arrays for 16 hours at 55°C. Subsequently, an alkalic phosphatase-linked digoxigenin antibody was incubated with the array and the phosphatase activity was initiated to start the chemiluminescent signal. The chemiluminescent (cRNA) and fluorescent (spot backgound) signals of the cRNA and standard controls spots were scanned for 5 and 25 seconds using an AB1700 Chemiluminescence Analyzer (Applied Biosystems). Using the software supplied with the AB1700 apparatus, the spot chemiluminescent signal was normalized over the fluorescent signal of the same spot (using the standard control signals) to obtain the normalized signal value that was used for further analysis. In addition, a signal to noise ratio for every spot was obtained, which needed to be at least 1 at each time point in order to use the spot for further analysis. In the analysis, the median value of the normalized signal of two independent arrays containing pooled RNA from 2 mice for each time point was calculated as an indication for the relative gene expression number at that time point. Genes whose mRNA levels were changed 2-fold or more were designated as differentially expressed genes. Differentially expressed genes were classified into functional categories based on the Panther (Protein ANalysis THrough Evolutionary Relationships) Classification System (Celera).

9.2.4 Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on isolated liver parenchymal cells was performed as described [13]. In short, total RNA was isolated according to Chomczynski and Sacchi [12] and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in Table 1. Hypoxanthine guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and acidic ribosomal phosphoprotein P0 (36B4) were used as the standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of HPRT, GAPDH, and 36B4 (Cthousekeeping) and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions.

Table 1
Primers for quantitative real-time PCR

	GenBank Accession	Forward primer	Reverse primer	Amplicon size
36B4	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
ACAS2	NM019811	GCTGTCGCAGAGGCAGCT	AAAGCAGTAGAGGCATTCGCC	66
ACC	XM109883	AGAATCTCCTGGTGACAATGCTTATT	GCTCTGTGAGGATATTTAGCAGCTC	87
ACLY	NM134037	AGGTACCCTGGGTCCACATTC	CTACGATCATCTTGACTCCTGGAGT	73
FABP5	BC002008	GGAAGGAGAGCACGATAACAAGA	GGTGGCATTGTTCATGACACA	73
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	91
L-PK	NM013631	AAGACAGTGTGGGTGGACTACCA	CGTCAATGTAGATGCGGCC	70
ME	NM008615	TTAAGGATCCACTGTACATCGGG	GGCGTCATACTCAGGGCCT	62
SCD1	NM009127	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA	65
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC	74

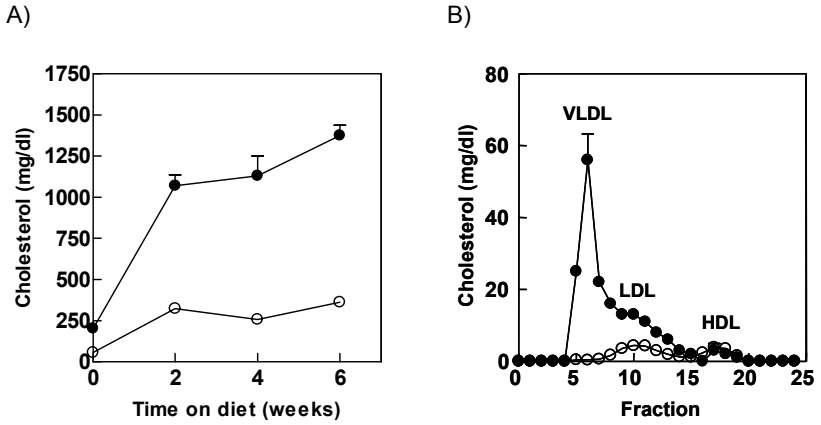

Fig.1. A) The effect of a Western type diet on serum free (○) and total (●) cholesterol levels in LDL receptor deficient mice. B) The effect of a Western type diet on the serum cholesterol distribution in LDL receptor deficient mice. Blood samples were drawn on a chow diet (○) and two weeks on a Western type diet (●). Sera from individual mice were loaded onto a Superose 6 column and fractions were collected. Fractions 3-7 represent VLDL; fractions 8-15, LDL; and fractions 15-19, HDL, respectively.

9.3 Results

9.3.1 Western type diet feeding induces an atherogenic serum lipoprotein profile

Feeding LDL receptor deficient mice a Western type diet containing 0.25% cholesterol and 15% fat resulted in a significant rise in free and total serum cholesterol levels (Fig.1A). In addition, the Western type diet induced an atherogenic lipoprotein profile as compared to the chow diet, with significant increases in serum LDL and VLDL cholesterol levels (Fig.1B).

9.3.2 Western type diet feeding induces changes in liver parenchymal gene expression

Liver parenchymal cells were isolated from LDL receptor deficient mice on a regular chow or Western type diet according to the method of Nagelkerke et al. [11]. Western type diet feeding had no effect on the viability or purity of the different cell fractions, since the parenchymal fractions consisted for >99% of parenchymal cells under both standard chow and Western type diet feeding conditions with a viability >95%. After isolation, parenchymal liver cell RNA was subjected to reverse transcription and second strand cDNA synthesis. Subsequently, using an IVT (amplification) reaction digoxigenin-labeled cRNA (chemiluminescent) was prepared, of which 10 μg was hybridized to Mouse Genome Survey Arrays containing 32.996 different spots consisting of 60-mer oligo's. Subsequently, the chemiluminescent (cRNA) and fluorescent (spot backgound) signals were determined and the spot chemiluminescent signal was normalized over the fluorescent signal of the same spot to obtain the normalized signal value that was used for further analysis. In addition, a signal to noise ratio for every spot was obtained, which needed to be at least 1 at each time point in order to use the spot for further analysis.
The gene expression levels of parenchymal cells isolated from mice fed the Western type diet were compared to that on chow diet. Only the genes whose mRNA levels were changed 2-fold or more were designated as differentially expressed genes. By these criteria, 244 genes were found to have significant changes in expression after two weeks of diet feeding; 158 genes were upregulated whilst 86 were downregulated in parenchymal cells of mice fed the Western type diet as compared to mice on a chow diet. By analyzing the biological processes in which these genes play a role, it appeared that most of the regulated genes were associated with lipid, fatty acid, and steroid metabolism (Table 2). In total 41 genes were involved in lipid, fatty acid, and steroid metabolism; 11 genes were 2-fold or more downregulated (Table 3) after two weeks on the Western type diet, whilst 30 genes were 2-fold or more upregulated (Table 4) as compared to the chow diet. The downregulated genes included members of the cytochrome P450 family, such as CYP4A14 (2-fold) and CYP7A1 (2-fold), enzymes involved in hepatic lipid metabolism i.e. fatty acid omega-oxidation [14] and the conversion of cholesterol to bile acids [15]. In addition, the expression of carnitine O-octanoyltransferase (Crot) and cytosolic acyl-CoA thioesterase 1 (Cte1), enzymes involved in hepatic fatty acid [16] and acyl-CoA metabolism [17], respectively, was also decreased 2.3- and 2.6-fold, respectively. Furthermore, retinol binding protein 1 expression was 2.8-fold decreased by the Western type diet. The single known function of this protein in rodents is to transport retinol, a derivative from vitamin A, from hepatic stores to target tissues, where it plays an essential role in many biological functions [18].

