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"Ο ρόλος της φλεγμονής στην έκφραση γονιδίων που συμμετέχουν στον μεταβολισμό της λιποπρωτεϊνης υψηλής πυκνότητας (HDL)"

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Ευχαριστίες

Για την πραγματοποιήση της διπλωματικής μου εργασίας ήταν πολύ σημαντική η συμβολή κάποιων ανθρώπων.

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Contents

Abstract

Atherosclerosis is the underlying cause of cardiovascular disease (CVD) which accounts for 50% of deaths in the western world. Various factors contribute to the development of atherosclerosis including dyslipidemia, lipid accumulation on the artery wall and chronic inflammation. High density lipoprotein (HDL) is a known atheroprotective factor, since it stimulates the reverse cholesterol transport RCT) and it also exerts both anti-inflammatory and anti-oxidative properties. Both proteins and lipds of HDL determine its atheroprotective functions. Dysfunctional, proinflammatory HDL, has been reported in patients with coronary artery disease (CAD), as well as in patients with chronic inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Therefore, in Part I of this thesis we examined the concentration of HDL and the plasma levels of apolipoproteins A-I and M in patients with SLE or RA. ApoM is a novel HDL binding protein with atheroprotective and anti-oxidative properties, which is downregulated by inflammatory stimuli such as tumor necrosis factor a (TNFα). We performed western blot analysis in serum from healthy controls and from patients with SLE or RA and we observed that apoM levels were decreased by approximately 20-65% in patients, while both HDL cholesterol and apolipoprotein A-I (apoA-I) levels remained unchanged. These findings suggest that the lack of apoM in HDL from RA and SLE patients could cause HDL dysfunction and loss of atheroprotection thus leading to accelerated atherosclerosis.

Liver X receptor α (LXRα) is a transcription factor that plays key roles in atheroprotection, since it controls the regulation of HDL genes participating in the reverse cholesterol transport pathway and it inhibits the expression of pro-inflammatory genes in macrophages. The mechanisms that regulate the expression of LXRα in macrophages, hepatocytes and other cell types are still not clear. Thus, in Part II of this thesis we focused on the regulation of the LXRα promoter in hepatic cells by inflammatory or metabolic stimuli. To identify regulatory elements in hLXRa promoter that are involved in these processes, we performed transient transfection assays both in human hepatoblastoma (HepG2) and human embryonic kidney (HEK) cells utilizing a series of luciferase reporter plasmids containing consecutive 5´ deletions of the hLXRα promoter along with inflammatory stimuli such as Toll Like Receptor 4 (TLR4) which is the natural receptor of LPS or metabolic stimuli such as Hepatocyte Nuclear Factor 4 (HNF-4). We observed that overexpression of TLR4 inhibited hLXRa promoter activity through the activation of Nuclear Factor κappa B (NF-κB) in an IKKβ (inhibitor of nuclear factor kappa-B kinase b)-independent pathway On the contrary we observed that overexpression of HNF4 in HepG2 cells

induced hLXRα promoter activity through the -111/-45 region of the promoter, indicating an antagonism between NF-κB and HNF4 in hLXRa promoter activity.

Περίληψη

Η αθηρωμάτωση είναι η βασική αιτία για την εμφάνιση της καρδιαγγειακής νοσου (Κ.Ν.), στην οποία οφείλεται το 50% των θανάυων στο δυτικό κόσμο. Αρκετοί παράγοντες συμμετέχουν στην ανάπτυξη της αθηρωμάτωσης όπως η δυσλιπιδαιμία, η συσσώρευση λιπιδίων στα αρτηριακά τοιχώματα και η χρόνια φλεγμονή. Η υψηλής πυκνότητας λιποπρωτεΐνη (HDL) είναι ένας αθηροπροστατευτικός παράγοντας, επειδή ενεργοποιεί το μηχανισμό της αντιστροφης μεταφοράς χοληστερόλης από τα περιφερικά κύτταρα στό ήπαρ ενώ επίσης έχει αντι-φλεγμονώδεις και αντι-οξειδωτικές δράσεις. Τόσο οι πρωτεΐνες όσο και τα λιπίδια που βρίσκονται στην HDL καθορίζουν τις αθηροπροστατευτικές της δράσεις. Μη λειτουργική, προφλεγμονώδης HDL έχει παρατηρηθεί σε άτομα με στεφανιαία νόσο, αλλά και σε ασθενείς με χρόνια φλεγμονώδη νόσο όπως η ρευματοειδής αρθρίτιδα (ΡΑ) και ο συστημικός ερυθηματώδης λύκος (ΣΕΛ). Συνεπώς, στο πρώτο μέρος αυτής της εργασίας, μελετήσαμε τη σύγκεντρωση της HDL χοληστερόλης καθώς και τα επίπεδα των απολιποπρωτεϊνών A-I και M στο πλάσμα ασθενών με ΣΕΛ και ΡΑ. Η απολιποπρωτεΐνη M είναι μια πρωτεΐνη που συνδέεται σε σωματίδια HDL με αθηροπροστατευτικές και αντι-οξειδωτικές ιδιότητες, ενώ η έκφραση της ελέγχεται αρνητικά από φλεγμονώδη ερεθίσματα, όπως ο TNFα. Με τη τεχνική Western blot, παρατηρήσαμε ότι τα επίπεδα της απολιποπρωεΐνης M μειώνονται στους ασθενείς σε σχέση με τους υγιείς σε ποσοστό 20-65%. Αντίθετα, τα επίπεδα της απολιποπρωτεΐνης A-I καθώς και τα επίπεδα της HDL χοληστερόλης παραμένουν ανεπηρέαστα στο πλάσμα των ασθενών σε σύγκριση με τους υγιείς. Αυτά τα ευρήματα δείχνουν ότι η έλλειψη της απολιποπρωτεΐνης M απο την HDL σε ασθενείς με ΡΑ και ΣΕΛ μπορεί να οδηγεί στη παραγωγή ελλατωματικής HDL, με αποτέλεσμα η τελευταία να χάνει τις αθεροπροστατευτικές τις δράσεις οδηγώντας στην ανάπτυξη της αθηρωμάτωσης.

Ο LXRα είναι ένας μεταγραφικός παράγοντας που παίζει πολύ σημαντικό ρόλο στην αθηροπροστασία, διότι αφενός ελέγχει τη ρύθμιση γονιδίων της HDL που συμμετέχουν στο μονοπάτι της αντίστροφης μεταφοράς χοληστερόλης και αφετέρου αναστέλλει την έκφραση προφλεγμονωδών γονιδίων στα μακροφάγα. Ωστόσο, οι μηχανισμοί που ρυθμίζουν την έκφραση του γονιδίου του LXRα στα μακροφάγα, στα ηπαττοκύτταρα καθώς και σε άλλους κυτταρικούς τύπους δεν έχουν εξακριβωθεί πλήρως. Έτσι, στο δεύτερο μέρος αυτής της εργασίας επικεντρωθήκαμε στο πώς ρυθμίζεται ο υποκινητής του ανθρώπινου LXRα γονιδίου από φλεγμονώδες ή μεταβολικό ερέθισμα. Για να ανιχνεύσουμε ρυθμιστικά στοιχεία στον υποκινητή του ανθρώπινου LXRa γονιδίου που συμμετέχουν σε αυτές τις διαδικασίες,

πραγματοποιήσαμε παροδικές διαμολύνσεις σε ανθρώπινα κύτταρα ηπατοβλαστώματος (HepG2) και σε ανθρώπινα εμβρυϊκά νεφρικά κύτταρα (HEK293) με πλασμίδια που φέρουν διαδοχικές ελλείψεις του υποκινητή του γονιδίου του LXRα και επίσης φέρουν το γονίδιο αναφοράς της λουσιφεράση. Σε αυτές τις διαμολύνσεις υπερεκφράσαμε επιπλέον είτε τον TLR4 υποδοχέα, οποίος είναι ο φυσικός υποδοχέας του LPS (φλεγμονώδες ερέθισμα), είτε τον μεταγραφικό παράγοντα HNF4 (μεταβολικό ερέθισμα). Παρατηρήσαμε ότι υπερέκφραση του TLR4 ανέστειλλε την ενεργότητα του υποκινητή του γονιδίου του LXRα μέσω της ενεργοποίησης του μεταγραφικού παράγοντα NF-κB, με εναν μηχανισμό ανεξάρτητου από τον IKKβ. Αντίθετα, η υπερέκφραση του HNF4 αύξησε την ενεργότητα του υποκινητή του γονιδίου LXRα μέσω της περιοχής -111/ -42 του υποκινητή, πτοτείνοντας μια ανταγωνιστική δράση μεταξύ του NF-κB και του HNF4 για τη ρύθμιση του γονδίου του LXRa στα ηπατοκύτταρα.

1. Introduction

Cholesterol biology

Cholesterol is an essential constituent of mammalian cell membranes modulating membrane fluidity and permeability. Cholesterol account for 20–25% of the lipid molecules in plasma membrane and is needed for the formation of caveolae and lipid rafts (Hooper, 1999). Cholesterol affects various cellular processes, including membrane trafficking and transmembrane signalling, by interacting both with other membrane lipids as well as with specific membrane proteins (Ikonen, 2008). The metabolites of cholesterol, such as steroids and bile acids, have important biological roles as signal transducers and solubilizers (Fernandez et al., 2005).

Although different tissues have characteristic patterns of cholesterol metabolism, the basic pattern is similar in all cells. Cells acquire cholesterol mainly from the diet and endogenous biosynthesis. However, the dietary intake of cholesterol is limited, so the physiological requirements for cholesterol are supplied mostly through de novo synthesis. Cholesterol is synthesized in the endoplasmic reticulum (ER) and the cytoplasm from acetyl-CoA (Goedeke and Fernandez-Hernando, 2012).

Almost all cells are involved in the synthesis of cholesterol. However, the quantity of cholesterol produced in the liver is as much as in the extrahepatic tissues combined (Dietschy et al., 1993). Therefore, cholesterol should be transported from the liver to other tissues via lipoproteins (Ikonen, 2008). In order to prevent over-accumulation of free cholesterol (FC) in the plasma and intracellular membranes, cholesterol is converted to cholesterol esters (CE) (Chang et al., 1997). Cholesterol esters are stored as cytosolic lipid droplets. This cholesterol/ cholesterol ester cycle occurs rapidly, and releases free cholesterol to be trafficked to other intracellular compartments (Brown and Goldstein, 1980).

