Regulation of ABCA1 in hepatocytes by the nuclear receptor PXR

German Tapia

Thesis for the Master of Science Degree in Molecular Biology

Department of Molecular Biosciences

University of Oslo, August 2005

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Acknowledgements

The present work was carried out from January 2004 to May 2005 in the laboratory of Professor Trond Berg at the department of Molecular Biosciences, University of Oslo.

First of all I would like to thank Professor Trond Berg for letting me study in his laboratory and his good humor. He has been of great help both during the master study and during the undergraduate courses. I deeply thank him and the rest of the staff at the University of Oslo for teaching me the fundamentals of biology and chemistry.

I am extremely grateful to Cand. Scient Marita Sporstøl for her enthusiasm and great help during this work. Without her encouragement and tutelage, this work would not have been possible. I would also like to thank the rest of the laboratory (Ali, Maria, Mehrdad) for their help and for the time we had together, and the rest of the department (including Professor Tor Gjøens Group) for all the help and friendliness. I would like to thank my family for support and encouragement.

And without my friends, this last five years would have been wasted.

Oslo, August 2005

German Tapia

Abbreviations

Acetylated LDL (Ac-LDL)
Activation Function (AF)
Apolipoprotein (apo)
ATP-Binding Cassette (ABC)
Bile Acid Export Pump (BSEP)
Cell Adhesion Molecules (CAMs)
Cholesterol 7α-hydroxylase (CYP7A1)
Cholesteryl Ester Transfer Protein (CETP)
Cholesteryl Esters (CE)
Cornary Artery Disease (CAD)
C-Reactive Protein (CRP)
Dexamethasone (DEX)
Direct Repeat (DR)
DNA Binding Domain (DBD)
Endothelial Cells (EC)
Endothelial NO synthase (eNOS)
Everted Repeat (ER)
Farnesoid X Receptor (FXR)
Free Cholesterol (FC)
High Density Lipoprotein (HDL)
High Density Lipoprotein Cholesterol (HDL-C)
Interleukin-1 (IL-1)
Intermediate Density Lipoprotein (IDL)
Intercellular Cell Adhesion Molecule (ICAM)
Inverted Repeat (IR)
Lecithin:cholesterol Acetyl Transferase (LCAT)
Lithocholic Acid (LCA)
Liver X Receptor (LXR)
Low Density Lipoprotein (LDL)
Low Density Lipoprotein Cholesterol (LDL-C)
Monocyte Chemoattractant Protein-1 (MCP-1)
Monocyte Colony Stimulating Factor (M-CSF)
Multi-Drug Resistance Protein 2 (MDR2)
Multidrug Resistance-associated Protein 2 (MRP2)
Nitric Oxide (NO)
Nuclear Receptor (NR)
Nucleotide Binding Domain (NBD)
Oxidized LDL (Ox-LDL)
Organic Anion-Transporting polypeptide (OATP2)
Paraoxonase (PON)
Peroxisome Proliferator-Activated Receptor (PPAR)
Phospholipid Transfer Protein (PLTP)
Phospholipids (PL)
Platelet-Activating Factor (PAF)
Platelet-activating Factor Acetylhydrolase (PAF-AH)
Pregnane X Receptor (PXR)
Pregnenolone 16α-carbonitrile (PCN)
Prostacyclin (PGI2)
Retinoid X Receptor (RXR)
Reverse Cholesterol Transport (RCT)
Scavenger Receptor A (SR-A)
Scavenger Receptor BI (SR-BI)
Scavenger Receptor B antigen (CD36)
Smooth Muscle Cells (SMC)
Steroid and Xenobiotic Receptor (SXR)
Sterol Regulatory Element Binding Protein-1c (SREBP-1c)
Tangier Disease (TD)
Transmembrane Domain (TMD)
Triacylglycerol (TG)
Tumor Necrosis Factor α (TNF-α)
Vascular Cell Adhesion Molecule-1 (VCAM-1)
Very Low Density Lipoprotein (VLDL)
Von Willebrand Factor (vWF)

General introduction

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial system that is influenced by genetical and environmental factors (reviewed in [1, 2]). Among these are genetic makeup, diet, smoking, sex, age and several other factors - and it is the primary cause of stroke and coronary artery disease (CAD). CAD is the leading cause of death in industrialized countries [3-5].

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements, called atherosclerotic lesions, in the large arteries [6]. These lesions are called the fatty streak, the intermediate fibrous cap or advanced complicated lesions [3] after how advanced the development is. Even though advanced lesions can grow large enough to block blood flow, the most important complication takes place when a thrombus or blood clot is formed as a result of a rupture or erosion of the lesion [2]. The rupturing of the plaques leads to thrombosis (reviewed in [7]), which is an acute clinical event that can lead to death or permanent damage by myocardial infarction or stroke.

Atherosclerosis and CAD are usually considered to be a result of an unhealthy lifestyle, but genetical makeup predisposes many people that live relatively healthy lives. Inflammation is recognized as playing a key role in atherosclerosis [8] and considered the primary risk factor. Only about half of the patients with atherosclerosis actually manifest hyperlipidemia, the major secondary risk factor that is usually targeted for intervention [9, 10].

Cholesterol is a well known risk factor for CAD, even among the general public. High plasma levels of cholesterol (>160 mg/dl [11]) increases the risk for complications of atherosclerosis. Although a simple preventative step against CAD would be to keep

plasma cholesterol levels reduced to below 150 mg/dl, this is unlikely to be achieved in the near future [12].

Cholesterol is an essential component of cell membranes, providing stability and allowing for transmembrane transport. Cholesterol is also the precursor of multiple metabolic pathways (adrenal steroids, sex hormones, vitamins and bile acids) [13, 14] and a major component of lipoproteins, but is not actually an essential part of the diet as it is synthesized from acetyl coenzyme A [15].

Because lipids are sparingly soluble in aqueous solutions they are transported as components of lipoproteins. Cholesterol is mainly transported in the blood in the form of cholesteryl esters (CE) associated with lipoproteins. Lipoproteins are globular, micelle-like particles that consist of a nonpolar core of triacylglycerols (TGs) and CE surrounded by an amphiphilic coating of protein, phospholipids and cholesterol [15]. Lipoproteins are classified in five broad categories depending on their functional and physical properties; chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons transports dietary TG and cholesterol from the intestines to the tissues. VLDL, LDL and IDL transport endogenous TG and cholesterol from the liver to the tissues. HDL transports endogenous cholesterol from the tissues to the liver [15], and has several important antiatherogenic properties described later. The apolipoproteins (apos) have a high helix content, which increases when they are incorporated in lipoproteins [16].

The excess cholesterol must be eliminated. Although the sterol core is not degradable in the body, cholesterol is a precursor for bile acids, which are small water soluble molecules with detergent-like properties [15]. The only quantitatively significant sink for excess cholesterol is the liver [17], owing to its unique ability to synthesize bile acids. The transport of cholesterol from extrahepatic tissues to the liver is termed *reverse cholesterol transport* (RCT) [18, 19] and will be discussed in more detail later. Both the input and output cholesterol pathways are regulated coordinately by nuclear receptors (NRs) to achieve homeostasis (reviewed in [20]).

Cellular and molecular interactions in atherosclerosis

Several factors that contribute to vascular damage have been identified. Hyperlipidemia, raised levels of homocysteine, cigarette smoking, viral infections and oxidized lipids and proteins (in particular oxidized LDL (ox-LDL)) [21] are some of these. Cells of the arterial wall secrete themselves oxidants that may oxidize LDL [11].

It is a known fact that high levels of low-density lipoprotein cholesterol (LDL-C) and low levels of high density lipoprotein cholesterol (HDL-C) are major contributing factors in the development of atherosclerosis [22-24]. On the other hand, several studies have shown that high levels of HDL-C are inversely associated with CAD risk [23, 25]. LDL in itself does not promote atherosclerosis, but with high levels of LDL in plasma LDL metabolism in monocytes and endothelial cells (EC) will deplete the antioxidant pool, resulting in mild oxidation of LDL [26]. LDL may also be oxidized through other means such as transition metals and cellular enzymes [27].

EC play an important role in the inflammatory process. Dysfunction and/or damage to the endothelium initiate a number of events that promote atherosclerosis, including increased endothelial permeability, platelet aggregation, leukocyte adhesion and generation of cytokines [28]. Cells in regions of arterial branching or curvature show increased permeability to macromolecules such as LDL and are the preferential sites for lesion formation. This is because fluid shear stress is among the important physical forces acting on EC, and arterial branching or curvature disturbs the flow [6]. Risk factors for atherosclerosis include typical markers such as C-reactive protein (CRP) [29] which also has a proinflammatory effect [30].

Injury or disease to the endothelium induces expression of cell adhesion molecules (CAMs), such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM), E-selectin and P-selectin [31, 32]. These act as chemoattractants for

monocytes and other leukocytes to adhere to the endothelium and then transmigrate into the intima (attracted by monocyte chemoattractant protein-1 (MCP-1) [33]). The inflammatory properties of lipoproteins stem from the fact that they may induce the activation of EC and expression of cell surface adhesion molecules; among these VCAM-1 and ICAM [34]. Circulating monocytes and T-lymphocytes bind tightly to these cell surface adhesion molecules and will then migrate into the artery wall [35]. Nitric oxide (NO) released from the endothelium normally suppresses expression of these adhesion molecules [36], but reduced NO levels are observed during endothelial dysfunction and inflammation. Decreased production of NO increases the oxidative modification of LDL, which is one of the major mechanisms of atherosclerosis [37]. Impaired activity or production of NO also leads to other events that promote atherosclerosis, as vasoconstriction, platelet aggregation, proliferation and migration of smooth muscle cells (SMC), leukocyte adhesion and oxidative stress [38].