Table 2
Number of genes differentially expressed in liver parenchymal cells on the Western type diet[a]

Molecular function	Number of genes
Biological process unclassified	65
Lipid, fatty acid and steroid metabolism	41
Immunity and defense	30
Signal transduction	30
Nucleoside, nucleotide and nucleic acid metabolism	20
Transport	19
Developmental processes	19
Carbohydrate metabolism	18
Protein metabolism and modification	16
Electron transport	12
Other metabolism	10
Cell structure and motility	10
Sensory perception	9
Cell cycle	8
Sulfur metabolism	8
Amino acid metabolism	6
Oncogenesis	6
Cell proliferation and differentiation	5
Coenzyme and prosthetic group metabolism	3
Apoptosis	3
Intracellular protein traffic	3
Cell adhesion	2
Homeostasis	2
Neuronal activities	2
Phosphate metabolism	1

[a] Genes designated as differentially expressed by the Western type diet were those for which the normalized signal was increased or decreased at least 2.0-fold after two weeks on the Western type diet. Classification of the differentially regulated genes by molecular function was based upon the Panther classification.

Table 3
Genes involved in lipid, fatty acid, and steroid metabolism that are *downregulated* in liver parenchymal cells on the Western type diet

Celera Gene ID	Gene name	Fold
mCG123382	*unassigned*; cytochrome P450 4A	0.28
mCG6017	cytochrome P450, family 4, subfamily a, polypeptide 14;Cyp4a14	0.32
mCG9784	retinol binding protein 1, cellular;Rbp1	0.35
mCG141486	*unassigned*; cytochrome P450 4A	0.37
mCG4926	cytosolic acyl-CoA thioesterase 1;Cte1	0.38
mCG17029	*unassigned*; retinol dehydrogenase-related	0.40
mCG5270	phospholipase A2, group XII;Pla2g12	0.44
mCG1181	carnitine O-octanoyltransferase;Crot	0.44
mCG131266	*unassigned*; L-carnitine dehydratase	0.46
mCG4371	cytochrome P450, family 7, subfamily a, polypeptide 1;Cyp7a1	0.49
mCG2997	*unassigned*; enoyl-CoA hydratase-related	0.50

Table 4
Genes involved in lipid, fatty acid, and steroid metabolism that are *upregulated* in liver parenchymal cells on the Western type diet

Celera Gene ID	Gene name	Fold
mCG141926	glycerol phosphate dehydrogenase 2, mitochondrial;Gpd2	2.0
mCG14176	mevalonate (diphospho) decarboxylase;Mvd	2.0
mCG5402	lipin 2;Lpin2	2.2
mCG145523	RIKEN cDNA 6330406P08 gene;6330406P08Rik; arylsulfatase	2.2
mCG142632	*unassigned*; glycerol-3-phosphate acyltransferase	2.2
mCG8819	*unassigned*; sterol regulatory element binding protein 1 (SREBP-1)	2.2
mCG19918	hydroxysteroid dehydrogenase-6, delta<5>-3-beta;Hsd3b6	2.3
mCG21999	cytochrome P450, family 17, subfamily a, polypeptide 1;Cyp17a1	2.3
mCG17020	retinol dehydrogenase 6;Rdh6	2.3
mCG21052	fatty acid binding protein 2, intestinal;Fabp2	2.4
mCG17550	RIKEN cDNA 1110011E12 gene;1110011E12Rik; phosphomevalonate kinase	2.4
mCG129300	*unassigned*; sialyltransferase	2.4
mCG14909	phospholipase C, beta 3;Plcb3	2.5
mCG14387	ELOVL family member 5, elongation of long chain fatty acids (yeast);Elovl5	2.7
mCG10969	apolipoprotein A-IV;Apoa4	2.7
mCG10998	ELOVL family member 2, elongation of long chain fatty acids (yeast);Elovl2	2.9
mCG17532	phospholipid transfer protein;Pltp	4.0
mCG141659	acetoacetyl-CoA synthetase;Aacs	5.3
mCG141310	unassigned; acetyl-CoA carboxylase	5.5
mCG21064	acetyl-Coenzyme A synthetase 2 (ADP forming);Acas2	5.9
mCG18119	*unassigned*; glycerol-3-phosphate acyltransferase	6.7
mCG131749	stearoyl-CoA desaturase 1;Scd1	7.4
mCG3541	*unassigned*; acetyl-CoA carboxylase	8.6
mCG125511	ELOVL family member 6, elongation of long chain fatty acids (yeast);Elovl6	8.6
mCG9729	*unassigned*; fatty acid binding protein	14.3
mCG7050	thyroid hormone responsive SPOT14 homolog (Rattus); Thrsp	16.5
mCG22653	*unassigned*; fatty acid binding protein	16.5
mCG5289	*unassigned*; fatty acid binding protein	16.6
mCG22278	*unassigned*; fatty acid binding protein	19.3
mCG1638	fatty acid binding protein 5, epidermal;Fabp5	22.3

The genes associated with lipid, fatty acid, and steroid metabolism that were upregulated after two weeks of Western type diet included predominantly genes involved in lipid and fatty acid biosynthesis, metabolism, and transport. Strikingly, the most highly regulated genes (>10-fold) included 4 members of the fatty acid binding protein family, such as epidermal fatty acid binding protein (E-FABP or FABP5). In addition, the

expression of Spot14 (S14), a protein involved in hepatic lipogenesis [19], was 16-fold higher in the parenchymal cells of mice on a Western type diet, while the acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase 1 (SCD1) genes were stimulated 5-fold by Western type diet feeding. ACC and SCD1 both play an important role in hepatic fatty lipogenesis, since they are key enzymes in the de novo production of malonyl-CoA (for fatty acid synthesis) [20], and oleate and palmitoleate [21], which are the major fatty acids found in triglycerides, cholesteryl esters, and phospholipids. The expression of a short-chain dehydrogenase that is expressed most intensely in liver and kidney, retinol dehydrogenase 6 (RDH6/CRAD1) [22], was 2.3-fold induced by the Western type diet as compared to chow diet. Interestingly, also the expression of two genes involved in HDL synthesis and metabolism were regulated upon Western type diet feeding. The expression of phospholipid transfer protein (PLTP) was upregulated 4.0-fold, whilst apolipoprotein A-IV (ApoA-IV) expression was stimulated 2.7-fold after two weeks of diet feeding as compared to control. PLTP is an important factor in the modulation of HDL lipid composition and size [23] and ApoA-IV levels influence the metabolism and anti-atherogenic properties of HDL [24].