Despite the beneficial effects of cholesterol, abnormal cholesterol levels can cause destructive effects in the cells. Excess of cholesterol is toxic for the cells, leading to diseases such as atherosclerosis and type II diabetes (Ikonen, 2006). Therefore, intracellular cholesterol levels are tightly controlled by feedback mechanisms that operate at both transcriptional and post-transcriptional levels. That is the reason why cholesterol homeostasis is among the most intensely regulated processes in biology (Goedeke and Fernandez-Hernando, 2012).

Lipoproteins

Cholesterol is a hydrophobic compound, unable to be dissolved directly in plasma. Therefore, in order to be transported in plasma, cholesterol is packaged into water-soluble lipid-protein assemblies called lipoproteins (Babin and Gibbons, 2009). The transport of cholesterol as free cholesterol or as cholesterol esters, as well as the transport of triglycerides (TG) and phospholipids (PL) in the circulation, is achieved by lipoproteins. Lipoproteins form either spherical or discoidal particles. Spherical particles consists of a core of non-polar neutral lipids (mainly CE and TG) and coats of relatively polar materials such as PL, FC and proteins, while discoidal particles consists mostly of polar lipids and proteins in a bilayer conformation (Babin and Gibbons, 2009; Zannis et al., 1993).

Apolipoproteins are the protein constituents of lipoproteins. Representative members of the apolipoprotein family are: apoA-I, apoA-II, apoB, apoC-II, apoC-III, apoE and apoM. Apolipoproteins provide structural stability and functional specificity to the lipoprotein particle, playing an essential role in the control of plasma and tissue lipid homeostasis. They are involved in specific binding to cellular receptors, in the regulation of lipolytic enzymes and in the process of lipid exchange and transfer (Zannis, 2004).

Plasma lipoproteins are categorized into five classes, based on their density (Babin and Gibbons, 2009; Zannis, 2004). These are:

1) chylomicrons,
2) very low-density lipoproteins (VLDL),
3) intermediate-density lipoproteins (IDL),
4) low-density lipoproteins (LDL), and
5) high density lipoproteins (HDL)

HDL and LDL are rich in cholesterol, whereas triglycerides constitute the major lipid in the remaining subclasses. apoB-48, apoB-100 and apoA-I are the major protein components of chylomicrons, VLDL/ IDL/ LDL and HDL, respectively (Zannis, 2004). The different lipoprotein classes and subclasses are depicted in Figure 1.1.

Figure 1.2 shows schematically the pathways of lipoprotein metabolism. Lipoproteins are synthesized and catabolized in three distinct but metabolically interrelated pathways: the chylomicron pathway, the VLDL/IDL/LDL pathway and the HDL pathway. Briefly, dietary lipids are absorbed by enterocytes of the small intestine where they are packaged, along with apoB-48, into chylomicrons. Chylomicron formation is facilitated by the microsomal triglyceride transfer protein (MTP). As the chylomicrons reach the circulation, are hydrolyzed by lipoprotein lipase (LPL), which is anchored on the surface of microvascular endothelial cells in muscle

and adipose tissue so that fatty acids enter these tissues. The chylomicron remnants, which are rich in cholesterol esters, are taken up by hepatocytes (Ikonen, 2008; Zannis, 2004).

In hepatocytes, lipids associate intracellularly with apoB-100 with the action of MTP, and form the VLDL particles. These particles are secreted in the circulation and hydrolyzed by lipoprotein lipase (LPL) into IDL, which in turn is further hydrolyzed to LDL by the action of hepatic lipase (HL). LDL is the main lipoprotein that delivers cholesterol to the peripheral cells. The uptake of VLDL, IDL and LDL by the cells is mediated through the LDL receptor (Goedeke and Fernandez-Hernando, 2012; Zannis, 2004).

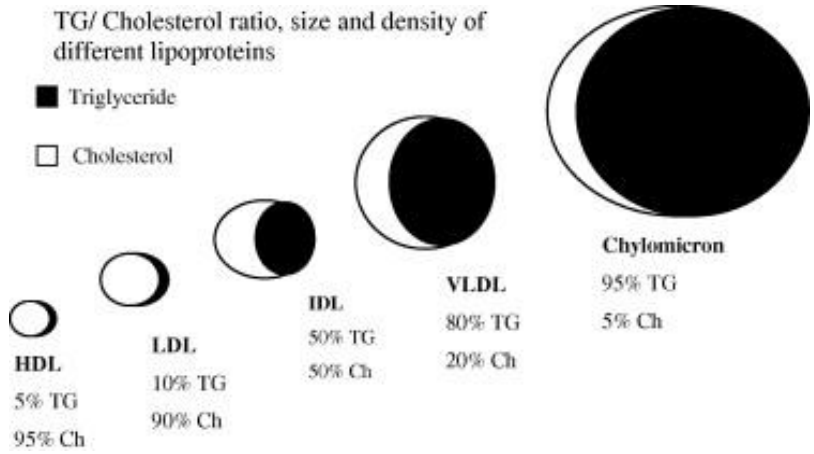


Figure 1.1 Lipoproteins classification based on their density and composition (Jain et al., 2007).

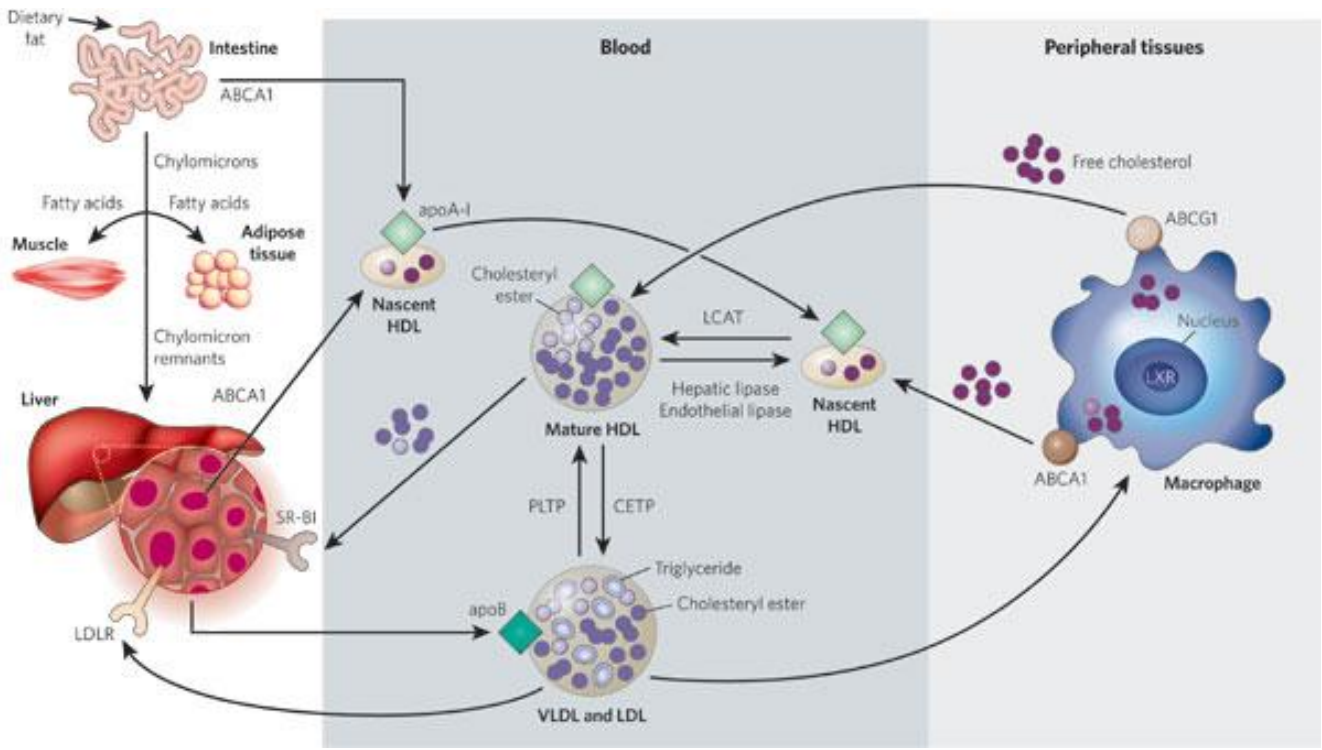


Figure 1.2 Lipoprotein metabolism (Rader and Daugherty, 2008).

HDL biology

HDL particles are very heterogeneous in terms of proteins and lipids composition. HDL is synthesized mainly by the liver and to a minor extent by the intestine through a complex pathway (Zannis et al., 2004). Briefly, hepatic cells synthesize and secrete apoA-I which interacts with the ATP-binding cassette transporter 1 (ABCA1) and facilitates the transport of cellular phospholipids and cholesterol to extracellular lipid-poor apoA-I. ApoA-I lipidation forms nascent HDL particles called pre-β. These particles, in turn promote additional cholesterol efflux from peripheral cells, resulting in the formation of discoidal HDL particles called βHDL. On HDL, the enzyme lecithin;cholesterol acetyl transferase (LCAT) catalyzes the esterification of free cholesterol leading to the conversion of the discoidal particles into the spherical ones called αHDL particles (Zannis et al., 2006).

HDL remodeling is a continuous process occurring in the plasma. The cholesteryl esters are transferred from HDL to VLDL/IDL/LDL by the cholesteryl ester transfer protein (CETP) in exchange of triglycerides, while phospholipids are transferred from VLDL/LDL to HDL by the phospholipid transfer protein (PLTP). Furthermore, cholesterol efflux from cells to βHDL or delivery of cholesteryl esters to cells from HDL is mediated by the ABCA1 transporter and the scavenger receptor class B type-I (SR-BI) respectively. Cholesterol efflux from macrophages to αHDL is

also mediated by the cell membrane transporter ATP binding cassette transporter G1 (ABCG1). Finally, hydrolysis of HDL lipids is mediated by various lipases including lipoprotein lipase (LpL), hepatic lipase (HL) and endothelial lipase (EL). HDL remodeling by plasma factors results in the formation of smaller α-HDL particles and pre-β HDL particles, which in turn promote more cholesterol efflux. The removal of excess cholesterol from the peripheral tissues, including macrophages and foam cells of the arterial wall, back to the liver for excretion into the bile is traditionally called the "reverse cholesterol transport" (RCT) pathway (von Eckardstein et al., 2005; Zannis et al., 2006). Figure 1.3 depicts the pathway of the biogenesis and catabolism of HDL (Zannis et al., 2006).