High levels of LDL and modified LDL are well known risk factors for atherosclerosis [22, 24]. LDL becomes trapped in a three-dimensional cage work of fibers and fibrils secreted by the cells in the artery wall after crossing the endothelium [39]. Interactions between apoB and matrix proteoglycans are thought to trap LDL, making apolipoproteins containing apoB atherogenic (LDL, VLDL and IDL) [6, 40]. Accumulation of LDL increases with increased circulating LDL levels, and LDL must be extensively modified to be taken up by macrophages. The extensively modified LDL is recognized by scavenger receptors such as the scavenger receptor B antigen (CD36) and scavenger receptor A (SR-A) [6]. Ox-LDL stimulates EC to secrete factors, like MCP-1 and monocyte colony stimulating factor (M-CSF) that promote differientation of monocytes to macrophages [27]. Macrophages are thought to contribute to atherogenesis though their production of inflammatory mediators and their interactions with modified lipoproteins in the arterial intima [41]. The macrophages modify LDL which may lead to a shift in receptor recognition away from the native LDL receptor to scavenger receptors localized at the macrophage surface [42-44]. Lipid loading activates peroxisome proliferator-activated receptor γ (PPARγ), which in turn up-regulates CD36 expression [41] and further uptake of Ox-LDL into the macrophages [45]. An Ox-LDL binding extracellular matrix is secreted

and engulfed by the macrophages, allowing further lipid accumulation and foam cell transformation [42].

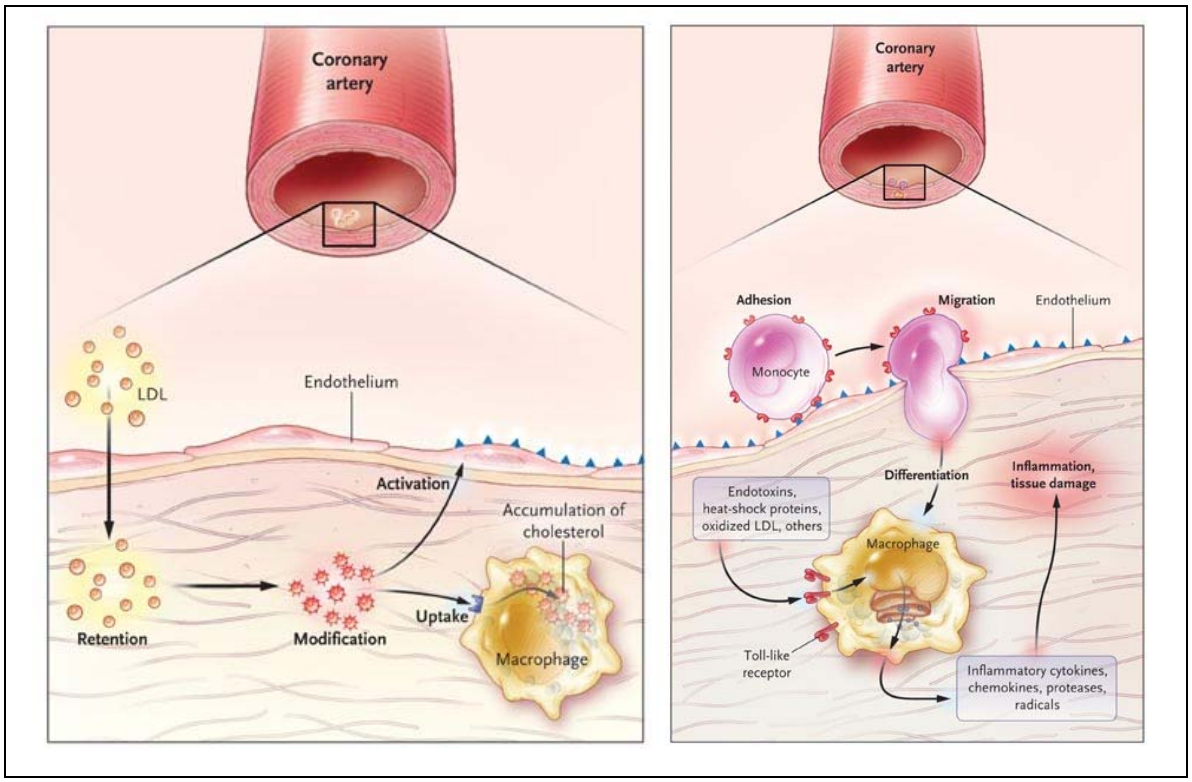

Fig. 1: Early steps in atherosclerosis. Lipoprotein, macrophage intrusion and retention in the endothelium, taken from [46]

In the intima, the monocytes proliferate and differentiate into macrophages that take up the lipoproteins to become foam cells. Activated foam cells produce growth factors and cytokines that influence atherosclerosis development, like the induction of proteases that may weaken the fibrous cap and lead to thrombosis [46, 47]. Antigens presented to T-cells by macrophages and dendritic cells trigger their activation, leading to further inflammatory reactions (cytokine production that activates macrophages and vascular cells) [46]. With time, these foam cells die and their lipid-filled contents contribute to the necrotic core of the lesion (reviewed in [2]).

Accumulation of foam cells leads to monocyte infiltration and smooth muscle cell migration and proliferation [48, 49]. Foam cells in atherosclerotic lesions accumulate free

cholesterol (FC) and CE, and have an increased rate of phospholipid biosynthesis. They accumulate intracellular phospholipids (PL) containing membrane structures called whorls [50]. These accumulated lipids generate extracellular cholesterol crystals within the intermediate fibrous cap lesions when the foam cells die by necrosis [51].

The formation of fatty streaks is due to lipoprotein transport into the artery wall [52] and accumulation of foam cells. The fatty streak is characterized by layers of foam cells and lipid droplets within intimal SMC [53]. The fatty streaks are prevalent in young people, never cause symptoms, and may progress or eventually disappear [46].

Simultaneously with the accumulation of foam cells and lipoprotein transport into the arterial wall, SMC proliferate and generate a fibrous layer of smooth muscle cell-secreted connective tissue that covers the fatty streak, in time forming a more complex lesion called the intermediate fibrous cap [54].

The last stage in atherosclerosis is called the advanced complicated lesions, and is characterized by calcification and hemorrhage into the plaque which may lead to complications [55, 56].

HDL and reverse cholesterol transport

HDL

Numerous studies have found an inverse association between HDL-C and CAD [23, 25, 57]. Several studies have shown that HDL particles, and HDL-associated proteins and lipids, may exert several potential anti-atherogenic effects [58]. Therefore, HDL is considered the "good" lipoprotein. The development of atherosclerotic lesions could be inhibited or even reversed in several genetic animal studies by an elevation of HDL-C concentrations [58].

HDLs are a structurally and functionally heterogeneous class of lipoproteins of high density (1.063 to 1.21 g/mL) and small diameter (5 to 17nm) [32]. HDL particles are multi-

shaped molecules with varying density, fluidity, charge and anti-genicity [59, 60]. Most of them contain apoA-I as the quantitatively most important protein constituent. Other associated proteins are apoA-II, apoC, apoE, lecithin:cholesterol acyltransferase (LCAT), paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH)[32]. The water soluble surface of HDL is formed by apoA-I, and possibly other amphipathic apos (which exert biological actions such as receptor binding and enzyme activation/inactivation [61]), together with PL and unesterified cholesterol [58]. The bulk of HDL is formed by spherical particles that contain a core of water-insoluble CE and to a lesser degree, TG [61].

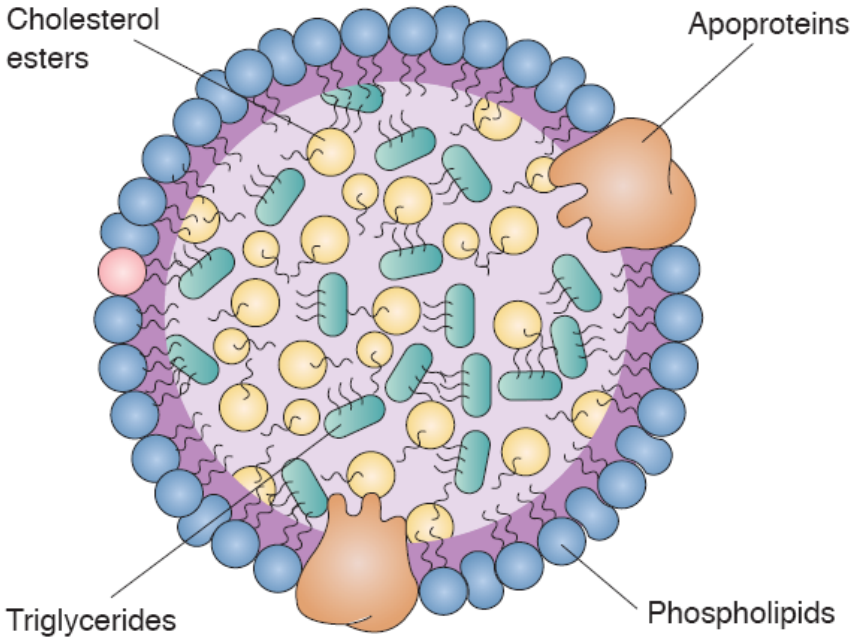

Fig. 2: General structure of a lipoprotein. The CE and TG are located in the hydrophobic core of the macromolecule, surrounded by phospholipids and apoproteins. Taken from an electronic resource page for the book [62] (http://connection.lww.com/Products/porth7e/documents/Ch24/jpg/24_002.jpg).

Two important functions are proposed to be carried out by HDL in the circulation; to serve as a reservoir of apolipoproteins necessary for the metabolism of TG-rich lipoproteins and to mediate cholesterol efflux from extrahepatic tissues to the liver (while apoB containing lipoproteins like VLDL and LDL transport cholesterol from the liver to the tissues) in a process termed reverse cholesterol transport (RCT) [19]. HDL has several other antiatherogenic properties in addition to its key role in RCT.

A critical process in response to injury is regeneration of the endothelium through the proliferation and migration of EC. HDL has been shown to enhanced bovine aortic EC proliferation in culture in a concentration-dependent, specific manner [63] and to stimulate the proliferation of human [64] and bovine [65, 66] vascular EC. HDL inhibits apoptosis of vascular EC, which is suggested to be one of the causes of endothelial injury contributing to various inflammatory disorders and cardiovascular dysfunction [67, 68], such as permeability of the endothelium, blood cell adhesion, proliferation of SMC and increased coagulation. HDL has been shown to protect against apoptosis stimulated by deprivation of growth factors [69] or cytokines [70] (reviewed in [68]).