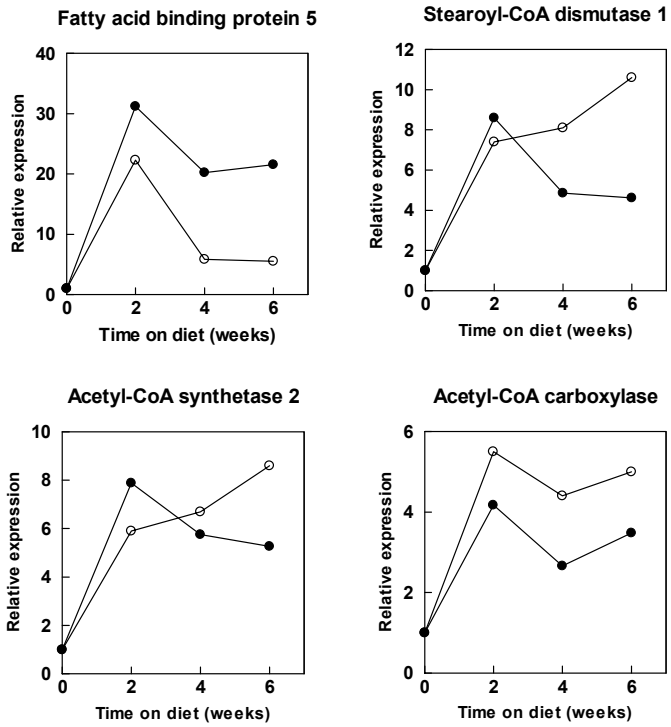

Fig.2. Time dependent effect of a Western type diet on parenchymal liver cell expression of fatty acid binding protein 5, stearoyl-CoA dismutase 1, acetyl-CoA synthetase 2, and acetyl-CoA carboxylase as determined by microarray (○) and validated by real-time quantitative PCR (●). Values (n=2-4) are expressed as fold induction on the Western type diet as compared to the chow diet (0 weeks).

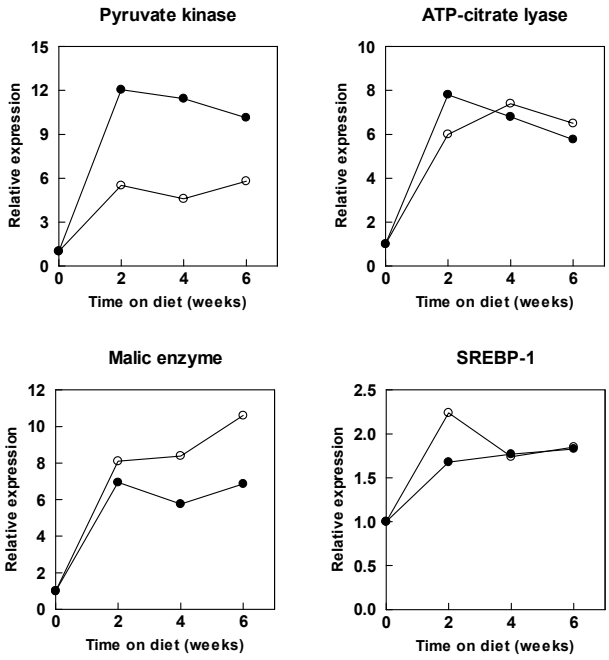

Fig.3. Time dependent effect of a Western type diet on parenchymal liver cell expression of key genes involved in glycolysis and fatty acid synthesis as determined by microarray (○) and validated by real-time quantitative PCR (●). Values (n=2-4) are expressed as fold induction on the Western type diet as compared to the chow diet (0 weeks).

9.3.3 Quantitative evaluation of the effect of the Western type diet on lipogenic pathways

Since the microarray indicated several genes associated with lipogenesis as most upregulated by the Western type diet, the effect of the Western type diet on pathways involved in lipogenesis was further examined. Changes in gene expression observed by DNA microarray analysis were confirmed with a selection of genes by real-time quantitative PCR (SYBR Green) technology, using the same (non-amplified) parenchymal cell RNA samples as used in the microarray hybridization (Fig.2). When gene expression profiles obtained by microarray analysis and real-time PCR were compared, their (time-dependent) patterns were very similar with regard to the direction (up- or downregulation) and degree of differences in expression. Strikingly, the expression of several enzymes needed for the biosynthesis of fatty acids (i.e. acetyl-CoA carboxylase, acetyl-CoA synthetase, stearoyl-CoA dismutase 1) was induced 4-fold or more in the first two weeks of feeding the Western type diet and subsequently stabilized at a relatively high level as compared to the control diet for 6 weeks. These data suggest that the lipogenesis rate was increased on the Western type diet. Accordingly, the expression of other key genes involved in the synthesis of fatty acids from glucose (i.e. pyruvate kinase, malic enzyme)

was upregulated by the Western type diet (Fig.3). Importantly, also the expression of a nuclear receptor that is a crucial factor in lipogenesis, sterol regulatory element binding protein 1 (SREBP-1), was increased after two weeks of Western type diet feeding and remained elevated in time (Fig.3). These combined data suggest that liver parenchymal cells stimulate the expression of genes involved in glycolysis and fatty acid synthesis to maintain their intracellular homeostasis in response to an increased dietary lipid level (Fig.4).

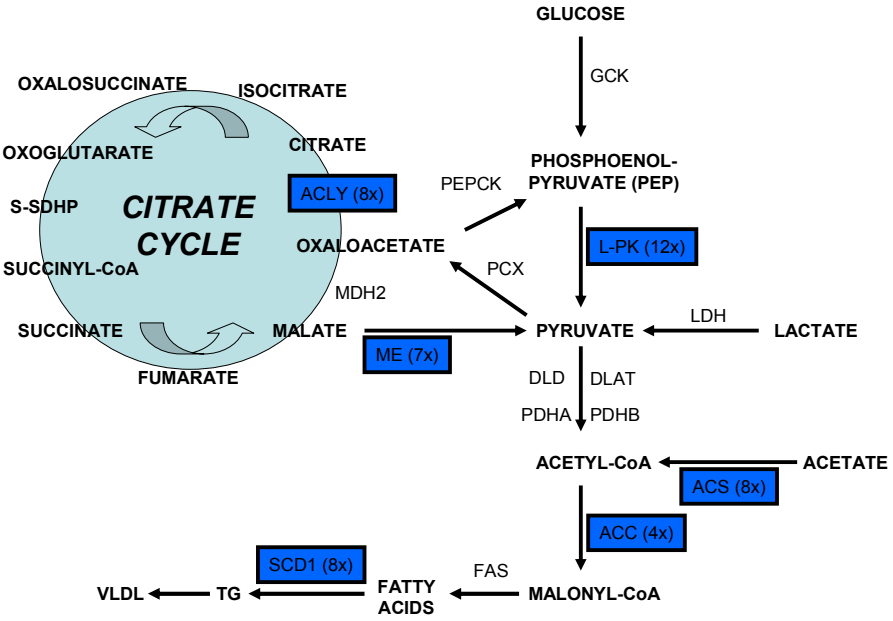

Fig.4. Schematic overview of the genes involved in glycolysis and fatty acid synthesis that are regulated by the Western type diet in parenchymal liver cells. Genes regulated by the Western type diet are boxed and the fold change in their expression on the Western type diet as compared to the chow diet is indicated.