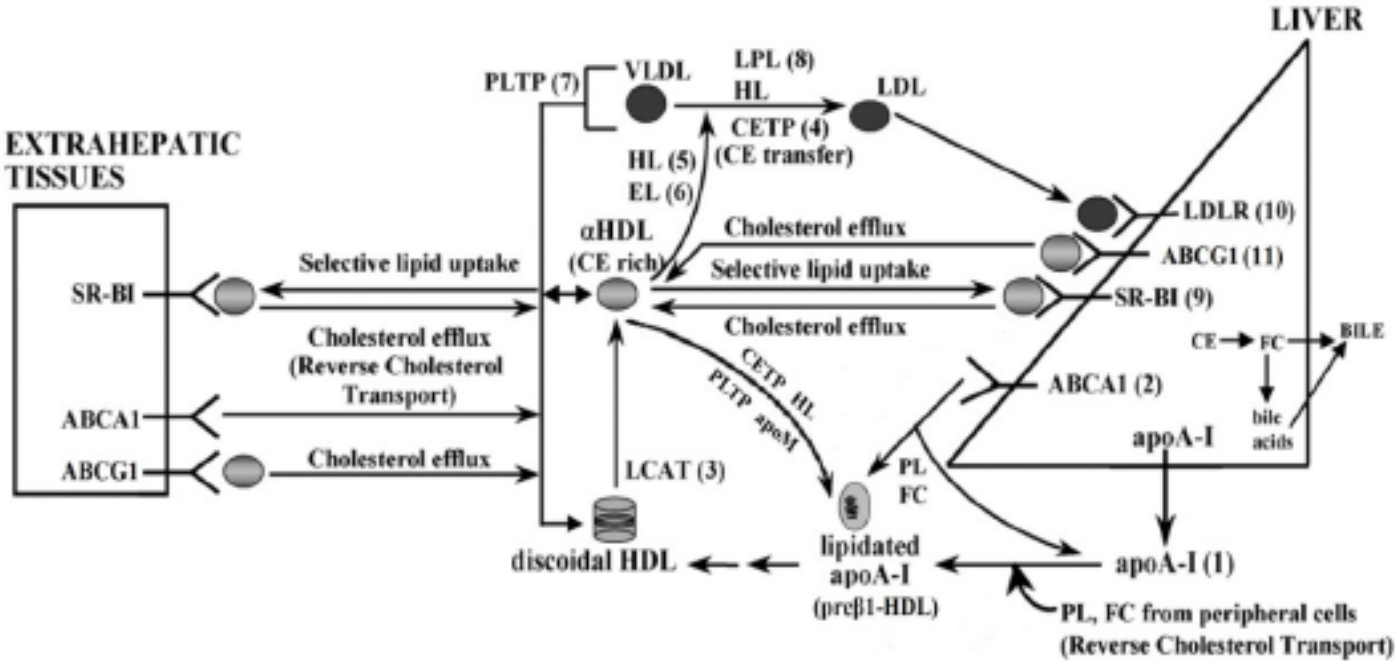


Figure 1.3. Schematic representation of the pathway of the biogenesis and the catabolism of HDL.

Human HDL isolation by ultracentrifugation, based on its density, results into two major subfractions, which have been designated HDL_2 (1.063<d<1.125g/mL,) and HDL_3 (1.125<d<1.21g/mL). Depending on its size, HDL is separated by non-denaturing polycaryamide gradient gel electrophoresis into five distinct subpopulations with diameters ranging from 7.6nm to 10.6nm (3c, 3b, 3a, 2a and 2b) (Blann et al., 2005).

HDL in human plasma contains two main apolipoproteins, apoA-I and apoA-II, which account for 70% and 20%, respectively, of the total HDL protein. Spherical HDL particles contain only apoA-I (A-I HDL), or both apoA-I and apoA-II (A-I/A-II

HDL). In most human subjects, apoA-I is distributed approximately equally between A-I HDL and AI/A-II HDL (Asztalos et al., 2005; Cheung and Albers, 1982, 1984). Apolipoprotein A-I exerts not only structural stability but also functional specificity into HDL function. ApoA-I interacts with ABCA-1,SR-B1 and ABCG1 promoting both the cholesterol efflux from cells to HDL and the cholesterol delivery from HDL to cells. Also, apoA-I interacts and subsequently activates LCAT (Sorci-Thomas and Thomas, 2012) . In addition to apoA-I and to apoA-II, apoE, apoA-IV, apoA-V, apoJ, apoC-I, apoC-II, apoM and apoC-III participate also in HDL formation (Karlsson et al., 2005). HDL particles also contain antioxidant enzymes such as the paraoxonase 1 (PON1) and the platelet-activating acyl hydrolase (PAF-AH) (Navab et al., 2004).

Atherosclerosis

Atherosclerosis is a pathological condition characterized by artery wall thickening, as a result of lipid accumulation. It is considered as a multifactorial, multiphase chronic inflammatory disease and is the underlying cause of most cardiovascular diseases (CVD), including hypertension, coronary artery disease and peripheral vascular disease (Hansson and Libby, 2006; Paoletti et al., 2004). CVD remains the leading cause of global morbidity and mortality. It is estimated that about 17.3 million people died of CVD in 2008 representing almost 30% of global mortality, while this number is considered to rise up to 23.6 million by 2030. Unhealthy diet, physical inactivity, smoking and alcoholism are important environmental risk factors for the disease (Samson et al., 2012).

Lipoprotein modifications play key role in atherogenesis. When LDL is trapped inside the artery wall is susceptible to modifications, such as oxidation, enzymatic cleavage, and aggregation. These modifications are inhibited in plasma, because of the existence of plasma antioxidants (Hevonoja et al., 2000). LDL oxidation is the most important modification converting normal LDL to proinflammatory LDL. This oxidation is facilitated by cell-generated reactive oxygen species (ROS), which are produced by several enzymes such as lipoxygenase, myeloperoxidase and NADPH oxidase in endothelial cells, macrophages and smooth muscle cells (Navab et al., 2004).

Oxidized LDL (oxLDL) promotes the inflammatory response leading to a chronic inflammatory reaction. In brief, endothelial cells are activated by oxLDL to produce chemoattractants, such as monocyte chemoattractant protein-1 (MCP1), and as a result monocytes are attracted into the artery wall. The release of the macrophage colony stimulating factor (M-CSF) from the endothelial cells after oxLDL-mediated stimulation promotes the differentiation of monocytes to

macrophages (Hansson and Libby, 2006; Young and McEneny, 2001). Macrophages in turn engulf oxLDLs. These cholesterol-loaded macrophages eventually will form foam cells which are major constituents of the growing atherosclerotic plaque (Navab et al., 1996). At the site of the plaque, endothelium injured and subsequently becomes dysfunctional by losing its integrity, exerting pro-coagulant instead of anti-coagulant properties and producing vasoactive molecules, cytokines, and growth factors into the growing plaque (Blann et al., 2005). As the atherosclerotic lesion evolves, other immune inflammatory cells such as T cells, dendritic cells, and mast cells accumulate in this region contributing to the innate immune response by generating free oxygen radicals, proteases, complement factors and cytokines attracting other immune cells (Boring et al., 1998; Gu et al., 1998). All these effects lead to the formation of a fibrous cap of variable thickness composed mainly of collagen, covering the lesion. The lesion consists of apoptotic and necrotic cells, cell debris, and cholesterol crystals which form the necrotic core over a long period of time (Hansson and Hermansson, 2011). Figure 1.4 depicts an atherosclerotic plaque.

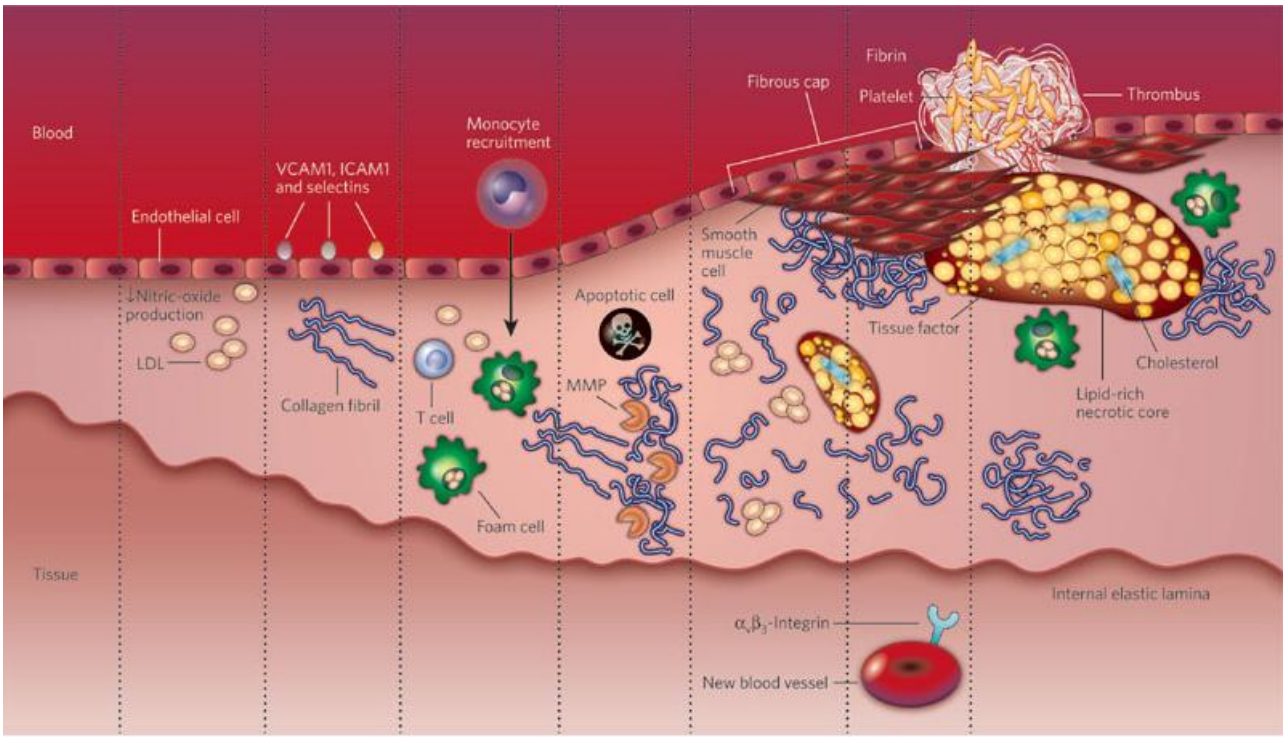


Figure 1.4. Both oxidized LDLs and inflammatory stimuli contribute to the development of atherosclerotic plaque (Xu and Dahlback, 1999).