HDL inhibits expression of E-selectin, P-selectin, ICAM-1 and VCAM-1 stimulated by cytokines (tumor necrosis factor α ((TNF-α), interleukin-1 (IL-1)) [71], Ox-LDL or CRP [30, 32]. HDL inhibits agonist-induced platelet-activating factor (PAF) expression [72]. PAF is a very bioactive protein, which stimulates vascular permeability, cell adhesion, aggregation and smooth muscle contraction [32]. HDL associated proteins like PAF-AH, LCAT and PON inactivates PAF (reviewed in [32]) which results in reduced macrophage homing to endothelium and reduced oxidative stress [73]. HDL and its associated lipids and enzymes inhibit oxidation of LDL. As mentioned, oxidation of LDL is one of the important pathogenic steps in atherosclerosis. PAF-AH [74] and gluthatione selenoperoxidase [75] are two enzymes in HDL that prevent the formation or degrade bioactive LDL oxidation products [76]. PON and apoA-I have antioxidant properties when associated with HDL [77, 78].

HDL increases endothelial NO synthase (eNOS) expression [79], thereby inducing formation of NO. HDL stabilizes prostacyclin (PGI2), a platelet inhibitor and vasodilator [80]. Both NO and PGI2 are antithrombotic because they inhibit platelet aggregation by increasing cGMP and cAMP, respectively [32]. Von Willebrand factor (vWF) is expressed by ECs and has an essential role in platelet adhesion and aggregration [32]. Circulating vWF levels are inversely correlated with plasma HDL [81] so it seems as HDL may inhibit vWF production [32].

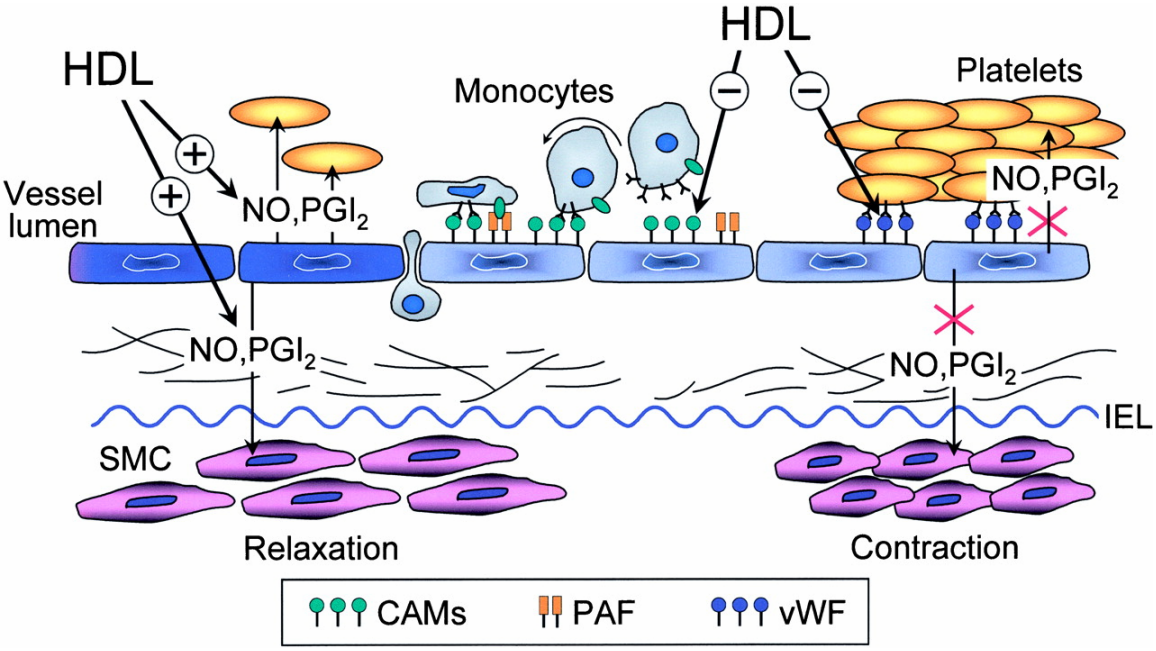

Fig. 3: Multiple biological actions of HDL on vascular endothelium. Functional EC are in dark blue; dysfunctional EC are in light blue. Taken from [32].

Due to these mentioned effects HDL is considered anti-inflammatory. However, HDL loses its anti-inflammatory properties (to the point of becoming pro-inflammatory) during the acute phase response and chronic inflammation [75, 82].

Reverse cholesterol transport

The fact that the sterol core is not degradable means that the body must maintain cholesterol homeostasis by cholesterol efflux. The cholesterol content of most cells is tightly regulated, but macrophages can accumulate large amounts of cholesterol by uncontrolled scavenger receptor uptake of modified lipoproteins and phagocytosis [61]. Efflux is the only mechanism by which macrophages can limit or reverse the cellular cholesterol accumulation, which results in foam cell formation if not held in check.

Cholesterol efflux is a complex process and multiple mechanisms may function depending on cell type, metabolic state, membrane cholesterol pools and the nature of acceptor particles [83]. There are three described mechanisms for cholesterol efflux; simple

(aqueous) diffusion, scavenger receptor BI (SR-BI) mediated (reviewed in [84]) and ATP-binding cassette (ABC) transporter - mediated efflux.

The simplest mechanism is diffusion, where cholesterol passively diffuses through the aqueous phase between plasma membrane to cholesterol acceptor molecules such as serum albumin [85] and HDL [86].

The transport of cholesterol can also be facilitated by SR-BI, which binds HDL with high affinity. It is most highly expressed in liver and steroidogenic tissue [87, 88]. It is suggested that SR-BI tethers HDL at the cell surface [89] and mediates the cellular uptake of CE [90, 91] or promotes HDL mediated cellular cholesterol efflux [89] depending on the gradient. The lipoprotein particle is subsequently released as a lipid-poor particle. Several ligands other than HDL have been shown to bind to SR-BI, among them Ac-LDL and Ox-LDL [92, 93].

The last mechanism, ABC transporter mediated cholesterol efflux, involves the release of cholesterol to lipid-free (mediated by ABCA1 [94, 95]) or lipid-poor (mediated by ABCG1 and ABCG 4 [96]) apolipoproteins, in particular apoA-I. Efflux to circulating apoA-I by ABCA1 generates nascent HDL and is the first stage in HDL biogenesis [97, 98]. LCAT and its cofactor apoA-I promote esterification of cholesterol [99-101] which then migrates into the hydrophobic core, leading to a larger HDL particle called HDL_2. Cholesterol efflux by ABCG1 and ABCG4 [96] further matures HDL. The CE in the core of HDL may be delivered to hepatocytes and steroidgenic tissues by several mechanisms [99, 100], among them SR-BI mediated uptake [90, 102, 103].

Bile acid production from cholesterol and the solubilization of cholesterol by bile acids in the liver are the final steps in RCT, facilitating cholesterol excretion from the body [104].

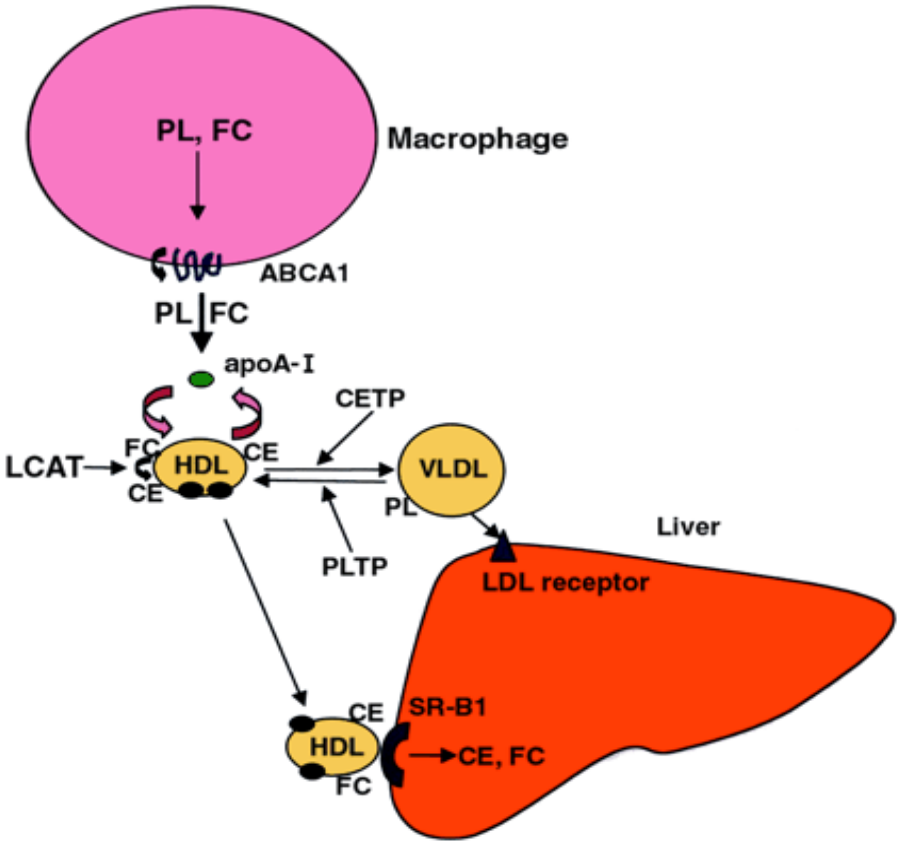

Fig. 4: HDL mediated reverse cholesterol pathway. ABCA1 is involved in transfer of FC and phospholipids (PL) from macrophages to apoA-I. LCAT converts FC to CE. Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) modify HDL by transferring CE and PL between HDL and TG-rich lipoproteins. HDL delivers cholesterol to liver via SR-BI–mediated selective uptake of the lipids. Taken from [105].

Tangier disease

Genetic disorders of HDL metabolism are rare in the general population. Mutations in genes coding for important components or enzymes that are critical for the formation or maturation of HDL, like apoA-I [106], ABCA1[107-110], LCAT[111] or CETP[112], are usually involved. Of course, it is also possible that mutations in these genes might even be beneficial and confer a protective effect. One such disorder is known as Tangier disease (TD) [113]. It was discovered in 1960 and is named after Tangier Island, where the index cases (a couple of siblings) lived [114].