9.4 Discussion

The purpose of the current study was to determine the effect of a Western type diet containing increased levels of cholesterol and fat on gene expression of liver parenchymal cells, which are the major player in hepatic metabolism, in an atherosclerotic mouse model. More specifically, the early (time-dependent) response of the parenchymal cells to the diet was studied using microarray technology. Most of the genes expressed differentially after two weeks of Western type diet were those involved in lipid, fatty acid, and steroid metabolism.

It has been shown in an earlier study that LDL receptor deficient mice on a Western type diet rapidly become hyperinsulinemic, hyperglycemic, and obese compared with chow-fed mice [25]. Since parenchymal cells are important in maintaining glucose homeostasis, the huge increase in the

expression of genes involved in the metabolism of glucose to acetyl-CoA such as pyruvate kinase (>10-fold) in parenchymal cells can be seen as a protective action to maintain homeostasis. The observed decrease in the expression of enzymes involved in fatty acid oxidation in liver parenchymal cells, as judged by the decreased expression of CYP4A14, carnitine O-octanoyltransferase and cytosolic acyl-CoA thioesterase, can also be seen as protective action of the liver to maintain homeostasis, since impaired hepatic fatty acid oxidation has been associated with decreased serum glucose levels due to reduced formation of ketone bodies, another source of energy for peripheral tissues [26]. Importantly, CYP4A14, carnitine O-octanoyltransferase and cytosolic acyl-CoA thioesterase expressions are transcriptionally controlled by the nuclear receptor peroxisome proliferator-activated receptor (PPAR) alpha [27-29]. The lowered expression of carnitine O-octanoyltransferase and cytosolic acyl-CoA thioesterase observed on the Western type diet therefore suggests that diet feeding results in an inhibition of PPARalpha activity. In agreement, the expression of two additional PPARalpha target genes, retinol binding protein (RBP) [30] and phospholipase A2 (PLA2) [31], was also decreased in the current study. Data from Horton et al. have indicated that PLA2-mediated release of arachidonic acid from membrane phospholipids contributes to cell injury and death [32]. The decreased PPARalpha-regulated PLA2 expression observed on the Western type diet might thus also be associated with an intracellular protective action of the parenchymal cells. Furthermore, data of Guerre-Millo et al. have suggested that hepatic PPARalpha plays a role in the development of insulin resistance in response to a Western type high-fat diet, since PPARalpha-mediated fatty acid oxidation is efficiently coupled to hepatic glucose production (gluconeogenesis) and a subsequent rise in serum glucose levels [33]. In conclusion, our data suggest that the decrease in parenchymal liver cell PPARalpha activation in LDL receptor deficient mice on a Western type diet may be considered as a protective reaction against cell injury, insulin resistance, and diabetes. Data from several studies have implied that PPARalpha also regulates the transcription of fatty acid binding proteins [34,35]. However, in the current study we observed that PPARalpha activity was decreased, whilst the mRNA expression of FABP5 and 4 novel fatty acid binding protein transcripts was drastically (~20-fold) increased. Schachtrup et al. have recently shown that the PPAR response element in the promoter of FABP5 is not functional, which suggests that the increase in the expression of FABP5 observed on the Western type diet was not due to a change in PPARalpha activation, but was likely due to the action of another upstream regulator [36].

A recent study by Ide et al. has shown that strong activation of the nuclear receptor liver X receptor (LXR) can interfere with PPARalpha mediated fatty acid degradation through competition for binding to their common heterodimeric partner, the retinoic X receptor (RXR) [37]. It is likely that the activity of LXR was stimulated in parenchymal liver cells on the Western type diet, since the expression of PLTP and ApoA-IV, two genes that in the liver are regulated by LXR [38,39], was upregulated by the diet. Strikingly, Schultz et al. imply that LXR, in addition to its role in cholesterol metabolism, plays a crucial role in lipogenesis control by the direct induction

of lipogenic gene expression via stimulation of SREBP-1 expression [40,41]. In agreement, the expression of SREBP-1 was stimulated by the Western type diet with a paralleled increase in the glycolytic and lipogenic enzymes L-type pyruvate kinase, Spot14, acetyl-CoA carboxylase, stearoyl-CoA dismutase 1, acetyl-CoA synthetase, malic enzyme, and ATP-citrate lyase. Several studies have provided evidence that these genes are all upregulated by SREBP-1 [42,43]. Recently also a SREBP-1 binding site in the promoter of retinol dehydrogenase 6 (RDH6), an enzyme that transforms vitamin A (retinol) in its active metabolite all-trans retinoic acid, was identified by Chai et al. [22]. In accordance with the observed increase in SREBP-1 expression and activity, the expression of RDH6 was also increased 2.3-fold in the parenchymal cells. Interestingly, Maxwell et al. have identified FABP5 as a novel putative SREBP-1 target gene [5]. The ~20-fold increase in FABP5 observed on the Western type diet may therefore be the result of the increased SREBP-1 expression/activity. Based upon these combined findings, it can thus be concluded that Western type diet feeding induces increased parenchymal liver cell LXR activation resulting in an increased glycolysis and lipogenesis via stimulation of SREBP-1 and a decreased fatty acid oxidation through inhibition of PPARalpha activity by LXR. Grefhorst et al. have also recently shown that stimulation of lipogenesis by pharmacological activation of LXR in C57BL/6 mice leads to the production of large triglyceride-rich VLDL particles, which are efficiently cleared, resulting in unchanged serum triglyceride levels [44]. In contrast, in the current study Western type diet-induced LXR activation in LDL receptor deficient mice resulted in a significant rise in VLDL cholesterol levels due to an impaired clearance of VLDL via the LDL receptor uptake system. These combined findings indicate that LXR activation of lipogenesis indeed does result in increased VLDL particle production. Accordingly, disruption of the LDL receptor gene in transgenic SREBP-1 mice results in the accumulation of these lipoproteins in the circulation, due to an increased synthesis and secretion and a blocked degradation via the LDL receptor [45].

Earlier studies by Recinos et al. [8] using microarray analysis of total liver RNA have indicated that feeding LDL receptor deficient or wild-type mice a high fat diet results in significant changes in the hepatic expression genes involved in cholesterol metabolism, but also in the expression of CD68 and CD63. These latter two proteins are not expressed in parenchymal cells, the main (metabolic) cell type of the liver, but in Kupffer and stellate cells [46,47], which makes it difficult to give a good interpretation of the exact effect on metabolic processes in the liver. In the current study, the effect of a Western type diet on the gene expression in one specific cell type of the liver was evaluated using microarrays, which gives a more clear-cut view on the specific biological process that are affected by the Western type diet. We therefore stress the importance of using an isolated single cell type for further studies using microarray analysis.