TLR4 signaling mediates atherosclerosis

Toll-like receptors (TLRs) are members of the Interleukin-1 superfamily and regulate innate immune responses. TLRs are essential for host defense by recognizing pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004). In addition to PAMPs, TLRs also recognize endogenous ligands that are related to atherosclerosis such as modified LDL and HSP-60, suggesting that TLR-mediated responses may contribute to the pathogenesis of atherosclerosis (Michelsen et al., 2004a). TLR4 is one of the best characterized TLRs, which binds to lipopolysaccharide (LPS) from Gram-negative bacterial cell walls (Akira and Takeda, 2004).

TLR4 signaling is triggered upon LPS binding to the corresponding TLR receptor. In brief, LPS interacts with circulating LPS-binding protein (LBP) and subsequently binds to TLR4 on the cell membrane. There, interactions between TLR4 with two co-receptors, the CD14 and the myeloid differentiation protein 2 (MD-2), are required to activate the downstream signalling cascade. Subsequently, both myeloid differentiation factor 88 (MyD-88)-dependent and (MyD-88-independent TLR4 signaling pathways are activated via different adaptor proteins. The MyD88-dependent pathway signals through activation of IκB kinase (IKK) and mitogen activated protein kinase (MAPK) pathways, which in turn lead to the activation of transcription factors nuclear factor (NF)-κB and activator protein (AP)-1, respectively, and control the expression of pro-inflammatory cytokines and other immune related genes. In addition, phosphatidylinositol 3-kinase (PI3K) and AKT/PKB are important factors downstream of MyD88 that mediate NF-κB activation. The MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing interferon-β (TRIF), which activates interferon regulatory 3 (IRF-3) and induces the expression of interferon (IFN)-β and IFN-responsive genes (Guo and Friedman, 2010). Figure 1.5 depicts TLR4 signalling pathway.

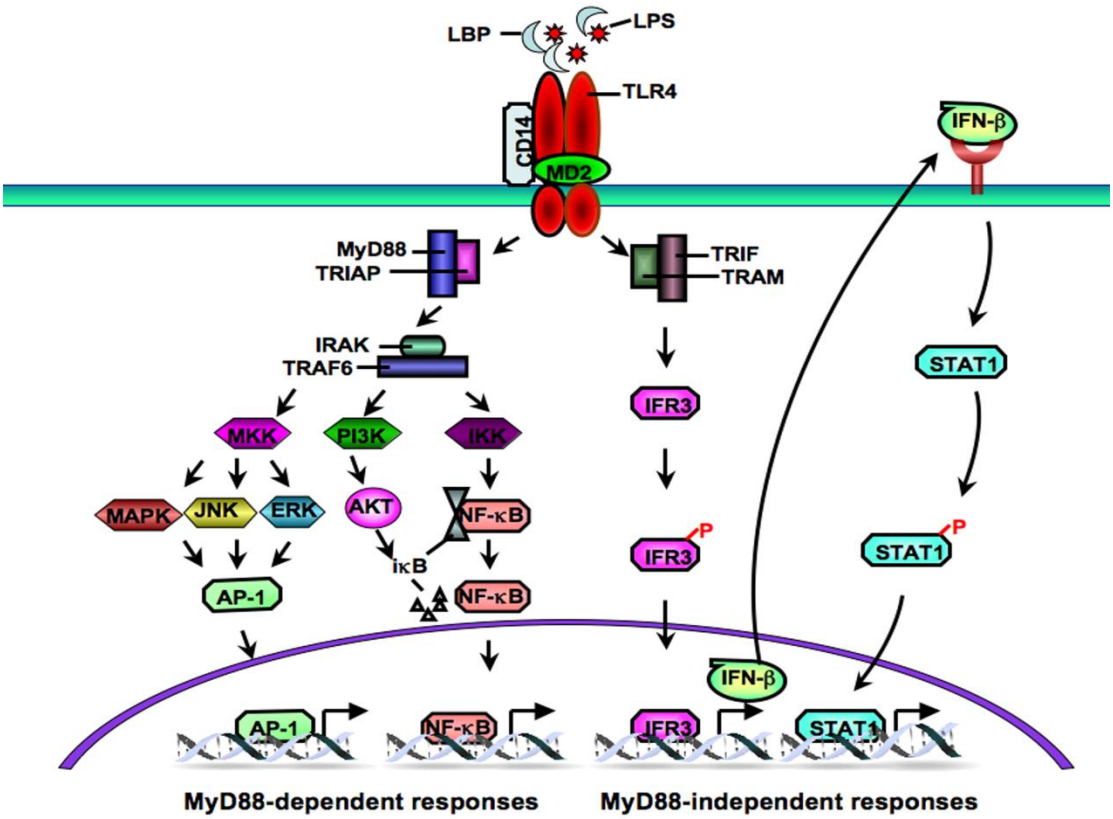


Figure 1.5. TLR4 signaling pathway (Guo and Friedman, 2010).

Numerous studies indicate that TLR4-signalling promotes the development of atherosclerosis. For example, in murine models of atherosclerosis, aortic plaque burden is significantly reduced in animals with genetically ablated TLR4, or the adaptor MyD88 (Michelsen et al., 2004b). Additionally, acceleration of atherosclerosis is observed in mice injected with purified ligands of TLR4 (Cuaz-Perolin et al., 2008).regarding human atherosclerosis, LPS has been identified in diseased, but not healthy arteries, using specific antibodies and PCR (Erridge et al., 2008). Recently, Lu et al showed that R. sphaeroids LPS (RS-LPS), a TLR4 antagonist, inhibited vascular inflammation and atherogenesis in diabetic apoE-/- mice (Lu et al., 2012). Furthermore, Wang et al observed that LPS promotes lipid accumulation via the up-regulation of adipose differentiation-related protein (ADRP) expression through TLR4 activated downstream of NF-kappaB in adventitial fibroblast (Wang et al., 2012). Polykratis et al 2012 reported that endothelial cell specific TRAF6 deficiency reduced the severity of atherosclerosis in female ApoE-/- mice, while ApoE-/- mice lacking TRAF6 specifically in myeloid cells showed more severe atherosclerosis, suggesting that TLR-mediated TRAF6 signaling acts in endothelial cells to promote atherosclerosis, but displays atheroprotective anti-

inflammatory and pro-survival functions in myeloid cells (Polykratis et al., 2012). This result highlights the TLR4 signaling complexity.

Atherosclerosis and autoimmunity

The prevalence of atherosclerosis is increased in several autoimmune inflammatory diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriatic arthritis, ankylosing spondylitis and vasculitis. However, SLE and RA are presented with the higher relevance of the disease (Hahn et al., 2008) . Clinical data indicate that there is a five-fold increased risk of atherosclerosis in the SLE population, while this number is increased in women up to 50-fold (Manzi et al., 1997). Atherosclerosis appears at a high rate in RA patients, in whom CVD is the major cause of morbidity and mortality (approximatey 70% increased risk of death compared with the general population) (Sattar et al., 2003).

It seems that the persistent inflammatory activity in patients with SLE or RA is the principal cause of accelerated atherosclerosis. SLE is characterized by both the production of auto-antibodies and the activation of immune complex in target tissues resulting in organ damage (Rahman and Isenberg, 2008). A plethora of autoantibodies in SLE including antibodies against endothelial cells, oxLDL, phospholipid and HDL have been reported. These auto-antibodies participate in the development of atherosclerotic lesions both by causing injury to the endothelium and by altering the lipoproteins metabolism. Additionally, immune complexes, are able both to upregulate adhesion molecules on endothelial cells, e.g. vascular cell adhesion molecule 1 (VCAM-1), and also to promote monocyte recruitment (Narshi et al., 2011).

Nowadays, it is clear that cytokines, produced at high levels in RA, promote vascular disease at the molecular level (Del Rincon et al., 2003). RA is associated with destructive, inflammatory arthritis. The primary site of inflammation is the synovial tissue, from which cytokines can be released into the systemic circulation. Tumor necrosis factor α (TNFα), IL-1β, and IL-6 are produced at high levels and affect the function of distant tissues, such as the adipose tissue, skeletal muscle, liver, and vascular endothelium generating a spectrum of proatherogenic changes including insulin resistance, dyslipidemia, prothrombotic effects, pro-oxidative stress, and endothelial dysfunction. These changes promote accelerated atherogenesis as presented in Figure 1.6 (Sattar et al., 2003).