TD is a rare autosomal genetic disorder, a HDL deficiency syndrome. It is characterized by severe HDL and apoA1 deficiency, sterol deposition in tissue macrophages and other reticuloendothelial cells (tonsils, thymus, lymph nodes, bone marrow, spleen, liver etc), and prevalent atherosclerosis [114]. Studies have revealed that a rapid degradation of apoA-I causes severe HDL deficiency. ApoA-I structure and synthesis is normal, but even HDL particles from normal patients are degraded at high rates when infused into TD patients [114]. Homozygotes have a higher incidence of CAD (4 to 6 fold higher, depending on the age group) than normolipidemic subjects [115], but it is not as high as one would expect from a virtual absence of HDL, and it may be that their below normal LDL levels protect them from atherosclerosis [114]. Studies showed that defective removal of cellular cholesterol and phospholipids by lipid-poor apoA-I led to the TD phenotype [116]. Several groups independently identified mutations in ABCA1 (then known as ABC1) as the cause of TD [107-110] in 1999, which led to a breakthrough in our understanding of HDL formation (specifically, the role ABCA1 played in the maturation of HDL).

ABCA1 mutations will not significantly affect the cholesterol levels of most cells, as cholesterol delivery and synthesis are tightly regulated by feedback mechanisms [117, 118]. Macrophages, however, ingest lipoproteins and membrane debris by phagocytic and endocytotic processes that are not repressed by excess cholesterol [119] and rely on efflux mechanisms to prevent massive accumulation of intracellular sterol [120] and development into foam cells.

ABC proteins and ABCA1

ABC proteins

ATP-binding cassette (ABC) transporters are one of the largest protein families and have been found in each kind of organism examined so far [121]. The ABC family has seven subfamilies, grouped by phylogenetic analysis, from ABCA to ABCG [122]. There are about 50 different ABC transporters in *homo sapiens*. ABCs are integral membrane proteins that use ATP as energy to transport various substrates across cell or organelle membranes [121]. The specific mechanism of action for ABCA1 is still unclear. The different ABC transporters have different ligands and functions, from drug resistance to cholesterol efflux, and several mutations in ABC transporters are associated with genetic diseases, some of the more famous being cystic fibrosis and TD [123].

ABC proteins are defined by the ABC unit, a nucleotide binding domain (NBD) that interacts with ATP [121]. The NBD harbors two conserved motifs known as Walker A and Walker B that are involved in ATP binding and are present in many other ATP binding proteins [124]. A third conserved sequence called "ABC signature" which defines the family and is located between the two Walker domains [121]. ABCs are integrated into the membrane via transmembrane domains (TMDs) composed of six transmembrane helices. The minimum requirement for a functioning ABC seems to be two ABC units and two TMDs [120, 121]. ABC genes are organized as full transporters (two TMD and two NBD) or as half transporters containing one TMD and one NBD [125].

Substrate specificity for each transporter is determined by the amino acid sequence in the TMD. Even if sequence similarity is noted, substrate similarity is not implied [123]

ABCA1

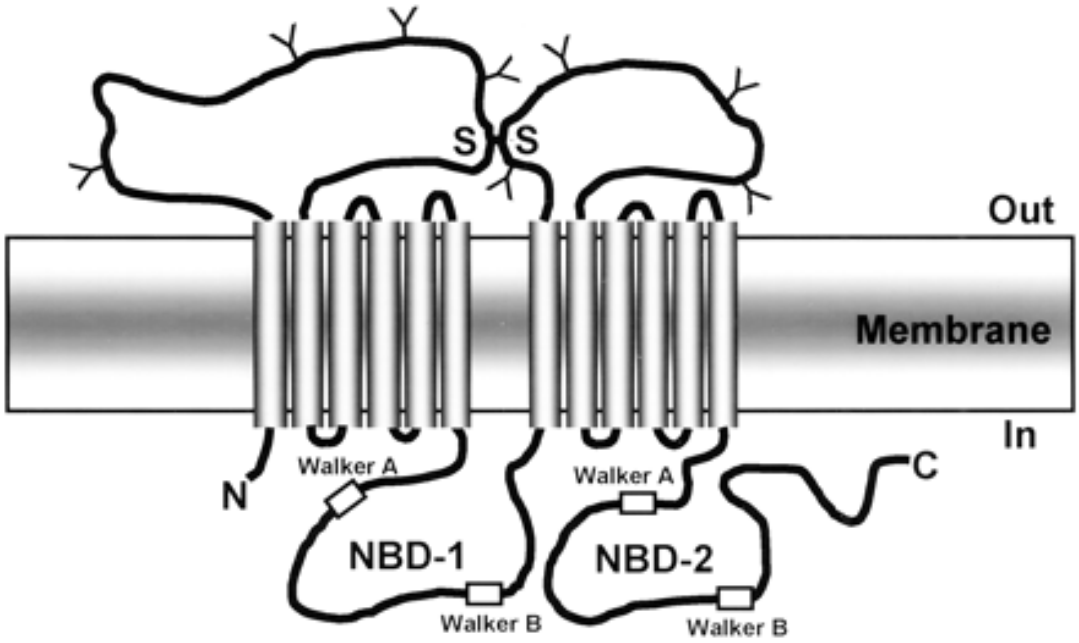

Fig. 5: Topological model of ABCA1, based on studies by [126, 127]. Y marks glycosylation sites; S-S marks a predicted disulfide bond. Taken from [128].

The ABCA subfamily is characterized by the presence of a stretch of hydrophobic amino acids thoughts to span the membrane within the putative regulatory domain [123] and has the classical arrangement (TMD/NBD/TMD/NBD) [129, 130]. The ABCA and ABCG subclasses are implicated in cellular homeostasis of cholesterol and phospholipids [129]. Overexpression of ABCA1 has been shown to increase apoA-I binding and cholesterol efflux [131, 132]. The loss of function in ABCA1 leads to TD, as described above.

ABCA1 is a 2261 amino acid integral membrane protein [107, 120, 133] containing 50 exons and spanning 149 kb [134]. ABCA1 is found as an oligomeric complex (dimers, tetramers and possibly higher order oligomerization) [135]. ABCA1 transports cellular cholesterol and PL, mostly phosphatidylcholine, to cell surface bound apolipoproteins [114, 136]. It is the first and rate-controlling step in the RCT pathway [108].

ABCA1 is expressed in most tissues (including macrophages) with the highest levels measured in placenta, liver, adrenal glands and fetal tissues [110], which all are critical

tissues in the cholesterol metabolism. ABCA1 is localized in the plasma membrane [108], in intracellular endocytic compartments [126, 132, 137] and in the Golgi [138]. It functions in the basolateral surface of hepatocytes [139]. ABCA1 is not restricted to the cell surface, but shuttles between the cell surface and early and late endosomes [140].

Interactions between lipid-poor apoA-I and ABCA1 lead to the efflux of PL and FC from many cells (among them macrophages and hepatocytes) and the subsequent formation of nascent discoial HDL particles [61, 108, 109, 138, 139, 141, 142]. They become mature, lipid-rich and spherical by esterification of cholesterol through the action of the enzyme LCAT, and increase in size through the acquisition of additional PL and unesterified cholesterol [61].

The underlying mechanism of action is not yet elucidated but it has been shown that ABCA1 and apoA-I recycles from the cell membrane to late endocytic compartments [140, 143] which appears to be critical in the movement of intracellular cholesterol to the cell surface for efflux. The first hydrophobic domain of ABCA1 contains an N-terminal signal anchor sequence that translocates the protein's first hydrophilic domain to the exoplasmic space [126]. It has been shown that ABCA1 has floppase activity which may enrich the exofacial leaflet of the bilayer with phosphatidylserine [85, 137, 144], but phosphatidylcholine is the predominating PL class in apoA-I [145]. Cross-linking experiments have shown direct interaction between ABCA1 and apoA-I [108, 146], but later studies suggest that there is not necessarily a direct binding but that the ABCA1 modification of lipid distribution by the phosphatidylserine exofloppase activity generates a biophysical microenviroment necessary for apoA-I binding to the cell surface [147]. An active ATPase is also necessary for apoA-I binding to cell surface [147, 148].

Both the view of direct binding and modification of membrane lipid composition may be correct, and a hybrid model of some sort is probably correct. [149]

Studies by Fitzgerald et al. [150] of five naturally occurring missense mutations in the extracellular loops of ABCA1 showed that four of these resulted in a marked decline in cross-binding to apoA-I, while the last showed cross-binding, but did not result in

cholesterol efflux [150]. These results suggest that apoA-I stimulated cholesterol efflux cannot occur without a direct interaction between the apolipoprotein and critical residues in the two extracellular loops of ABCA1, and that although binding may be necessary, it is not sufficient for stimulation of cholesterol efflux [150].

The fact that several apolipoproteins can bind to ABCA1 without a common ABCA1 binding sequence implies that the interaction is not highly specific [132]. Mechanisms other than direct binding have been proposed by Burgess *et al.* [151] and Panagotopulos *et al* [152]. Burgess *et al.* suggest that PL in the extracellular matrix of macrophages act as an initial tether for apoA-I, bringing it close to ABCA1 (which supplies the PL through its floppase activity [151]). Panagotopulos *et al* suggest that helix 10 of apoA-I may function to tether lipid-free apoA-I to the ABCA1 generated lipid domain of the cell membrane in close proximity to ABCA1. Tethered apoA-I could then diffuse within the plane of the membrane to ABCA1 where a protein/protein interaction could lead to the lipidation of apoA-I which would be released from the membrane possibly because of conformational changes [152]. Although speculative, the hybrid models seem to fit better than the straight protein/protein of lipid interaction hypotheses [152].

ABCA1 is tightly regulated by the cholesterol status of the cell. Cholesterol loading of macrophages increases ABCA1 mRNA and protein levels [108, 153]. In addition, several other compounds have a regulatory effect on ABCA1.