In summary, in the present study, genes differentially expressed in liver parenchymal cells on a Western type diet were examined using microarray analysis and real-time PCR. The microarray data indicate that the Western type diet affects transcriptional programs involving lipid, fatty acid, and steroid metabolism such as glycolysis and lipogenesis.

In conclusion, we suggest that liver parenchymal cells in response to an increased dietary lipid level stimulate glycolysis and lipogenesis to maintain (intracellular) homeostasis. In addition, our studies suggest a very important role for SREBP and LXR transcription factors in the (initial) response of the parenchymal cells to the increased dietary lipid levels (Fig.5)

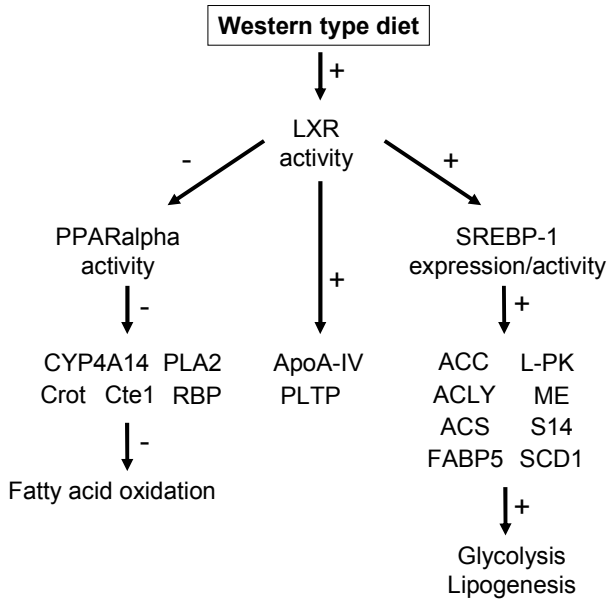

Fig.5. Mechanistic overview of the Western type diet effect on pathways involved in glucose and lipid metabolism in isolated parenchymal liver cells. The Western type diet induces the activity of the nuclear receptor LXR in parenchymal liver cells. The increased LXR activation results in 1) an inhibition of the activity of the nuclear receptor PPARalpha leading to a subsequently decreased expression of genes involved in fatty acid oxidation, 2) an increased expression of the LXR target genes PLTP and ApoA-IV, and 3) an increased expression/activity of SREBP-1 resulting in stimulation of the expression of genes involved in glycolysis and lipogenesis.

9.5 Acknowledgements

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10

Summary and Perspectives

10.1 Summary

High serum VLDL and LDL, and low serum HDL levels are important risk factors for the development of atherosclerosis, the main cause of cardiovascular diseases and thus death in the Western world [1,2]. Regulation of serum lipid levels is exerted by the liver since this organ is able to remove lipid from the blood circulation by mediating the uptake of lipoproteins [3,4]. In addition, the liver is responsible for the production/secretion of VLDL and HDL [5,6]. Inside the liver, control of intra-hepatic cholesterol homeostasis is maintained by an appropriate balance between the regulatory free cholesterol and the more inert cholesterol ester pool and by secretion of cholesterol into bile and/or conversion into bile acids. In this thesis the modulation of hepatic gene expression in response to diet induced metabolic changes is studied. Furthermore, the specific role of the HDL receptor SR-BI in these responses is analysed.

10.1.1 Multiple roles for hepatic SR-BI in metabolism

The liver is able to remove HDL cholesterol via several different routes; the LDL receptor/LRP1 uptake system mediates whole particle uptake of ApoE-enriched HDL [7] and more importantly SR-BI is able to mediate the selective uptake of HDL cholesterol esters into the liver without internalization and degradation of the HDL particle [8].
Since its discovery and cloning in 1994, SR-BI has been characterized as a multi-ligand receptor which plays an important role in the selective uptake of cholesterol esters from HDL. However, no quantitative data on the importance of HDL cholesterol ester uptake via SR-BI were available. In **chapter 2** data are presented which indicate that absence of functional SR-BI in mice results in a significant decrease in the clearance rate of HDL cholesterol esters from the circulation. In addition, we show that the uptake of cholesterol esters in the liver and adrenals is greatly affected (~90% decrease), with a total absence of selective uptake of cholesterol esters. We therefore conclude that SR-BI is the sole molecule involved in the selective uptake of cholesterol esters in the liver and adrenals from HDL.
Interestingly, recent data from Out et al. [9] have suggested a role for SR-BI in the removal of ApoB-containing lipoproteins by mediating the initial association of remnants while the subsequent internalization occurs via other receptor systems such as the LDL receptor and LRP1. In **chapter 3** it

is shown that hepatic overexpression of SR-BI results in decreased serum VLDL levels and a lower postprandial serum triglyceride response upon an intragastric fat load. These data establish that upregulation of SR-BI expression will lower the level of atherogenic chylomicron remnants. Type II diabetes is a disease with a heterogeneous phenotype, which is primarily characterized by an increase in serum glucose levels [10]. However, patients suffering from diabetes usually also have lipid abnormalities, such as hypertriglyceridemia. Interestingly, Osgood et al. have recently observed that there appears to be a significant interaction between variation at the SR-BI locus and type II diabetes in determining the lipoprotein profile [11]. Since SR-BI might thus play a role in the pathology of diabetes, in **chapter 4** the role of SR-BI in glucose metabolism was evaluated. Strikingly, SR-BI deficiency, due to a hampered hepatic uptake of lipoprotein-associated fatty acids, results in a decreased hepatic expression of HMG-CoA synthase, the rate-limiting enzyme in ketogenesis. The lower production of ketone bodies leads to a subsequent reduction in fasting serum glucose levels. In addition to its role in lipid metabolism, our data indicate that functional SR-BI is important for the maintenance of adequate fasting serum glucose levels.