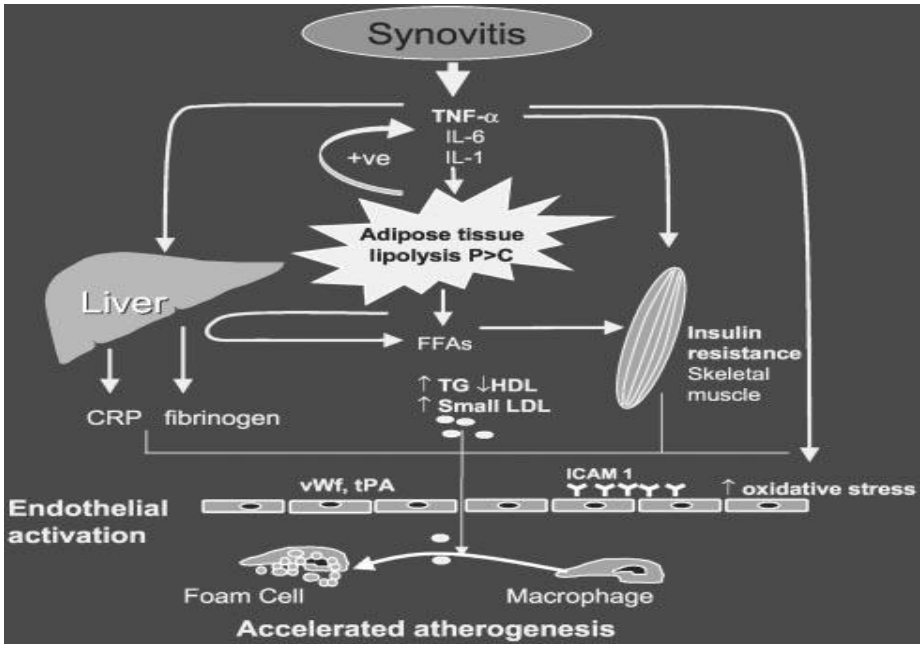


Figure 1.6. Cytokines production from an inflamed synovial tissue contributes to atherogenesis (Sattar et al., 2003).

The atheroprotective role of HDL

It is well known that HDL levels are reversely correlated to the risk of atherosclerotic events (Miller and Miller, 1975). HDL exerts atheroprotective role by participating in diverse biological functions ranging from lipid transport to innate immune functions. The main mechanism, by which HDL prevents atherosclerosis, is the process of reverse cholesterol transport. As mentioned above, excess of cholesterol in cells is toxic. HDL is responsible for removing excess cholesterol from peripheral tissues, including tissue macrophages, foam cells, and artery walls and transporting it back to the liver for excretion into bile (Zannis et al., 2006).

HDL exerts atheroprotection through its antioxidative properties. Both proteins and lipids in LDL are protected from oxidative modifications *in vivo* in the presence of normal HDL (Navab et al., 2004). The antioxidative capacity of HDL depends on several antioxidant enzymes and several apolipoproteins. ApoA-I plays a key role not only by removing oxidized phospholipids both from LDL and from arterial wall cells, but also by stabilizing antioxidant enzymes in HDL, including PON1, platelet-activating factor acylhydrolase (PAF-AH), LCAT and glutathione peroxidase (Navab et al., 2004; Navab et al., 2000). These enzymes hydrolyze LDL derived short-chain oxidized phospholipids (Navab et al., 2004).

HDL interactions with lipids in human arterial endothelial cells are an additional atheroprotective mechanism. Oxidized lipids such as 1-palmitoyl- 2-arachidonyl-sn-3-glycero-phosphorylcholine (ox-PAPC) and its component

phospholipid, 1-palmitoyl-2-5,6 epoxyisoprostanoyl)- sn-glycero-e-phosphocholine (PEIPC), are present in atherosclerotic lesions activating endothelial cells to induce inflammatory and pro-oxidant responses. These responses include the induction of genes regulating chemotaxis, sterol biosynthesis, the unfolded protein response, and redox homeostasis. It has been observed that addition of normal HDL to the arterial endothelial cells *in vitro* inhibits the proinflammatory responses, resulting in reduced chemotactic activity and monocyte binding (Gharavi et al., 2007). Also, it has been shown that HDL stimulates endothelial cell NO production promoting endothelial repair mechanisms (Seetharam et al., 2006; Yuhanna et al., 2001)

Furthermore, cellular contact between stimulated T cells and monocytes is inhibited by HDL-associated apoA-I. This results in decreased monocyte activation and subsequently decreased release of the highly proinflammatory cytokines IL-1β and TNF-α (Hyka et al., 2001).

Pro-inflammatory HDL

More and more studies indicate that the quantity of HDL in serum alone does not determine its atheroprotective role, but also the quality of HDL is equally important. It seems that HDL is a “chameleon-like” lipoprotein with the capacity to be antiinflammatory in the basal state and proinflammatory during acute-phase responses (Navab et al., 1996). During acute or chronic inflammation, several changes occur in HDL affecting their protein cargo and as a result change their functional capacity (Hahn et al., 2008).

During acute phase responses, several plasma proteins carried by HDL are decreased, including PON, LCAT, CETP, PLTP, hepatic lipase, and apoA-I. Also, acute-phase HDL are depleted in cholesterol ester but enriched in free cholesterol, triglycerides, and free fatty acids, none of which can participate in reverse cholesterol transport or antioxidation. Additionally, lipids in acute-phase HDL are oxidized. On the contrary, the levels of the pro-oxidant protein serum amyloid A (SAA) levels are increased in these HDL particles. These changes result in the conversion of the anti-inflammatory HDL to proinflammatory HDL (piHDL). This HDL not only is unable to prevent LDL oxidation, but also enhances this oxidation and therefore induces the monocyte chemotactic activity. Furthermore, piHDLs are much less effective than normal HDLs in removing cholesterol from macrophages and delivering cholesterol esters to hepatocytes (Hahn et al., 2008)

Numerous studies correlate atherogenesis with the presence of dysfunctional, proi-inflammatory HDL. Recently, Besler et al presented that HDL from patients with stable CAD or an acute coronary syndrome (HDL-CAD) did not have endothelial

antiinflammatory effects and did not stimulate endothelial repair because it fails to induce endothelial NO production (Besler et al., 2011). In other reports, CAD patients was shown to have proinflammatory properties, which promoted, rather than prevented, the accumulation of oxidized phospholipids in LDL (Ansell et al., 2003). Additionally, piHDL levels have been shown to be increased in patients with systemic lupus erythematosus and RA (Charles-Schoeman et al., 2009; McMahon et al., 2006)

The measurement of HDL functionality is based on the ability of normal HDLs to prevent oxidation of LDLs *in vitro* (Navab et al., 2001). Detection of piHDL has been identified as a potentially useful marker for gauging susceptibility to atherosclerosis. Interestingly, patients with piHDL have high serum levels of HDL suggesting that assessment of HDL functionality may be a better predictor of atherosclerosis than standard measurements of plasma lipids (Ansell et al., 2003)

Apolipoprotein M

Apolipoprotein M (apoM) is a novel apolipoprotein, which was first identified by Xu and Dahlback in 1999 (Xu and Dahlback, 1999). In plasma, human apoM is mainly part of HDL, but it is also present at lower quantities in other lipoprotein classes including chylomicrons, VLDL and LDL (Christoffersen et al., 2006). The mean molar plasma concentration of apoM is approximately 0.9μM (23mg/L) which is 2-3% of that of apoA-I. In humans, apoM constitutes a minor apolipoprotein found only in 5% and 2% of total HDL and LDL respectively (Christoffersen et al., 2006).

ApoM is synthesized mainly in the liver and to a smaller degree in the kidney. (Xu and Dahlback, 1999). The human apoM gene (*APOM*) is located on chromosome 6 at position p21.3 in the region of major histocompatibility complex (MHC) III. This is a highly conserved chromosomal region rich in genes involved in innate immunity and inflammation, including TNF and lymphotoxins A and B (Deakin et al., 2006). *APOM* expression is directly regulated by transcription factors, including hepatic nuclear factor-1α (HNF-1α), HNF4-α, liver receptor homolog-1 (LRH-1), forkhead box A2 (Foxa2), and liver X receptor (LXR), all of which contribute to hepatic lipid and glucose metabolism (Dahlback and Nielsen, 2009; Mosialou et al., 2010). Interestingly, Mosialou et al in 2011 showed that the activator protein 1 (AP-1) induces apoM downregulation via activation of the protein kinase C (PKC) and the Jun transcription factors (both c-Jun and JunB). Specifically, they identified and characterized an AP-1 element inside the binding site for HNF-1α and they showed that apoM downregulation by pro-inflammatory cytokines is facilitated by the competition between HNF-1α and Jun proteins for binding to the same regulatory site on the proximal apoM promoter. This observation indicates a direct link between

inflammation and atheroprotective lipoproteins gene regulation (Mosialou et al., 2011).

APOM encodes a 22kDa protein (188 amino acid residues in length) that belong to lipocalin protein superfamiy. Members of this superfamily exhibit diverse properties such as lipid binding, transport, and immunological functions (Duan et al., 2001). The recent elucidation of the crystal structure of human recombinant apoM (r-apoM) demonstrated a typical lipocalin fold characterized by an eight-stranded antiparallel β-barrel enclosing an internal binding pocket that probably facilitates binding of small lipophilic ligands (Sevvana et al., 2009). A noteworthy property of the apoM protein is that its sequence lacks a signal peptidase cleavage site. This means that the retained signal peptide probably serves as a hydrophobic anchor, localizing apoM to the single phospholipid layer of lipoproteins. This unusual feature is shared only with paraoxonase-1 (PON-I) and haptoglobin-related protein (HRP), both being HDL-associated proteins (Axler et al., 2008).

Apolipoprotein-M functions have been associated with atheroprotection. In LDL-receptor knockout mice, overexpression of the human apoM gene resulted in protection against atherogenesis (Hu et al., 2010). Moreover, Wolfrum et al, showed that silencing of *apoM* gene, with a spefic siRNA against *apoM*, resulted both in the accumulation of cholesterol in large HDL particles and in the absence of pre-β-HDL particles in the plasma (Wolfrum et al., 2005). Also, Christoffersen et al showed that apoM-containing HDL inhibits LDL oxidation by Cu^{+2} (Christoffersen et al., 2006). However, the mechanism by which apoM exerts these atheroprotective functions, was not clear until recently.