Unsaturated fatty acids and cholesterol can increase ABCA1 degradation, while saturated fatty acids have no effect on ABCA1 mRNA or protein levels [154]. On the other hand, binding of apoA-I stabilizes ABCA1 by inhibiting calpain-mediated degradation [155]. The direct mechanism of sterol stimulation of gene transcription is by transactivation of the ABCA1 promoter by liver X receptor (LXR) and retinoid X receptor (RXR) [156-158] which are activated by oxysterols and retinoid ligands, respectively. One LXR ligand (oxysterol) is intracellular cholesterol, which leads to LXR activation and ultimately increased cholesterol efflux. Cyclic AMP analogs activate ABCA1 transcription through other, distinct pathways/mechanisms [141, 146, 159, 160]. The promoter contains no obvious

cAMP response elements that can account for the stimulatory effect of cholesterol efflux by cAMP, suggesting that there could be more remote enhancers [161] or maybe that ABCA1 is activated by cAMP dependent protein kinases [162].

Nuclear receptors

The nuclear receptor (NR) superfamily is believed to be derived from a common ancestor and is divided into six subfamilies: RXR-heterodimer receptors, dimeric orphan receptors, steroid receptors, half receptors, monomeric orphan receptors and orphans lacking DNA binding domain (DBD) [163]. It consists of about 48 known receptors (and some not yet verified that would bring the number closer to fifty) [164], that modulate gene expression in response to lipophilic ligands [165, 166]. They play important roles in cell growth, differentiation and general metabolism [167: Francis, 2003 #225].

NRs are organized into regions called A/B, C, D and E. Some have also a F region at the C terminal of unknown function. C and E regions are highly conserved (with only a few exceptions) in the family [163].

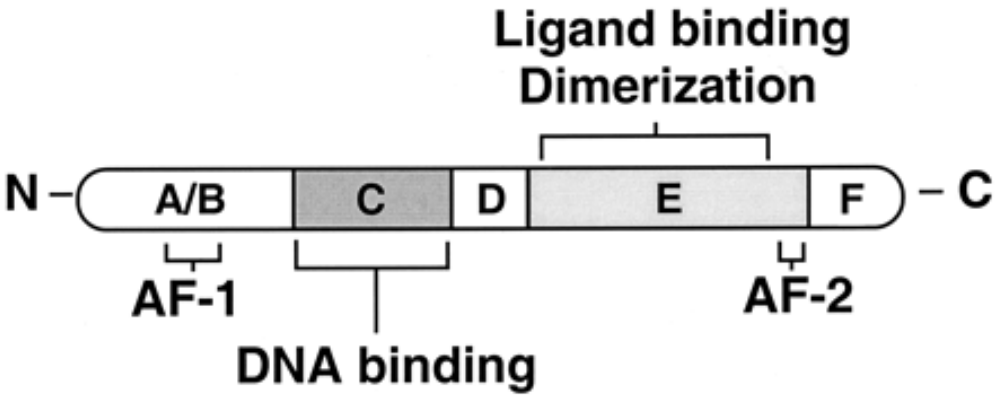

Fig. 6: Nuclear receptor domains. Taken from [168]

The A/B region, which is in the N-terminal, contains a powerful ligand-independent transactivation domain called activation function 1 (AF1) [169]. The C region is the highly conserved DBD and has two zinc finger modules that interacts/binds with DNA [163]. Each

zinc finger contains four conserved cysteines that coordinate binding of a $zinc^{2+}$ ion [15] followed by a linker or hinge domain (the D region) that harbors nuclear localization signals in many cases and also gives it flexibility for simultaneous binding of DNA and dimerization [168]. The length of the hinge varies between NRs and may contribute to the specific receptor-DNA interaction and dimerization through minor groove interaction [170]. The large carboxyl-terminal domain (the E region) contains the ligand-binding domain [171, 172], dimerization domain [173] and a ligand-dependent activation function (AF-2) [174]. The AF-2 domain in the E region is necessary for transactivation and interaction with corepressors and coactivators [175-177]. The F region is at the extreme carboxyl end and is not well characterized [168].

NRs interact with the regulatory domains of their target genes through their DNA-binding domain. They recognize sequences called response elements. Most often these are present in two copies (as most NRs bind as dimers) in the promoter region of their target genes [168]. Different types of NR recognize different response elements. Most NRs bind these as homodimers or heterodimers with RXR, although there are some that form other heterodimers or work as monomers [163, 168, 178].

These response elements can be palindromes, direct repeats or inverted palindromes [179, 180], spaced 1 to 5 nucleotides apart [165]. The response elements are written after how they are repeated (direct repeat (DR), inverted repeat (IR) and everted repeat (ER)) followed by the number of spacer nucleotides. For example, the two repeats of a direct repeat response element spaced 4 nucleotides apart are written as DR-4. The spacing and adjacent sequences to the response elements confer specificity [181].

The metabolic NR act as regulators of diverse processes such as energy, lipoprotein, fatty acid and TG metabolism by the PPARs, reverse cholesterol transport and cholesterol absorption by the LXRs, bile acid metabolism through the farnesoid X receptor (FXR) and LXRs and defence against xeno- and endobiotics by pregnane X receptor (PXR) [182] which form obligate heterodimers with RXR [182]. Ligand binding to one or the other stabilizes the dimer [165].

NRs are usually in complex with corepressors. Binding of ligand and/or phosphorylation induces a conformational change that cause dissociation of repressors and facilitates the recruitment of coactivator complexes [174] which facilitates transcription [182, 183]. This conformational change is necessary for DNA-binding, making the NR more compact and active [184-188].

In addition, cofactors that are associated with DNA-bound transcription factors determine whether a target gene is induced or repressed [189]. Coactivators work by remodeling chromatin and exposing binding sites for the general transcription complex [168] while repressors work by compacting the nucleosomal structure, making it less available for binding of transcription factors [190].

RXR

There are three RXR isotypes; α, β and γ [165, 178, 191]. These are encoded by separate genes and give rise to numerous alternatively spliced variants [192]. RXR binds as heterodimer to direct repeats (DRs) [193, 194] spaced 0 to 5 nucleotides apart, depending on the dimerization partner [165]. RXR also binds to IR-0 elements as a homodimer, but it is uncertain whether separable RXR responses exist or RXR only serves as a partner for other NRs [192]. RXR works as a dimerization partner [165] for most of the adopted orphan receptors. Among these are the PPARs, LXRs, FXR and PXR. Other dimerization partners include thyroid hormone, retinoic acid, vitamin D and ecdysone receptors [174]. The RXR heterodimers serve as regulators of several pathways involved in lipid homeostasis, embryogenesis [195], cell growth and differentiation [196].

LXR

LXR seems to be central in the regulation of lipid metabolism. LXR exists in to forms: LXRα and LXRβ. LXRβ is ubiquitously expressed [197] while LXRα is expressed in tissues involved with lipid metabolism such as the liver, macrophages, kidney, lung, intestine, adrenals and adipose tissue [198].

The natural ligands of LXRs are oxysterols, mostly metabolic derivates of cholesterol [199-201]. LXR acts as a cholesterol "sensor", responding to elevated cholesterol (oxysterol) levels. The constitutive activation of LXR is dependent on endogenous lipid synthesis (in particular, mevalonate biosynthesis products such as cholesterol and oxysterols) [202].

LXRα and LXRβ form heterodimers with RXR [198]. The result is transactivation by LXR of several genes which have roles in transport, storage, absorption, catabolism, elimination of cholesterol (reviewed in [198]) and in fatty acid metabolism [203, 204]. ABCA1 has two promoters that both contain LXR binding elements [205] that bind both LXRα and LXRβ and mediate transcriptional activation by LXR and PXR [156-158]. Other LXR target genes are apoE [206], SR-BI [207], CETP [208], sterol regulatory element binding protein-1c (SREBP-1c) [203], cholesterol 7α-hydroxylase (CYP7A1) [209] (CYP7A1 is the rate limiting enzyme in the neutral bile acid biosynthetic pathway [210-212], one of the principle means of eliminating cholesterol), ABCG1 [213], ABCG5 and ABCG8 [214], which are all up-regulated. The *de novo* synthesis of cholesterol is reduced [215, 216].

Sterol absorption in the gut is regulated by LXR [104] by upregulating ABCA1, ABCG5 and ABCG8 expression [217, 218]. To conclude, LXR works as a regulator of cholesterol metabolism.

FXR

The biological ligands for the FXR are bile acids and their conjugated metabolites [219-221]. FXR is highly expressed in liver, gut, kidney, adrenals and testis [222, 223]. FXR forms dimers with RXR [223] and preferentially binds to response elements in the IR-1 configuration [223, 224]. FXR becomes activated by bile acids and is involved in the negative feedback regulation of bile acid synthesis in hepatocytes. FXR/RXR represses transcription of CYP7A1 [225] which is the rate limiting enzyme in bile acid synthesis [210-212]. The FXR/RXR dimer stimulates expression of Ileal bile acid binding protein [226, 227], bile acid export pump (BSEP, ABCB11) [228, 229], phospholipid transfer protein (PLTP)[224,

[230] and multi-drug resistance protein 2 (MDR2, ABCC2)[231]. Both ABC transporters are involved in transport of bile acids into the bile canaliculus, while ileal bile acid binding protein promotes uptake of bile acids [221, 226, 227].

PPAR

PPARs are NRs that work as heterodimer partners with RXR and function as ligand-activated transcription regulators of lipid and glucose metabolism [232, 233]. There are three PPAR members: α, γ and δ [234-237]. These receptors are activated by polyunsaturated (and saturated) fatty acids, eicosanoids and some synthetic ligands [238]. Fatty acid binding proteins seem to act together with PPARs, presenting fatty acids to the NR [239]. Each PPAR has a distinct expression pattern and a specific function in fatty acid metabolism [158]. PPARα is a global regulator of fatty acid catabolism [240]. PPARγ is a regulator of metabolism, differientation and cell growth (reviewed in [241]) and is activated by fatty acids [41, 240, 242]. PPARγ induces ABCA1 in macrophages through the LXR pathway [158, 243].