10.1.2 Nuclear receptors modulate hepatic lipid metabolism

Diets enriched in lipid have been widely used to study the metabolic effect of changes in serum lipid levels on total body lipid metabolism and atherogenesis. Several orphan nuclear receptors have been suggested to play a role in the regulation of (hepatic) lipid metabolism. Recent data have indicated that a specific diet containing 0.25% cholesterol and 15% fat does induce atherogenesis in ApoE [12], SR-BI [13], and LDL receptor [12,14,15] deficient mice, but not in wild-type mice [13]. Since the liver plays an important role in lipid metabolism, in **chapter 5** it was evaluated whether hepatic nuclear receptor-mediated regulation of gene expression plays a role in the absence of atherosclerotic lesion formation in wild-type mice. Feeding C57BL/6 mice the high cholesterol/high fat diet induces an increased hepatic activation of the nuclear receptors LXR and PPARalpha resulting in significant changes in the hepatic expression of genes involved in the maintenance of hepatic and total body lipid homeostasis, such as ABCA1 (HDL production), BSEP, CYP3A11, CYP7A1, SHP (bile acid synthesis and metabolism), HMG-CoA reductase and SREBP-1 (lipid synthesis), CD36, PPARgamma (adipogenesis), and SCD1 (VLDL production). The net result of these adaptive changes leads to a non-atherogenic serum lipoprotein profile, thus preventing the development of atherosclerotic lesions in the vessel wall.
In contrast, adding taurocholic acid, a naturally occurring bile acid that is able to activate FXR, to a regular chow diet results in a markedly increased atherosclerotic lesion formation in ApoE deficient mice, a well-established atherosclerosis mouse model (**chapter 6**). Recent data have suggested that therapeutic activation of FXR might be beneficial in treating or preventing cholesterol gallstone disease [16,17]. In agreement, in chapter 6 it is shown that taurocholic acid does inhibit hepatic bile acid synthesis by decreasing CYP7A1 expression ~20-fold through stimulation of the

expression of nuclear receptor SHP. However, due to a subsequent rise in the intra-hepatic free cholesterol level, taurocholic acid treatment also resulted in a downregulation of the hepatic LDL receptor expression and a concomitant rise in the serum total cholesterol level of the pro-atherogenic lipoprotein VLDL leading to the enhanced atherosclerotic lesion development. In the light that FXR agonists are promising therapeutic agents for treating or preventing gallstone disease, the observed increase in atherosclerotic lesion formation can be considered as contraindication for pharmaceutical application.

10.1.3 Cell-type specific expression and regulation of genes involved lipoprotein metabolism

The liver consists of different cell types, which have their specific localization and functions. It is of interest to see whether differences in localization and/or functions are associated with differences in the expression of genes involved in lipid metabolism. In **chapter 7** the relative gene expression of a set of genes involved in hepatic lipid metabolism was investigated in rat liver parenchymal, endothelial, and Kupffer cells (tissue macrophages). In agreement with their metabolic phenotype, parenchymal cells contain high expression levels compared with endothelial and Kupffer cells of genes involved in the uptake of cholesterol (SR-BI), the metabolism of cholesterol to bile acids (CYP7A1) and the direct efflux of cholesterol to bile (ABCG5/G8). Strikingly, under normal conditions, the expression of a novel gene that has been proposed to play a role in hepatic (parenchymal) biliary cholesterol efflux, ABCG1, is particularly high in liver endothelial and Kupffer cells as compared to parenchymal cells. In addition, the expression level of nuclear receptors (**chapter 7**) and their regulation (**chapter 8**) in the specific cell types of the liver is quite different. A relatively high expression of PPARalpha and PPARgamma is observed in parenchymal cells, whilst LXRalpha, LXRbeta, and PPARdelta are equally expressed in parenchymal, endothelial, and Kupffer cells. Furthermore, atherogenic diet induced lipid loading of parenchymal and Kupffer cells results in markedly different responses in the specific cell types. Lipid loading of parenchymal cells leads to moderate gene expression changes, while Kupffer cells respond highly adaptive by stimulating the expression of genes involved in (regulation of) cholesterol metabolism and transport. These findings stress that is very important to determine the expression and nuclear receptor-mediated regulation of genes involved in lipid metabolism not only in complete liver, but also to attribute this regulatory effect to the specific cell types of the liver. In parallel, in **chapter 9** the effect of a Western type diet on gene expression patterns specifically in the parenchymal cells was evaluated to gain insight into the response of parenchymal cells to a rise in dietary lipid levels. Microarray analysis data suggest that liver parenchymal cells in response to an increased dietary lipid level stimulate the expression of genes involved in glycolysis (pyruvate kinase) and lipogenesis (ACLY, ACC, ME, SCD1) to maintain (intracellular) homeostasis. In addition, based upon the gene expression profile, it is suggested that SREBP and LXR transcription factors play a very prominant role in the (initial) response of the parenchymal cells to the increased dietary lipid levels.

10.2 Perspectives

In this thesis it has been shown that hepatic gene expression is highly adaptive and can lead to significant alterations in (hepatic) lipid metabolism. This knowledge can lead to novel approaches to treat lipid disorders such as atherosclerosis and consequently cardiovascular disease. In particular the role of nuclear receptors in the modulation of gene expression is of interest, since hepatic activation of LXR and PPARalpha may prevent atherosclerotic lesion formation, whilst hepatic FXR activation results in a stimulation of atherogenesis. Based on these findings, it can be suggested that development of drugs that stimulate the activity of LXR and PPARalpha might be beneficial in the treatment of atherosclerotic lesion formation. In this respect, it is very promising to see that co-administration of a LXR agonist and a PPARalpha agonist in mice reduces concentrations of circulating triglycerides while simultaneously raising HDL concentrations, generating a more anti-atherogenic lipoprotein profile [18]. However, treatment with synthetic LXR ligands also induces lipogenic enzymes such as SREBP-1 and fatty acid synthase resulting in increased hepatic triglyceride synthesis, hypertriglyceridmia, and hepatic steatosis, which can be considered as a contraindication for pharmaceutical application. Interestingly, the recent finding by Miao et al. [19] that tissue and gene selective modulation is possible with selective LXR modulators opens up the possibility that selective LXR modulators will only affect genes involved in HDL production (e.g. ABCA1), but not lipogenesis, which offers the potential of anti-atherogenic applications in combination with PPARalpha activators.

Importantly, in addition to its role in hepatic lipid metabolism, activation of PPARalpha in the vessel wall has also been associated with local inhibition of the progression of atherosclerosis, since PPAR activators were shown to inhibit the activation of inflammatory response genes (such as IL-2, IL-6, IL-8, TNF alpha and metalloproteases) by negatively interfering with the NF-kappa B, STAT and AP-1 signalling pathways in cells of the vascular wall (reviewed by Fruchart et al. [20]). Furthermore, PPARalpha activation leads to inhibition of the expression of monocyte-recruiting proteins such as adhesion molecules [21], which are very important for the initiation of atherosclerosis.

In conclusion, modulation of the activity of nuclear receptors in the liver might provide a new tool to treat lipid disorders and consequently the initiation and progression of atherosclerotic lesions. It will be interesting to see whether indeed gene selective nuclear receptor modulators can be developed, opening new approaches to treat cardiovascular disease.

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11

Samenvatting voor niet-ingewijden

Hart- en vaatziekten zijn doodsoorzaak nummer 1 in Nederland en andere westerse landen en de verwachting is dat in de nabije toekomst het aantal sterfgevallen door hart- en vaatziekten sterk zal toenemen doordat mensen gemiddeld genomen minder bewegen en ongezonder eten. Een belangrijke oorzaak van hart- en vaatziekten is de vernauwing van de bloedvaten door een verdikking van de wand van het bloedvat als gevolg van een plaatselijke ophoping van vetten (lipiden), ook wel bekend onder de term “atherosclerose”.