In a study that was published recently, Christoffersen et al found that apoM is the physiological carrier of sphingosine-1-phosphate (S1P) in HDL (Christoffersen et al., 2011). S1P is suggested to be responsible for many protective effects of HDL on endothelial cells. For instance, HDL-S1P inhibits the migration of vascular smooth muscle cells and promotes both vasodilatation and endothelial cell growth and survival. Furthermore, it has been demonstrated that HDL–S1P inhibits reactive oxiygen species production, while it also interferes with the recruitment of monocytes and lymphocytes into the intima of the arterial wall by inhibiting endothelial cell expression of cell adhesion molecules (Sattler and Levkau, 2009). Christoffersen et al. observed that not only S1P was lacking in apoM-free HDL, but this HDL was dysfunctional, since apoM-free HDL did not elicited the characteristic S1P effects on endothelial cells. Also, they showed that lungs from apoM-deficient mice were characterized by a reduction in basal endothelial barrier function (Christoffersen et al., 2011)

In addition to its atheroprotective functions, Elsoe et al showed that apoM is also able to bind oxidized phospholipids allowing HDL to serve as a sink for oxidation products. Specifically, they observed that recombinant apoM is capable of binding oxidized phospholipids including PAPC (1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine), a major arachidonoyl phospholipid, oxidation products, such as POVPC (1-(palmitoyl)-2-(5-oxovaleroyl)-sn-glycero-3-phosphatidylcholine) and PGPC (1-palmitoyl-2-glutaryl-phosphatidylcholine), while it could not bind native PAPC. This ability of apoM to trap oxidized phospholipids may both prevent lipoprotein oxidation and adverse the effects of oxidized phospholipids on the arterial wall (Elsoe et al., 2012).

Nuclear hormone receptors

Nuclear receptors (NRs) are a superfamily of eukaryotic ligand-depedent transcription factors that regulate important processes in response to a broad array of physiological stimuli. In humans, 48 NRs have been identified, and their function has been implicated in nearly every biologic process, including development, homeostasis, metabolism, circadian rhythms, endocrine function, reproduction, inflammation, and immunity (Kidani and Bensinger, 2012). NRs respond to lipophilic hormones, vitamins, dietary lipids, or other intracellular signals, most of which have turned out to be intermediates or end products of metabolic pathways (Chawla et al., 2001b). Dysfunction of nuclear receptor signaling leads to proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity, type 2 diabetes and cardiovascular disease.

Members of the NR superamily are characterized by a similar structure that is highly conserved through evolution. This structure is divided in mainly four functional domains: 1) a N-terminal, ligand-independent transactivation domain (A/B domain) called activation function (AF-1), 2) a conserved DNA binding domain (DBD or C domain) composed of two zing fingers , 3) a domain adjacent to DBD called D or hinge domain containing nuclear localization signals and protein-protein interaction sites and 4) a C-terminal ligand binding domain (LBD or E domain) that accommodates small lipophilic molecules and harbors a ligand-dependent activation function 2 domain (AF-2) (Castrillo and Tontonoz, 2004). Figure 1.6 depicts the basic structure of NR

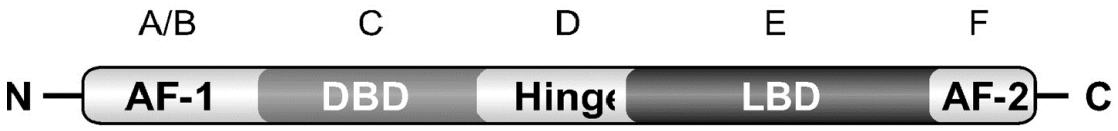


Figure 1.6 The basic structure of nuclear receptors (Christoffersen et al., 2006).

NR members can be divided into three main subfamilies (Gonzalez and Castrillo, 2011):

1) The steroid hormone receptors that mediate most of the biological actions of steroid hormones, such as estrogen receptor and glucocorticoid receptor.
2) The “orphan” receptors, such as the nerve growth factor IB (Nur77) and the neuron-derived orphan receptor (NOR1), whose endogenous ligands are still not identified and it remains unclear if these NRs require ligand binding to activate transcription. Many of these receptors rely on other physiologic stimuli, such as growth factor signaling, for activation.
3) The “adopted” orphan receptors, that were identified prior to the discovery of their regulatory ligands and now have been deorphanized with the aid of chemical, structural and genomic technologies, including the liver-X-receptors (LXRs) and the peroxisome proliferator-activated receptor gamma (PPARγ).

NRs bind to hormone response elements (HREs) in their target promoters through their DBD. HREs variously contain direct repeats (DRs), inverted repeats (IRs) or palindromic repeats (PRs) of the sequence 5’-AG(G/T)TCA-3’. These repeats are separated by one, two, three, four, or five nucleotides and are designated DR1, DR2, etc. (for direct repeats), IR1, IR2, etc. (for the inverted repeats), and PR1, PR2, etc. (for the palindromic repeats) (Mangelsdorf and Evans, 1995). The exact sequence of the repeats and the spacing as well as the 5’ extension of the HREs define the specificity of different nuclear receptor heterodimers. Additionally, it has been reported that NRs regulate the expression of genes lacking canonical HREs through physical and functional interactions with other promoter bound transcription factors such as specificity protein 1 (Sp1), acting as superactivators, or AP1, acting as transrepressors (Glass, 2006).

Steroid-activated nuclear receptors form homodimers and bind to HREs, while the third subclass of nuclear receptors form heterodimers with Retinoid X Receptor (RXR) and bind to HREs (Mangelsdorf and Evans, 1995). In the absence

of ligand, NRs bind to DNA and associat with corepressor proteins, such as silencing mediator of retinoic acid, thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR) resulting in the repression of transcription (Perissi et al., 2010). Upon ligand binding heterodimeric NRs undergo conformational changes that lead to the dissociation of correpressor complexes and the subsequent recruitment of coactivators resulting in gene activation (Bourguet et al., 2000).

Liver X receptor (LXR)

LXRs are nuclear receptors involved in lipid metabolism regulation, as well as in innate and adaptive immunity. Two isoforms have been identified, LXRα (*NR1H3)* and LXRβ *(NR1H2)*, whose genes are located in different chromosomes. LXRα and LXRβ proteins have considerable sequence homology, sharing about 77% identity in their DBD and LBD domains both in humans and rodents (Bensinger and Tontonoz, 2008). However, LXR tissue distribution differs significantly. LXRβ is more ubiquitously expressed, while LXRα is highly expressed in metabolic tissues such as the liver, adipose tissue, intestine, kidney and tissue macrophages (Repa and Mangelsdorf, 2000). Both isoforms are activated by sterol metabolites such as 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol, as well as by synthetic high affinity agonists such as TO901317 and GW3965 (Lehmann et al., 1997). LXRs bind to characteristic DNA sequences termed LXR response elements (LXRE) of DR-4 type as obligate heterodimers with RXR (Willy et al., 1995).

Nowadays it is clear that LXRα and LXRβ are crucial regulators of both cholesterol and lipid homeostasis, by controlling several genes involved in these pathways. Specifically, LXRs by inducing the expression of cholesterol efflux transporters ABCA1 and ABCG1, the lipoprotein remodeling enzyme PLTP, and apolipoproteins ApoC and ApoE in peripheral tissues and in macrophages, control the mechanism of reverse cholesterol transport (Kidani and Bensinger, 2012). Interestingly, Zelcer et al showed that LXRs induce IDOL expression, a novel LDLr degrader, resulting in the decrease of LDL uptake (Zelcer et al., 2009). In the liver, LXR activation regulates genes involved in cholesterol processing and excretion including cytochrome P450 7a1 (Cyp7a1), the rate-limiting enzyme for the conversion of cholesterol to bile acids. Intestinal LXR inhibits cholesterol absorption by upregulating the expression of cholesterol transporters, ABCG5 and ABCG8. Additionally, LXRs increase hepatic lipogenesis through transcriptional upregulation of both sterol regulatory element-binding protein 1c (SREBP1c), the master lipogenic regulator and fatty acid synthase (FAS) (Kidani and Bensinger, 2012). Also, it has

been reported that LXRα activates adipocyte lipolysis by downregulating the peripilin-1 (PLIN1) gene that encodes a lipid droplet coating protein, which inhibits lipolysis (Stenson et al., 2011).

As mentioned above, macrophages participate in the development of atherosclerosis, a disease characterized by chronic inflammation. In this regard, the role of LXRs in macrophage biology has great importance. LXRs by activating a gene program that increases lipid efflux (e.g. ABCA1, ABCG1, ApoE) and decreases lipoprotein uptake (e.g. IDOL), protect macrophages against excessive lipid accumulation and lipotoxicity (Gonzalez and Castrillo, 2011). In addition to this, LXRs have been reported to have anti-inflammatory effects since they inhibit macrophage inflammatory gene expression, including iNOS, COX-2, cytokines IL-6 and IL-1β and MCP-1 (Joseph et al., 2003). Detailed analysis of the promoter regions of these genes demonstrated that many of them have regulatory regions containing NFκB, AP-1 and ISRE consensus binding sites but not HREs, indicating that the anti-inflammatory properties of these receptors may, to a large extent, be mediated through transrepression mechanisms (Ogawa et al., 2005). It has been shown that ligand-activated LXRs preserve the integrity of the corepressor complexes at the promoters of inflammatory genes by interacting with HDAC4 E3 ligase and SUMO2/3 to prevent inflammatory signal dependent activation (Ghisletti et al., 2007).

These data helped to explain the intimate link between TLR signaling and LXR activity. While it is widely understood that LXR activity can repress NF-κB-dependent inflammatory gene expression, it is not known whether TLR signalling can repress LXR. In 2003, Castrillo et al showed that bacterial or viral infection inhibits the function of LXRs resulting in reduced expression of ABCA1, ABCG1 and apoE genes and subsequently decreased cholesterol efflux. These effects were mediated by both TLR3 and TLR4. Interestingly this inhibition was mediated by the transcription factor IRF3 and was independent of MyD88 and NFκB (Castrillo et al., 2003).