PXR

The pregnane X receptor (PXR), also known as steroid and xenobiotic receptor (SXR) in humans, responds to many drugs, contaminants, steroids and toxic bile acids (mainly xenobiotics and steroids) [244]. PXR is highly expressed in liver, small intestine and colon [245, 246]. Among the chemicals that activate PXR is the antibiotic rifampicin (a human agonist), the glucocortioid dexamethasone (DEX) and the antiglucocorticoid pregnenolone 16α-carbonitrile (PCN, a murine agonist) [247]. The secondary bile acid lithocholic acid (LCA), which is toxic in high concentrations, is also a PXR agonist [248, 249]. PXR has a large spherical ligand-binding cavity which is believed to account for its precise but promiscuous ligand-binding properties [244]. PXR works to protect the body by regulating the detoxification and elimination of xenobiotics and toxic endogenous lipids [174], like bile acids. PXR upregulates CYP enzymes [250, 251] and other genes like ABCA1 [252], multidrug resistance-associated protein 2 (MRP2) [231] and organic anion-transporting polypeptide (OATP2) (which both transport bile acids) [246]. PXR-RXR heterodimer

response elements in target genes include DR-3, DR-4, ER-6, ER-8 and IR-0 configurations [253]

The aim of the present study

ABCA1 is a transporter that has a central role in HDL formation and cholesterol efflux from cells. As such, the mechanisms for underlying its regulation are important to study. We wanted to investigate the regulation of ABCA1 in hepatocytes by the nuclear receptor PXR.

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Manuscript

Regulation of hepatic ABCA1 by the nuclear receptor PXR

German Tapia

From the Programme for Cell Biology, Department of Molecular Biosciences,

University of Oslo, P.O Box 1051 Blindern, 0316 Oslo, Norway

Abstract

Nuclear receptors that work as heterodimers with RXR control several aspects of lipid metabolism. One such nuclear receptor, pregnane X receptor (PXR) is the molecular target for a wide variety of endogenous and xenobiotic compounds. PXR regulates the expression of genes central to the detoxification and excretion of potentially harmful compounds. Accumulation of intracellular cholesterol will lead to foam cell formation and necrosis if left unchecked. ATP-binding cassette transporter A1 (ABCA1) mediates the efflux of cholesterol from cells to apoA-I to produce HDL, which transports cholesterol to the liver for excretion. The aim of the present investigation was to determine the role of PXR in regulation of hepatic ABCA1 expression. Expression analyses were performed using Western blotting and quantitative real time RT-PCR. Luciferase reporter gene assays were used to measure promoter activities. Total cholesterol was measured enzymatically after lipid extraction (Folch's method). The expression of ABCA1 was inhibited by the PXR activators rifampicin in HepG2 cells and pregnenolone 16α-carbonitrile (PCN) in primary rat hepatocytes and Hepa1c1c-7 cells. Thus, PXR appears to be a regulator of hepatic cholesterol transport.

Keywords: ABCA1; PXR; Cholesterol; Liver

Introduction

ATP-binding cassette transporter A1 (ABCA1) is a protein with well documented protective effects on atherosclerosis development [1, 2]. ABCA1 was discovered as the mutated protein giving rise to the Tangier disease (TD) phenotype [3-6]. TD is, among other characteristics, characterized by high density lipoprotein (HDL) deficiency and prevalent atherosclerosis [7, 8]. The human ABCA1, cloned in 2000 [9], is expressed in most tissues (including macrophages) with the highest levels measured in placenta, liver, adrenal glands and fetal tissues [6] which all are critical tissues in the cholesterol metabolism.

Atherosclerotic risk is inversely proportional with HDL levels [10]. HDL plays a key role in cholesterol homeostasis by transporting excess peripheral cholesterol to the liver [11]. The protective effect of ABCA1 comes from its role in HDL formation and cholesterol efflux. ABCA1 mediates the efflux of cholesterol and phospholipids from cells to poorly lipidated apoA-I to form nascent HDL particles [12], the first and rate-limiting step in HDL formation [13] and is also believed to be the rate-limiting step of the reverse cholesterol transport pathway [14]. For this reason, ABCA1 is called the gatekeeper of the reverse cholesterol transport pathway [3, 12, 15]. ABCA1 has been shown to be regulated by nuclear receptors [16, 17] of the steroid hormone receptor superfamily, which are ligand activated transcription factors [18].

The retinoid X receptor (RXR) works as a common heterodimerization partner for adopted orphan receptors [19], and many nuclear receptors which are involved in control of fat, cholesterol, glucose, bile acid and xenobiotic metabolism (liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR)) form obligate heterodimers with RXR [20]. The LXR-RXR heterodimer is a known activator of ABCA1 [21]. PXR is thought to work as a xenobiotic sensor [20, 22] that protects the body from accumulation of toxic substances. PXR is expressed primarily in the liver and intestine [23] and is activated by a variety of drugs, contaminants, steroids and toxic bile acids [22]. The PXR-RXR heterodimer prefers direct repeat-4 (DR-4) response elements [24-26] but has also been shown to bind to DR-3, everted repeat-6 (ER-6), ER-8 and inverted repeat-0 (IR-0) configurations [27].

We have studied the effects of rifampicin and PCN, two well-known PXR agonists on ABCA1 expression in hepatoma cells and hepatocytes. The metabolism of bile acids, cholesterol and fatty acids are generally coordinately regulated by RXR and its heterodimerization partners [20]; therefore it is conceivable that PXR is involved in regulation of ABCA1.

Materials and methods

Plasmids and constructs

Expression plasmids for human RXRα (RXRα-pCMV) and β-galactosidase-pSV were kindly provided by Dr. H. Nebb (Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway). Human PXR-pSG5 was a kind gift from Dr. B. Goodwin (Nuclear Receptor Discovery Research, GlaxoSmithKline, Five Moore Drive, Research Triangle Park, NC 27709, USA). The human ABCA1 (−928 bp/+101 bp) promoter-luciferase reporter construct was a kind gift from Dr. P. Costet (INSERM U 539, Centre de Recherche en Nutrition Humaine de Nantes, France). Deletion promoter constructs were made by restriction enzyme digestion (New England Biolabs). The full length (-928 bp/+101 bp) human ABCA1 promoter pGL3 reporter construct was deleted to -604 bp/+101 bp and -264 bp/+101 bp by BstXI-cutting and SacII-cutting, respectively. The ABCA1-pGL3 reporter constructs were sequenced to verify DNA sequence fidelity by GATC Biotech AG (Germany).

Isolation and culturing of rat primary liver cells

Rats used in the experiments were treated according to established guidelines for the use of experimental animals. Primary rat hepatocytes were isolated from adult Wistar rats as described elsewhere [28]. After isolation, cells were grown in Dulbecco`s modified Eagle`s medium (DMEM) supplemented with 2 mM L-glutamine, 100 U penicillin, 100 μg/ml streptomycin (Bio Whittaker, Europe), and 3% fetal bovine serum (Sigma) at 37 °C. After 3-4 hours cells were stimulated with the given concentrations of pregnenolone-16α-carbonitrile (PCN) (Sigma) or vehicle (ethanol) in their respective media containing 0.5%

charcoal-treated fetal bovine serum. Lipoprotein-depleted serum was prepared as described [29].

Cell cultures

Murine hepatoma cells (Hepa1c1c-7) were grown in DMEM supplemented with 2 mM L-glutamine, 100 U penicillin, 100µg/ml streptomycin (BioWhittaker, Europe) and 5% fetal bovine serum (Sigma) (medium A) and human hepatoma cells (HepG2) were grown in medium A containing non-essential amino acids (Bio Whittaker, Europe) at 37 °C. After reaching 70% confluence, cells were stimulated with the given concentrations of rifampicin (HepG2 cells) or PCN (Hepa1c1c-7 cells) (Sigma) or vehicle (methanol or ethanol, respectively) in their respective medium containing 0.5% charcoal-treated fetal bovine serum (medium B).

Western blot

HepG2 cells and primary rat hepatocytes were grown in their respective media and incubated with vehicle (methanol or ethanol, respectively) or the indicated concentrations of rifampicin (Sigma) or PCN (Sigma), respectively. The cells were washed twice in phosphate-buffered saline (PBS) prior to being harvested in non-reducing SDS lysis buffer (20nM Hepes, 300 mM NaCl, 0.2mM EDTA, 1.5mM $MgCl_2$, 2% SDS and 1% Triton X-100) containing 2% phenylmethylsulforylfluoride. Protein concentrations were determined using BCA protein assay (Pierce). 25 µg of cell lysates were reduced with 5% 2-mercaptoethanol and heated at 50 °C for 10 min prior to SDS-polyacrylamid gel electrophoresis on 7.5% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Millipore, USA) and pre-incubated with blocking buffer (0.5xPBS-1% Tween20 containing 5% skim milk) to prevent unspecific binding. Monoclonal rat anti-ABCA1, kindly provided by Dr. K. Ueda (Lab. Cell. Biochem., Div. Applied Life Science; Kyoto, Japan), was diluted (1:400) in blocking buffer and interacting anti-rat HRP-IgG (Sigma) was detected by chemiluminescence ECL blot detection system (Amersham Biosciences). The blots were stripped (0.8% 2-mercaptoethanol in 0.5xPBS-1% Tween20 at 50 °C for 30 min) and re-hybridized with monoclonal mouse anti-β-actin (clone AC-74, Sigma) and detected with anti-mouse

HRP-IgG (Amersham Biosciences). Immunoreactive ABCA1 bands were quantified using a Gel Doc 1000 scanner (Bio-Rad) and normalized to the intensity of the immunoreactive β-actin bands.

Real time RT-PCR

Total RNA was isolated from Hepa1c1c-7 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Four micrograms of total RNA were reverse transcribed using SuperScript II RNase H- Reverse Transcriptase (Invitrogen) and oligo $dT_{(15)}$ primers (DNA Technology A/S, Denmark). The cDNA was used as template for qualitative real time RT-PCR on a LightCycler (Roche) using SYBR Green I technology (Roche). The primers used were amplified in the same PCR conditions using 60 °C as the annealing temperature. The primers used were designed such that they do not amplify from genomic DNA. This was tested by using RNA samples reversed transcribed without the enzyme which was more than 10 Cp-values different than RNA samples reversed transcribed using the enzyme. The primers were designed by the Primer 3 Output program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in table I.