Cholesterol, een van de belangrijkste vetten in het lichaam, is een component van celmembranen en een precursor voor de aanmaak van steroïdhormonen, regulatoren van fysiologische functies, en galzouten, die noodzakelijk zijn voor de galvorming en opname van vetten en vetoplosbare vitaminen in de darm. Cholesterol en andere vetten zijn niet oplosbaar in bloed en worden daarom door het lichaam vervoerd in bolvormige deeltjes, de lipoproteïnen. Afhankelijk van de grootte, samenstelling en dichtheid kunnen 4 verschillende soorten lipoproteïnen worden onderscheiden: chylomicronen, zeer lage dichtheids lipoproteïnen (VLDL), lage dichtheids lipoproteïnen (LDL), en hoge dichtheids lipoproteïnen (HDL). Een hoog VLDL en LDL cholesterolgehalte vormt een belangrijke *risicofactor* voor het ontstaan van hart- en vaatziekten, terwijl een hoog HDL cholesterolgehalte *beschermt* tegen hart- en vaatziekten.
In het lichaam speelt de lever een belangrijke rol in de huishouding van cholesterol en andere vetten. Ten eerste maakt de lever lipoproteïnen zoals VLDL en HDL. Echter de lever kan ook lipoproteïnen (en dus vetten) uit het bloed kan verwijderen om ze vervolgens uit te scheiden in de gal door middel van diverse eiwitten, receptoren en enzymen.
In dit proefschrift wordt de regulatie van receptoren en enzymen die van belang zijn voor de vethuishouding bestudeerd, aangezien meer inzicht in de regulatie kan leiden tot de ontwikkeling van geneesmiddelen die het vetmetabolisme optimaliseren.

De lever verwijdert cholesterol uit het bloed via verschillende receptoren en routes. De LDL receptor en LRP1 zijn voornamelijk betrokken bij de opname van VLDL en LDL deeltjes, terwijl scavenger receptor BI (SR-BI) een belangrijke receptor is voor de *selectieve* verwijdering van cholesterol uit HDL zonder opname van het gehele deeltje. Eerdere studies hadden al aangetoond dat SR-BI een rol speelt in de *selectieve* opname van cholesterol uit HDL, maar de kwantitatieve bijdrage van SR-BI in dit proces was nog onbekend. In **hoofdstuk 2** worden kwantitatieve data

gepresenteerd die aantonen dat SR-BI het *enige* molecuul is dat een rol speelt bij de selectieve opname van HDL's cholesterol uit het bloed. Alleen de lever en bijnier tonen selectieve opname van cholesterol uit HDL, de twee organen waar het SR-BI eiwit onder normale condities in grote hoeveelheid aanwezig is. Muizen die het functionele SR-BI eiwit missen, zogenaamde SR-BI knockout muizen, laten een zeer verminderde opname van HDL cholesterol in de lever en bijnieren zien wat tevens leidt tot een sterk geremde verwijdering van HDL cholesterol uit het bloed. Recent is aangetoond dat SR-BI ook een rol speelt bij de opname van andere lipoproteïnen dan HDL, omdat de binding van VLDL en chylomicronen aan levercellen sterk is geremd in SR-BI knockout muizen. **Hoofdstuk 3** beschrijft proeven om deze gepostuleerde nieuwe functie van SR-BI te ondersteunen. De hoeveelheid SR-BI eiwit in de lever van muizen is met behulp van een SR-BI bevattend adenovirus (een verkoudheidsvirus dat eiwitten tot expressie kan brengen) sterk verhoogd, waardoor de opname van VLDL en chylomicronen sterk werd verhoogd. Dit impliceert dat SR-BI, naast zijn rol in HDL cholesterol opname, inderdaad ook een rol speelt in de verwijdering van VLDL and chylomicronen uit het bloed door de lever. Opmerkelijk genoeg hebben SR-BI knockout muizen onder gevaste condities een lagere bloed glucose spiegel dan muizen die het SR-BI eiwit wel hebben. In **hoofdstuk 4** wordt aangetoond dat dit waarschijnlijk een gevolg is van de lagere opname door de lever van vetzuren (getransporteerd in lipoproteïnen). Het blijkt dus dat SR-BI niet alleen een rol speelt in de huishouding van vetten, maar dat dit ook consequenties heeft voor de glucosehuishouding. Verstoringen van de glucosehuishouding zijn geassocieerd met verschillende aandoeningen zoals diabetes (door te veel glucose in het bloed) en coma in kinderen na vasten (door te weinig glucose in het bloed).

In muizen kan vaatvernauwing bij de mens soms worden nagebootst door muizen specifieke diëten te geven met een hoog cholesterol gehalte. Teneinde de dieet geïnduceerde veranderingen in genetische expressie te analyseren werd in **hoofdstuk 5** bestudeerd wat het effect was van een cholesterol/vet rijk (westers) dieet op de aanwezigheid van receptoren en enzymen in de lever van muizen. Het Westerse dieet induceerde significante veranderingen in de activiteit van de transcriptiefactoren: liver X receptor (LXR) en peroxisome proliferator-activated receptor (PPAR) alpha, leidend tot veranderingen in de genactiviteit van genen betrokken bij HDL en VLDL aanmaak, galzuur synthese en transport, en vetophoping in de lever. Echter, de veranderingen in genexpressie in de lever zorgden ervoor dat in normale muizen de pro- en anti-atherosclerotische balans in de concentratie van de verschillende lipoproteïnen in het bloed onveranderd was, wat verklaart dat de normale muizen op het Westerse dieet in tegenstelling tot mensen geen atherosclerose ontwikkelen. Activatie van een andere transcriptiefactor, farnesoid X receptor (FXR), in de lever van een muis model dat wel atherosclerose ontwikkelt op het Westerse dieet (ApoE knockout muizen) leidde tot meer vorming van atherosclerose, hetgeen verklaard kan worden door een 50% daling van de LDL receptor in de lever, (**hoofdstuk 6**). Activatoren van FXR zijn recentelijk aangedragen als mogelijke geneesmiddelen tegen galstenen. De toename in de

hoeveelheid atherosclerose zoals geconstateerd in muizen, en dus mogelijk hart- en vaatziekten, biedt geen goed perspectief dat FXR activatoren toepassing kunnen vinden als geneesmiddelen ter behandeling/voorkoming van galstenen.