The mechanisms that control the expression of both LXR isoforms are not completely understood. Whereas LXRβ seems to be constitutive expressed in many cell types, the regulation of LXRα expression seems to be of great importance. Inflammatory mediators (LPS) and cytokines (TNFα, IL-1β) have been reported to promote a reduction in LXRα mRNA levels in adipocytes (Lu et al., 2006), hepatocytes (Fang et al., 2004), kidney cells (Wang et al., 2005), as well as in 3T3-adipocytes (Lu et al., 2006), in the human hepatoma cell line Hep3B (Kim et al., 2007; Kim et al., 2010) and in the human kidney cell line HK-2 (Wang et al., 2005). However, the regulation of LXRα in macrophages is more complicated. Castrillo et al

showed that E.coli treatment of mice inhibits LXRα target gene induction in thioglycolate-elicited peritoneal macrophages without downregulating the LXRα expression (Castrillo et al., 2003). On the contrary, Park et al showed that LPS downregulates LXRα expression in thioglycolate-elicited peritoneal macrophages, but this effect was not observed in RAW264.7 macrophages (Park et al., 2012). Additionally, in mouse macrophages, PPARα and PPARγ have been shown to induce the expression of LXRα through a PPRE present in the promoter of LXRα gene (Tobin et al., 2000). Furthermore, in humans but not in mice, LXRα gene is subject to an autoregulatory loop, through three functional LXREs in the LXRα gene promoter, in which both LXRα and LXRβ can bind (Laffitte et al., 2001; Whitney et al., 2001). However, this autoregulation does not seem to apply in the case of the LXRβ gene. Together, these studies show that the expression of LXRα is not only species and tissue specific, but also it is likely to be regulated at several levels in response to certain metabolic and inflammatory signaling pathways.

Hepatocyte nuclear factor 4α (HNF-4α)

HNF-4α is a liver-enriched transcription factor that plays a significant role in hepatocyte differentiation and function. HNF4 controls many liver specific functions including lipid and glucose metabolism, bile acid synthesis and serum protein production, by regulating the expression of specific genes, such as apoB, MTP, apoA-II, apoA-IV, apoC-II, apoCIII, PXR, HNF-1α, and HNF-4α itself. HNF-4α is also expressed in intestine, kidney and pancreatic beta- islet cells at lower levels (Sladek, 1994). HNF4 deficient mice are embryonically lethal, while liver-specific inactivation of the HNF-4α gene caused weight loss, increased mortality and lipid abnormalities (Hayhurst et al., 2001; Li et al., 2000). Furthermore, heterozygous mutations in the HNF-4α gene are associated with an early onset form of type II diabetes called maturity onset diabetes of the young 1 (MODY1) characterized by impaired triglyceride metabolism and insulin secretion (Shih et al., 2000) whereas genome wide association studies (GWAs) identified a common variant in the coding region of HNF-4α gene (rs1899861) associated with low HDL concentrations in humans (Kathiresan et al., 2009)

HNF-4α belongs to nuclear receptors and was initially believed to be an orphan receptor. However, crystallographic studies showed that fatty acids may be endogenous ligands of this factor (Sladek, 1994). HNF-4α binds to DNA as a homodimer and activates target gene transcription via the recruitment of coactivators such as steroid receptor coactivator 1 (Src-1), glucocorticoid receptor interacting

protein -1/transcriptional intermediary factor 2 (GRIP1/TIF2) as well as histone acetyltransferase CREB binding protein (CBP) and peroxisome proliferator activated receptor γ coactivator 1 (PGC-1) (Viollet et al., 1997). Interestingly, protein-protein interactions with other transcription factors such as Smads, NF-κB or SHP affect its transcription activity. NF-κB inhibits while Smads increase the transcriptional activity of HNF4α (Lee et al., 2000). Additionally, post-translational modifications are essential for its transcriptional activity. Both phosphorylation by PKA (Viollet et al., 1997) , MAPK and JAKs (De Fabiani et al., 2001) and acetylation by CBP (Soutoglou et al., 2000) affect its DNA binding activity and consequently its interaction with coactivators.

Purpose of study

Numerous studies indicate that patients with chronic inflammatory diseases such as rheumatoid arthritis or chronic kidney disease are characterized by increased concentration of proinflammatory cytokines in the plasma (TNFα, IFNg) and by the presence of proinflammatory HDL which contributes to the pathogenesis of atherosclerosis in these patients. Apolipoprotein M, a novel protein that is present in a subfraction of HDL particles, is expressed mainly in the liver and exerts various anti-inflammatory and anti-oxidative actions due to its role as the sole carrier of sphingosine 1 pshosphate (S1P) in HDL. We have shown previously that proinflamamtory cytokines such as TNFα inhibit the expression of apoM gene in hepatic cells via a novel mechanism that involves competition between HNF-1α and AP1 factors for a common regulatory element in the proximal promoter. Thus, in Part I of this thesis we examined the levels of HDL, apoA-I and apoM in plasma samples that were taken from patients with inflammatory disease (SLE, RA) that were presented in the Rheumatology Clinic of the University Hospital of Crete.

Liver X Receptors (LXRs) are important regulators of cholesterol homeostasis contributing to cholesterol efflux and subsequently exert atheroprotection. LXRS are activated by certain hydroxylated products of cholesterol (oxysterols) and to regulate the expression of various HDL genes including ABCA1, ABCG1 and apoE among others. Previous reports have shown that the expression of LXRα is subject to a negative feedback loop but the details of this mechanism are still unknown. Furthermore, the expression of LXRα was shown previously to be downregulated during inflammation or infection. Given the significant role of LXRa in HDL metabolism, in Part II of this thesis we focused on the mechanisms that control the expression of the human LXRα gene in hepatic cells. Specifically, we attempted to identify and characterize hormone response elements (HREs) in the promoter of the LXRα gene and to elucidate the mechanism by which lipopolysaccharide (LPS) and its receptor, Toll Like Receptor 4 (TLR4), inhibit the activity of the LXRα promoter.

2. Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and trypsin/EDTA for cell culture were purchased from Invitrogen/Life Technologies. Fetal bovine serum (FBS) was purchased from BioChrom Labs.

The luciferase assay system and the cell lysis buffer were purchased from Promega Corp. ONPG (*o*nitrophenyl *β*-D-galactopyranoside) and anti-goat peroxidase-conjugated secondary antibody was purchased from Sigma-Aldrich. Anti-apoM antibody was purchased from Novus Biologicals. Anti-apoA1 antibody was purschased frm Chemicon. Protran nitrocellulose membranes of 0.45 μm size pore were purschaced from Schleicher & Schuell. ECL substrate (Super Signal West Pico chemiluminescent substrate) was purchased from Pierce-Thermo.

Cholesterol kit for measuring cholesterol levels on human serum was purchased from Thermo Scientific. Dextran for HDL isolation from human serum was purchased from MP Biomedicals

Bact-tryptone and yeast extracts for LB liquid media preparation were purschached from Difco. Plasmid kit for plasmid DNA purification was purschased from Qiaqen.

The reporter plasmid bearing the firefly luciferase gene under the control of the human LXRa promoter between nucleotides -2625/+385 has been described previously (Stender et al., 2011). The following hLXRα promoter deletion mutants had been generated by Aristidis Anestis (Anestis A. Diploma thesis, University of Crete, 2011): (-844/+384)-hLXRa-luc, (-457/+384)-hLXRa-luc, (-300/+384)-hLXRa-luc, (-176/+384)-hLXRa-luc, (-111/+384)-hLXRa-luc and (-42/+384)-hLXRa-luc. Plasmids (3X)-kB-luc, (-890/+24)-apoCIII-luc, CMV-p50/p65, CMV-bgal, pMTE-HNF4a, pCDNA3-IKB-DN, pCDNA3-IKK (Δ34) were described previously (Prokova et al. J Biol Chem. 2002 Mar 15;277(11):9342-50; (Mosialou et al., 2010; Prokova et al., 2002). Plasmid vectors expressing TLR4, the full-length IRF3 (FL-IRF3) and a dominant negative form of IRF3 (DN-IRF3) were kindly provided by Dr. Antonio Castrillo (University of Las Palmas, Spain).

Samples from human blood were obtained from the Rheumatology Clinic of the University Hospital of Heraklion, Crete.

Methods

Cell cultures and transient transfections

Cell cultures

For the purpose of this study, we used the human hepatoblastoma HepG2 and the Human Embryonic Kidney (HEK293T) cell lines. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and 1% penicillin/streptomycin (P/S) (growth medium) at 37 ^{o}C in a 5 % CO_2 atmosphere. The growth medium was replenished every second day. When the cells were 80 to 90% confluent, they were subcultured. For this process we used trypsin/EDTA and growth medium.

Transient transfections

Transient transfections were performed using the calcium phosphate [$Ca_3(PO_4)_2$] co-precipitation method. Briefly, one day before transfection, cells are plated in 6-wells in the appropriate amount of growth medium so that they will be 60% confluent at the time of transfection. Therefore, $0.25*10^6$ HEK293T cells and $0.50*10^6$ HepG2 cells are plated per well. The transfection samples are prepared as follows:

- dilute $CaCl_2$ (2M) and DNA in ddH_20
- add equal volume of 2x Hepes Buffered Saline (HBS) while vortexing
- incubate for 15min at RT
- add transfection complexes to cells. Mix gently by rocking the plate back and forth
- incubate the cells at 37 ^{o}C in a CO_2 incubator for 8-18h
- replace medium with fresh medium
- incubate the cells at 37 ^{o}C in a CO_2 incubator for 24h
- harvest cells

It should be mentioned, that the optimal amount of transfected DNA is up to 6 µg per well for 6-well plates. Salmon sperm DNA (ssDNA) is added to equalize the total amount of the transfected DNA in each well.

Reporter assays

For reporter assays, cells were plated in 6-well plates and were subsequently transfected by the calcium phosphate method. Luciferase assays were performed using the luciferase assay kit from Promega Corp. according to the manufacturer's instructions. Normalization for transfection efficiency was performed by β-galactosidase assays.

Cell lysis:

- wash cells with 1 ml ice-cold PBS
- add 200 μl 1 x lysis buffer (Promega) to the HepG2 cells
- or 500 μl to the HEK293T cells
- incubate for 5 minutes at RT in a shaking platform
- scrape and collect cells
- vortex and freeze at -80 ^{o}C for 10-30 minutes
- thaw at 37 ^{o}C for 2 minutes, vortex
- centrifuge for 5 minutes at 13.000 rpm at RT
- keep supernatant (cell extract)

Measurement of luciferase activity:

- Mix 60 μl of cell extract with 60 μl luciferase substrate (Promega) and measure relative light units (RLU) in the luminometer.