Transient transfection

300,000 HepG2 cells were cultured in 6-wells plates with medium A the day prior to transfection. The cells were transiently transfected with 0.3 µg ABCA1 promoter-pGL3 reporter constructs using FuGENE 6 Transfection Reagent according to the manufacturer (Roche). Transfection efficiency was assessed measuring the activity of co-expressed β-galactosidase (0.5µg). In some experiments, 0.3 µg human PXR-pSG5 and 0.3 µg RXRα-pCMV were cotransfected. The amount of DNA was adjusted with carrier DNA (pcDNA3.1, Stratagene) to a total of 1.4 µg. Sixteen hours after transfection, medium A was replaced with medium B containing the given concentrations of PCN or vehicle (ethanol) for 24 hours. The cells were washed twice with PBS and lysed with Reporter Lysis Buffer (Promega). Luciferase activities and β-galactosidase activities were measured according to the manufacturer's instructions (Promega). Luciferase activities

were normalized to β-galactosidase activities to account for variations in transfection efficiency.

Cholesterol Efflux

A lipid soluble fraction was isolated from HepG2 cells and primary rat hepatocytes using a modified version of Folchs method [30] using the procedures described in [31]. Total and free cholesterol components were assayed using the commercial kit Cholèsterol RTU kit based on the cholesterol oxidase method [32] according to the manufacturer`s instructions (Kits BioMérieux, Marcy l'Etoile, France).

Densitometry and statistics

Semi-quantitative results of ABCA1 protein expression were obtained using a Gel Doc 1000 scanner (BIO RAD). Statistical significance of the data was evaluated by Student's t test. Probability values $p < 0.05$ was considered statistically significant.

Table I

Primers for quantitative real time RT-PCR analysis

Gene	GeneBank accession	Forward primer	Reverse primer	Amplicon size
ABCA1[a]		AACAGTTTGTGGCCCTTTTG	AGTTCCAGGCTGGGGTACTT	157
*m*GADPH	BC023196	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC	171

[a] Primer sequence based on an alignment of human (AF285167), rat (NM178096), and mouse (NM013454) ABCA1 mRNA

Results

PXR agonists reduce ABCA1 expression

A common activator of human PXR (rifampicin) or murine PXR (PCN) was added to HepG2 cells or primary rat hepatocytes, respectively, to test their ability to influence ABCA1 protein expression. As indicated in Figure 1A human ABCA1 protein expression decreased by 30% and 34% in response to 1 μM and 10 μM rifampicin, respectively. In

primary rat hepatocytes, a significant reduction (37%) in ABCA1 protein expression could only be observed after the addition of 10 µM PCN (Fig. 1B). Furthermore, quantitative real time RT-PCR revealed that PCN decreases the ABCA1 mRNA expression in Hepa1c1c-7 cells. As indicated in Figure 2 the ABCA1 mRNA expression was significantly reduced by 57% after PCN-treatment.

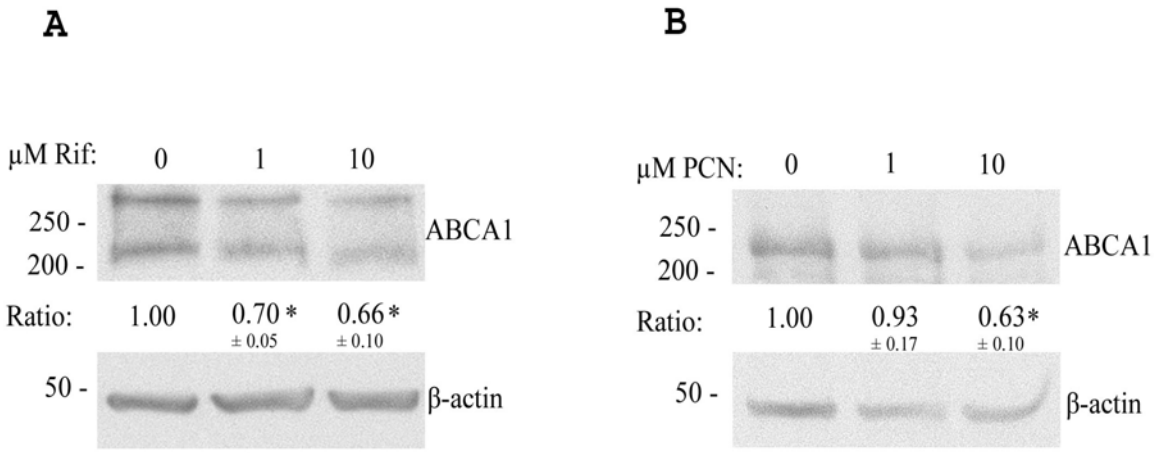

Fig. 1: PXR activators reduce hepatic ABCA1 protein expression*. HepG2 cells (A) or primary rat hepatocytes (B) were incubated with the indicated concentrations of rifampicin or PCN, respectively, in lipid-deficient medium for 24 h. Immunoreactive ABCA1 protein levels after rifampicin-treatment (A) or PCN-treatment (B) were measured by densitometer scanning, normalized to the intensity of the immunoreactive β-actin bands, and expressed relative to the vehicle-treated controls (±S.D). The results represent three separate experiments. *p < 0.05 indicates significant difference from control.*

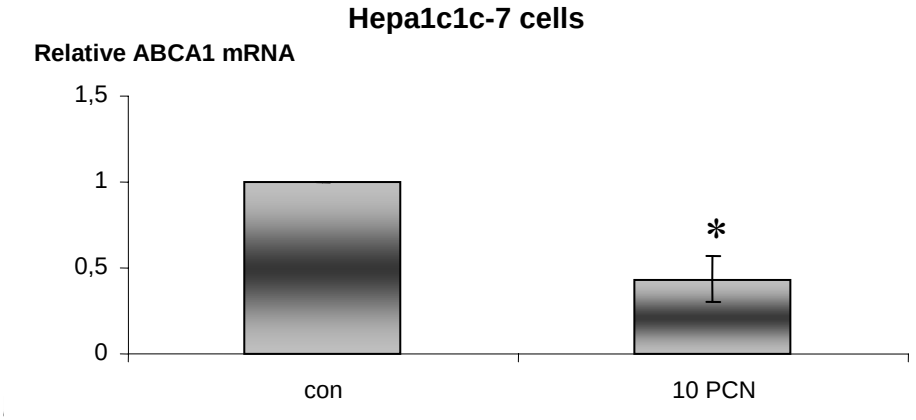

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Fig. 2: The PXR-activator PCN inhibits ABCA1 mRNA expression. *Hepa1c1c-7 cells were incubated with vehicle or 10 μM PCN in lipid-deficient medium for 24 h. ABCA1 mRNA levels were examined by real time RT-PCR. The results were normalized to GADPH expression and expressed relative to the vehicle-treated controls (±S.D). The experiment was performed at least three times, in triplicates. *p < 0.05 indicates significant difference from control.*

PXR/RXR represses ABCA1 promoter activity in HepG2 cells

HepG2 cells were transiently transfected with a vector expressing the reporter gene luciferase under the control of the human ABCA1 promoter. In Figure 3 the experimental data show that the addition of 10 μM rifampicin alone does not significantly affect ABCA1 promoter activity. Co-transfection with the expression plasmids for RXR and PXR, however, decreased the promoter activity by 53% with a further decrease (73%) in the presence of 10 μM rifampicin. Furthermore, deletion analysis of the human ABCA1 promoter showed that none of the constructs generated lost their ability to be regulated by rifampicin-activated PXR/RXR (Fig. 4) making it plausible that the binding site is located downstream from -264 bp. However, no putative PXR response element could be identified in the region spanning from -264 bp to +101 bp.

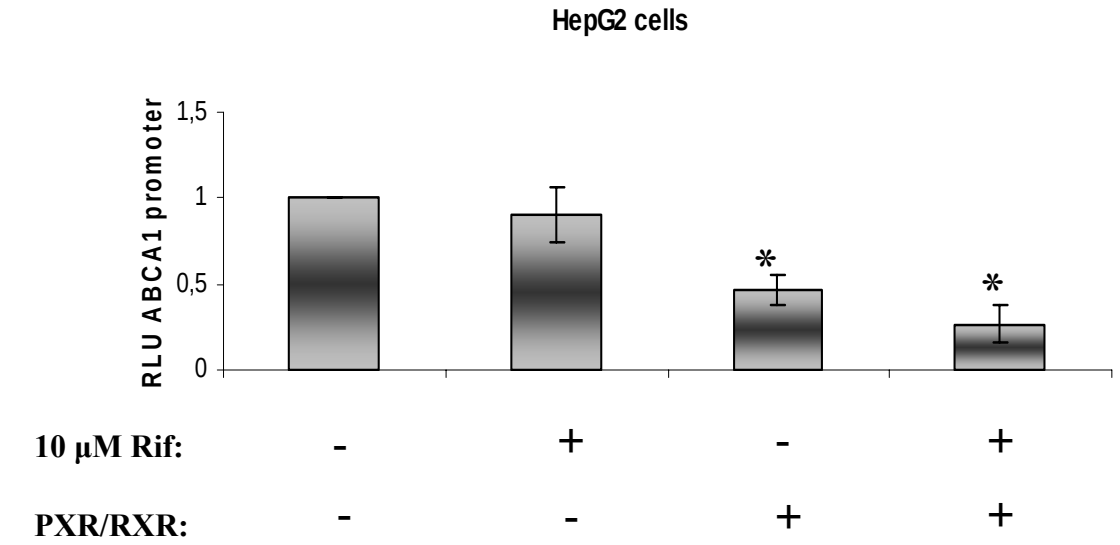

Fig. 3: PXR/RXR reduces ABCA1 promoter activity in HepG2 cells. *300,000 cells were transiently transfected with ABCA1 promoter/luciferase reporter construct (0.3 µg) together with an expression plasmid for β-galactosidase (0.5 µg) with or without the expression plasmids for PXR (0.3 µg) and RXR (0.3 µg). β-galactosidase was used as an internal control to account for any variation in transfection efficiency. Sixteen hours after transfection, the cells were treated with 10 µM rifampicin or vehicle (methanol) for additional 24 h. The luciferase activity was normalized to the β-galactosidase activity. The relative change in promoter activity (RLU) is related to vehicle-treated controls (±S.D). The results represent experiments performed in triplicates and repeated at least three times. *p < 0.05 indicates significant difference from control.*

Intracellular cholesterol levels are maintained after PXR agonist treatment

Intracellular cholesterol levels were measured in HepG2 cells or primary rat hepatocytes after rifampicin- or PCN-treatment, respectively. No significant change in cholesterol levels was observed in rifampicin-treated HepG2 cells (Fig. 5A) or PCN-treated primary rat hepatocytes (Fig. 5B) compared to vehicle-treated cells.