De lever is een complex orgaan bestaande uit verschillende soorten cellen. Kupffercellen (macrofagen in de lever) en endotheelcellen beschermen de lever tegen schadelijke stoffen en bacteriën, terwijl de lever parenchymcellen de opname van cholesterol uit het bloed en de daaraan gekoppelde uitscheiding in de gal voor hun rekening nemen. Omdat de verschillende celtypen zulke specifieke functies hebben, is het goed mogelijk er tussen de cellen ook verschillen zijn wat betreft de activiteit van genen/eiwitten betrokken bij de vethuishouding. In **hoofdstuk 7** is de genexpressie van genen betrokken bij de opname en verwerking van cholesterol tot galzuren in de verschillende celtypen onder normale condities bestudeerd. In overeenstemming met hun rol in de cholesterol huishuishouding bevatten parenchymcellen een hoge genexpressie van SR-BI, cholesterol 7α-hydroxylase (CYP7A1) en de ATP-binding cassette transporters G5 en G8 (ABCG5/G8). Deze eiwitten zijn betrokken bij de opname van cholesterol uit het bloed en de daarop volgende uitscheiding in de gal. Opvallend genoeg was de expressie van ABCG1, een eiwit waarvan met aannam dat het een rol speelt in het cholesterol metabolisme, relatief zeer hoog in de endotheel- en Kupffercellen in vergelijking met de parenchymcellen. Ook de aanwezigheid van transcriptiefactoren betrokken bij de regulatie van cholesterol huishouding (LXR, PPAR) was zeer verschillend tussen de diverse cellen onder normale condities. In **hoofdstuk 8** wordt verder bewijs geleverd dat de verschillende celtypen in de lever specifieke functies vervullen in het cholesterol metabolisme. Parenchymcellen reageren met relatief kleine veranderingen in genexpressie wanneer muizen een hoog cholesterol dieet ondergaan, terwijl in Kupffercellen de aanwezigheid van meerdere genen betrokken bij de verwijdering van cholesterol uit de cellen (ABCA1, ABCG5/G8, en CYP7A1) sterk vermeerderd wordt. Teneinde een verklaring te vinden voor de beperkte response van de parenchymcellen is in **hoofdstuk 9** het tijdsafhankelijke effect van een Westers dieet op de activiteit van genen in de lever parenchymcellen bepaald. Grootschalige genexpressie profielen (>30.000 genen) werden verkregen met behulp van zogenaamde microarray (gen-chip) arrays. Uit de analyses blijkt dat de aanwezigheid van eiwitten betrokken bij de afbraak van glucose (glycolyse) en de daaraan gekoppelde aanmaak van vet (lipogenese) sterk verhoogd was in parenchym cellen op het westerse dieet. Interessant genoeg bleek verder dat de initiële veranderingen door het dieet voornamelijk aangestuurd werden door de transcriptiefactoren LXR en SREBP. Het grote adaptatievermogen van de complexe metabole paden in de leverparenchymcel verklaart de relatief kleine veranderingen in genexpressie van specifieke genen op een hoog cholesterol dieet.

Perspectief

In dit proefschrift is beschreven dat de lever zich zeer snel kan aanpassen aan veranderingen in haar omgeving door de expressie van bepaalde regulatoren te veranderen, leidend tot significante aanpassingen in de (lever) vethuishouding. Deze kennis kan goed van pas komen bij de zoektocht naar nieuwe behandelingsmethoden voor aandoeningen zoals atherosclerose en dus hart- en vaatziekten. Vooral de identificatie van de transcriptiefactoren LXR en PPARalpha als cruciale eiwitten in de regulatie van de vethuishouding is belangrijk. Recentelijk is al veel onderzoek gedaan naar de effecten van activatoren van de beide transcriptiefactoren LXR en PPARalpha op hart- en vaatziekten in muizen, omdat deze eiwitten een grote rol spelen in de vethuishouding door de lever en omdat PPAR activatoren ook plaatselijk de ontstekingsreactie in de vaatwand kunnen remmen. Uit deze studies is duidelijk geworden dat het belangrijk is om activatoren te ontwikkelen die lokaal de aanwezigheid van specifieke genen/eiwitten in de lever of in de vaatwand kunnen moduleren. Hierdoor kunnen nadelige bijeffecten (bv. een leververvetting) worden voorkomen. Het zal interessant zijn om te zien of daadwerkelijk lokaalwerkende gen-selectieve activatoren kunnen worden ontwikkeld, als nieuwe effectieve therapieën voor de bestrijding van hart- en vaatziekten.

Abbreviations

18SrRNA	18S ribosomal RNA
36B4	Acidic ribosomal phosphoprotein P0
ABC	ATP-binding cassette transporter
Apo	Apolipoprotein
BSEP	Bile salt efflux pump
CETP	Cholesterol ester transfer protein
CYP7A1	Cholesterol 7α-hydroxylase
EC	Endothelial cell
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDL	High-density lipoprotein
HMGCR	HMG-CoA reductase
HMGCS	HMG-CoA synthase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
FXR	Farnesoid X receptor
KC	Kupffer cell
KO	Knockout
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LRP1	LDL receptor-related protein
LXR	Liver X receptor
MTP	Microsomal triglyceride transfer protein
PC	Parenchymal cell
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
SHP	Small heterodimer partner
SR-BI	Scavenger receptor class B type I
SREBP	Sterol regulatory element binding protein
VLDL	Very low-density lipoprotein
WT	Wild-type

Full papers

M. Van Eck, J. Twisk, M. Hoekstra, B.T. Van Rij, C.A. Van der Lans, I.S.T. Bos, J.K. Kruijt, F. Kuipers, and Th.J.C. van Berkel (2003) Differential effects of scavenger receptor BI deficiency on lipid metabolism in cells of the arterial wall and in the liver. *J Biol Chem*. **278**:23699-23705

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 16 december 1977 te Leiderdorp. In juni 1996 werd het VWO diploma behaald aan het Stedelijk Gymnasium te Leiden. In datzelfde jaar werd begonnen met de studie Biofarmaceutische Wetenschappen aan de Universiteit van Leiden. Hiervan werd het propedeutisch examen behaald in mei 1998. Van juli 1999 tot en met juni 2000 werd in het kader van de hoofdvakstage onderzoek verricht binnen de vakgroepen Toxicologie en Medische Farmacologie van het Leiden/Amsterdam Center for Drug Research (LACDR) onder leiding van Prof.dr. G. Mulder en Dr. E. Vreugdenhil. Onderwerp van de stage was het bepalen van de rol van CaMK-VI in adenosine geïnduceerde apoptose. Van juni 2000 tot april 2001 werd een tweede stage gevolgd bij de afdeling Vascular Biology van GlaxoSmithKline (GSK) te Harlow, Engeland onder begeleiding van Dr. P.H. Groot en Dr. L. Patel. Tijdens deze stage werd onderzoek verricht naar de genexpressie van 7TM receptoren en hun liganden in humane atherosclerotische plaques, aneurysma's en gezonde bloedvaten. Het doctoraal examen Biofarmaceutische Wetenschappen werd behaald in juni 2001. Van mei 2001 tot mei 2005 werd als assistent-in-opleiding het in dit proefschrift beschreven onderzoek uitgevoerd bij de vakgroep Biofarmacie van het Leiden/Amsterdam Center for Drug Research (LACDR) onder leiding van Prof.dr. Th.J.C. van Berkel binnen het door NWO gesubsidieerde project 'The role of scavenger receptor BI (SR-BI) in HDL metabolism and the prevention/regression of atherosclerosis'.