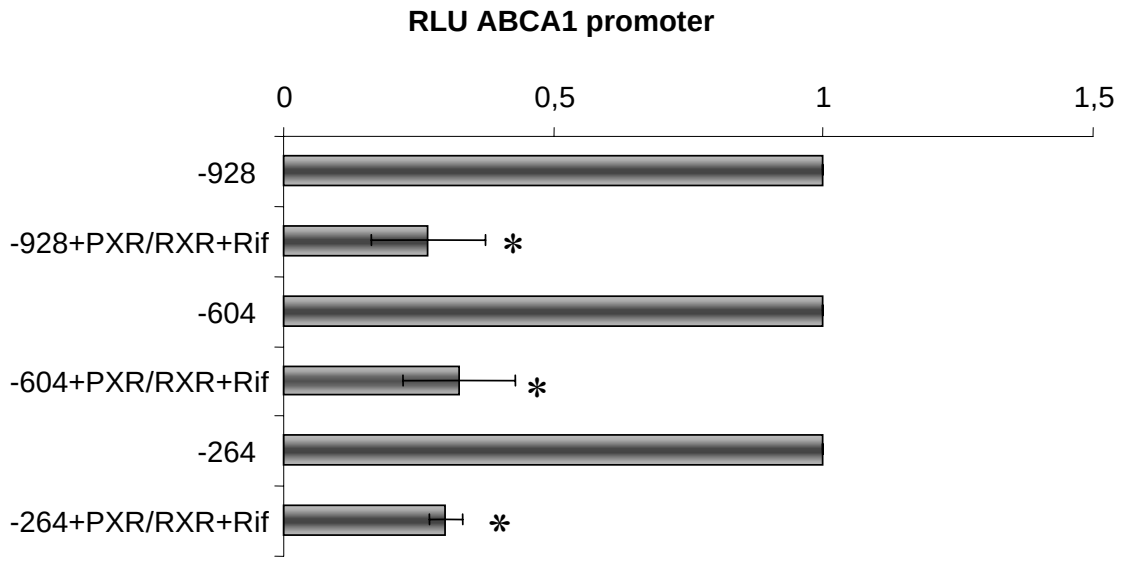

Fig. 4: No PXR/RXR response element could be identified within the region spanning from -928 bp to -264 bp of the human ABCA1 promoter. *300,000 cells were transiently transfected with ABCA1 promoter/ luciferase reporter constructs (0.3 μg) together with an expression plasmid for β-galactosidase (0.5 μg) with or without the expression plasmids for PXR (0.3 μg) and RXR (0.3 μg). β-galactosidase was used as an internal control to account for any variation in transfection efficiency. Sixteen hours after transfection, the cells were treated with 10 μM rifampicin or vehicle (methanol) for additional 24 h. The luciferase activity was normalized to the β-galactosidase activity. The relative change in promoter activity (RLU) is related to vehicle-treated controls (±S.D). The results represent experiments performed in triplicates and repeated at least three times. *p < 0.05 indicates significant difference from control.*

Discussion

The present data show that hepatic ABCA1 expression is repressed by PXR agonists. Promoter activity studies in HepG2 cells demonstrated that the inhibitory effects observed by PXR-agonists are dependent on PXR/RXR co-expression in order to repress human ABCA1 promoter activity. In order to map potential response elements, the promoter was fragmented into shorter deletion reporter constructs and transiently transfected into HepG2 cells. The data obtained show that none of the generated

constructs lost the ability to respond to agonist-activated PXR/RXR suggesting that the response element is located in the region spanning from -264 bp to +101. By use of

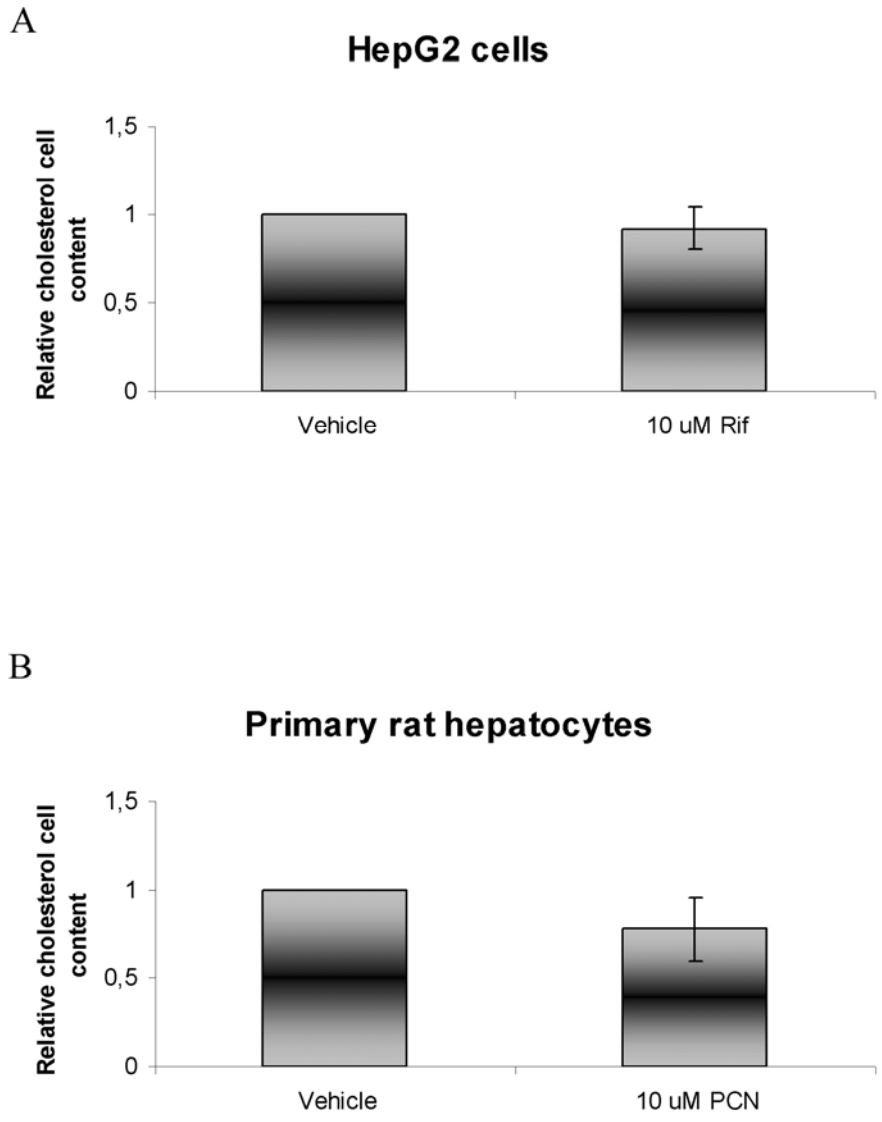

Fig. 5: Intracellular cholesterol levels are not changed by PXR agonists. *HepG2 cells (A) or primary rat hepatocytes (B) were incubated with 10 μM agonist in lipid-deficient medium for 24 h, followed by cell-lipid extraction and enzymatic measurement of cholesterol content. The results are presented as relative to the vehicle-treated controls (±S.D). The experiments were performed at least three times, in triplicates.*

clustalW multiple sequence alignment program we were not able to locate any putative PXR response elements, but cannot rule out the possibility that the PXR/RXR heterodimer may bind to a yet non-identified response elements in this region since PXR has been shown to bind a wide range of response elements. One cannot, however, exclude the possibility that ligand-activated PXR/RXR regulates the human ABCA1 promoter through indirect mechanisms. Other nuclear receptors such as PPARα and PPARγ have been shown to regulate ABCA1 through the LXR pathway (since LXRα is a PPAR target gene) [33, 34] but the detailed mechanisms are at the time unknown.

PXR is a master regulator of genes involved in the detoxification and excretion of potentially harmful substances. Among these target genes are other ABC transporters like multidrug resistance-associated protein-2 (MRP2/ABCC2), MRP3/ABCC3 (bile acid and drug transporter) [35-37], and ABCG5/G8 [38] (which mediates the efflux of cholesterol to the bile [39, 40]). Bile acids, which are toxic in high concentrations, have been demonstrated to activate the PXR-pathway [41-43], and activated PXR has been shown to negatively regulate the production of bile acids by inhibiting the expression and activity of the first and rate-limiting enzyme in the production of bile acids, namely CYP7A1 [42, 44-47]. Furthermore, PXR agonists have been demonstrated to increase plasma HDL cholesterol as well as hepatic apoA-I expression [48] and increase biliary cholesterol output [49] in rodents. We have in our study identified another ABC transporter, ABCA1, as a target gene for PXR thus assigning PXR as an important regulator of cholesterol trafficking in hepatocytes. Based on the observations that PXR-activation stimulates apical cholesterol efflux (in a ABCG5/G8-dependent manner) [38, 49] reduces endogenous cholesterol synthesis (by decreasing HMG-CoA reductase expression), and reduces ABCA1 expression, we measured intracellular cholesterol levels. The data obtained shows that the intracellular cholesterol levels are unchanged suggesting that the down-regulation of ABCA1 is part of a mechanism the hepatocytes possesses in order to maintain cholesterol levels.

In conclusion, the present work demonstrates that PXR negatively regulates hepatic ABCA1 expression which can be supported by the observation made in FXR/PXR knockout mice displaying enhanced hepatic ABCA1 mRNA levels [50]. In addition to

being a master regulator of the defence against endo- and exogenous toxicity [51], data obtained this far point to a role of PXR as being antiatherogenic (by increasing HDL and apoA-I levels [48, 52]) and anti-cholestatic (by being a bile acid sensor and negative regulator), but further studies will be needed in order to settle its role in atherosclerosis development.

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