Measurement of β-galactosidase activity:

- add to 5 μl of cell extract to a microplate
- add 195 μl of the following mix per plate:
    - 456 μl sodium phosphate buffer (P buffer)
    - 132 μl ONPG (8 mg/ml)
    - 2 μl Mg^{2+} buffer (100Xsalt buffer)
- incubate at 37 oC until the samples are colored yellow
- measure OD at 410 nm

In detail, Sodium phosphate buffer (P buffer) consists of Na_2HPO_4 0.1M (adjusting the pH of Na_2HPO_4 to 7.3 with NaH_2PO_4 0.1M). The appropriate amount of ONPG is resuspended in P buffer to a final concentration of 8 mg/ml. 100Xsalt buffer consists of: 1M KCl, 0.1M $MgCl_2$ and 5M β-mercaptoethanol. Finally, for this process, we utilized a plate reader from TECAN, which can incubate the samples and in

parallel can measure OD at different time points. The optimal OD is among 0.2 to 0.9.

Cloning

For the purpose of this study, plasmid vectors containing the ideal DNA sequence were amplified and purified from bacteria. To do so, we utilized techniques of molecular cloning.

Transformation

We performed transformation of plasmid DNA into bacterial DH10β competent cells using the heat shock method. The process is as depicted below:

- add 0.5 ng of plasmid DNA to 100 λ DH10β competent cells
- incubation for 30 min on ice
- incubate for 45sec at 42 ^{o}C (heat shock)
- cool on ice for 3 min
- add 900μl growth medium (LB) without any antibiotic
- incubate for 1h at 37 ^{o}C
- spread 100μl of the mix on an LB agar plate
- incubate at 37 ^{o}C o/n

Purification of plasmid DNA (midiprep)

For the preparation of sufficient amount of plasmid DNA (100-300 μg), QIAGEN-tips-100 were used according to the protocol provided by QIAGEN (QIAGEN Plasmid Midi Protocol):

- pick a single colony from the plate with the transformation and inoculate it into a 200 ml LB medium containing the appropriate selective antibiotic. Incubate at 37°C, o/n, with vigorous shaking
- harvest the bacterial cells by centrifugation at 3.500 rpm for 30 minutes at 4°C
- for a 100 ml culture, resuspend the bacterial pellet in 4 ml buffer P1 supplemented with RNase A (100 μg/ml)
- add 4 ml buffer P2, mix gently but thoroughly by inverting 2-3 times, and incubate at RT for 5 minutes
- add 4 ml of buffer P3, mix immediately but gently by inverting 2-3 times, and incubate on ice for 15 minutes

- centrifuge at 3.500 rpm for 30 minutes at 4°C. Keep supernatant containing plasmid DNA
- equilibrate a QIAGEN-tip 100 by applying 4 ml buffer QBT, and allow the column to empty by gravity flow
- apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow
- wash the QIAGEN-tip with 2 x 10 ml buffer QC
- elute DNA with 5ml buffer QF into a freshly tube.
- precipitate DNA by adding 3.5 ml RT isopropanol to the eluted DNA. Mix and centrifuge immediately at 11.000 rpm for 30 minutes at 4°C. Carefully decant the supernatant
- wash DNA pellet with 2ml RT 70% EtOH, and centrifuge at 11.000 rpm for 10 minutes
- airdry pellet and redissolve the DNA in 30μl WFI (water for injection)
- determine DNA concentration from the absorbance at 260nm

Human serum manipulation techniques

Serum isolation from human peripheral blood

Whole blood is collected in covered test tube containing no anticoagulant. Then, the blood is allowed undisturbed to clot at room temperature for about 30 minutes. The clot is removed by centrifuging at 2500 rpm for 15 minutes. After centrifugation, the supernatant (serum) is transferred immediately into a clean polypropylene tube. Serum is stored at -80 ^{0}C.

HDL purification

In order to measure HDL-cholesterol and to identify proteins binding to HDL, the HDL fraction should be isolated from whole serum. The process is the below:

- add 3.5 μl $MgCl_2$ (1M, ph 7) into 70 μl serum
- vortex
- add 3.5 μl Dextran (20 μg/L)
- vortex
- incubate at RT for 10 minutes
- centrifuge at 1500g for 30 minutes at 4°C

The supernatant, containing HDL fraction, is transferred into a clean tube. It should be mentioned that the pellet of the centrifugation can be diluted in saline and corresponds to HDL free serum fraction.

Cholesterol measurement

For the measurement of HDL cholesterol, we utilized a cholesterol assay kit provided by Thermo scientific according to the manufacturer's instruction.

Immunoblot analysis

To identify the levels of specific proteins on human serum, we performed western blot analysis. Human serum samples were diluted 1:2 in sterile ddH_20. Samples are boiled at 100^oC for 10 minutes in 2 x sample buffer. 2µl of samples, each of them contains 0.5µl of human serum, were loaded on SDS polyarylamide gels followed by electrotransfer onto nitrocellulose membranes.

SDS-PAGE:

Based on the protein size, samples are run on 12.5 % polyacrylamide gels consisting of the separating and stacking gel. For the electrophoresis 1 x TGS buffer is used. Electrophoresis is occurred at 160V for about 1h.

Separating gel consists of:

- 3.2 ml ddH2O
- 4.2 ml 30% acrylamide
- 2.5 ml separating buffer
- 160µl 10% APS
- 8µl TEMED

Stacking gel consists of:

- 3.6 ml ddH2O
- 0.9 ml 30% acrylamide
- 1.5 ml separating buffer
- 60 µl 10% APS
- 6 µl TEMED

1L of 10X TGS consists of

- 30.3 gr Tis
- 144.2 gr Glycine
- 10 gr SDS

For the preparation of separating buffer, 18.165 g of 1.5M SDS are diluted in 100 ml. For the preparation of stacking buffer, 0.05 g of 0.5M SDS are diluted in 100 ml.

Western Blot:

Following SDS-PAGE, proteins are transferred onto nitrocellulose membranes using transfer buffer. Proteins are then visualized by probing the membrane with appropriate monoclonal or polyclonal antibodies and subsequent incubation with horseradish-peroxidase-conjugated secondary antibodies, as follows:

- wash membrane for 5 minutes with TBS-T (1X TBS with 0.05% Tween)
- incubate for 1 hour at RT in 5 % w/v non-fat milk in TBS-T (blocking)
- incubate with the primary antibody o/n at 4 ^{o}C (1:1000 in 5 % w/v non-fat milk in TBST 0.05 %, 0.02 % NaN3)
- wash 3 times for 10 minutes with TBS-T 0.05 %
- incubate with the secondary antibody for 1 hour at RT (a-goat IgG HRP 1:10000 in 5 % w/v non-fat milk in TBS-T)
- wash 3 times for 10 minutes with TBS-T
- wash once for 5 minutes with 1XTBS
- detect signals by enhanced chemiluminescence

1L of Transfer buffer consists of:

- 100 ml 10XTGS
- 200 ml Methanol
- 700 ml ddH2O

1 L of 10XTBS consists of:

- 180 gr NaCl
- 121.14 gr Tris
- pH 7.3 (with HCl)

3. Results-Discussion

PART I: Plasma apolipoprotein-M and A-I levels in patients with chronic inflammatory diseases

Apolipoprotein M plays an important role in HDL metabolism and atherosclerosis because it is the sole carrier of the bioactive lipid Sphingosine 1 phosphate (S1P) in HDL (Christoffersen et al., 2011). We have shown recently that pro-inflammatory cytokines such as Tumor Necrosis Factor α inhibit the expression of the human apoM gene in hepatic cells via a mechanism that involves transcription factors HNF-1α and AP-1 competing for the same regulatory element on the proximal apoM promoter (Mosialou et al., 2010). We also showed that the mouse apoM promoter bears a nucleotide substitution within the dual HNF-1/AP1 element that prevents binding of AP1 factors to this site. As a consequence, the apoM gene is not inhibited by pro-inflammatory stimuli in mouse cells and thus mouse models are not useful for the validation of our in vitro findings. To verify that the human apoM gene is downregulated during inflammation *in vivo*, we sought to collaborate with the research team of Professor Dimitrios Boumpas at the Department of Internal Medicine of the University of Crete Medical School in order to obtain access to samples from human patients with chronic inflammatory diseases. Our hypothesis was that if the expression of the apoM gene in the liver of patients were downregulated, this should reflect decreased amounts of apoM in the HDL fraction and reduced atheroprotection.

Thus, in collaboration with Professor Boumpas, we obtained serum from patients with Systemic Lupus Erythematosus (SLE) or Rheumatoid arthritis (RA) that were presented at the Rheumatology Clinic of the university Hospital of Heraklion.
Some of these patients were receiving medications for the disease such as methotrexate (inhibitor of DHFR) hydroxychoroquine (inhibition of lysosomal acidic proteases), prednisone (synthetic corticosteroid) or anti-TNFα, but others were not. Table 3.1 shows the clinical characteristics of the patients participating in the study (disease activity status, treatment). SLE and RA activity in the patients were measured in the Rheumatology Clinic of University Hospital of Heraklion, using the SLEDAI score for SLE disease and the DAS28 score for RA disease. Also, RA and SLE patients fulfilled the ARA classification criteria. As controls we used healthy subjects.

Table 3.1. *Clinical characteristics of the participants in the study.*

	condition	Disease activity	treatment
1.	Healthy (control)	-	-
2.	Healthy (control)	-	-
3.	Healthy (control)	-	-
4.	Patient, SLE	inactive	-
5.	Patient, SLE	active	-
6.	Patient, SLE	active	methotrexate
7.	Patient, RA	active	Anti-TNF
8.	Patient, SLE	inactive	-
9.	Patient, SLE	inactive	methotrexate
10.	Patient, SLE	inactive	hydroxychoroquine
11.	Patient, SLE	active	hydroxychoroquine
12.	Patient, SLE	active	prednisone, hydroxychoroquine
13.	Patient, SLE	active	methotrexate

Apolipoprotein-M serum levels were decreased in patients with active inflammatory disease

To determine the levels of apoM in the plasma of patients with RA or SLE, we performed Western blot analysis as described in the “Materials and Methods” section of this thesis. As shown in Fig. 3.1, a reduction in the levels of plasma apoM was observed in essentially all patients. Specifically, in patients not receiving any medication (patients 4, 5, 8), apoM levels were decreased by approximately 60%. In addition, in patients under medical treatment, apoM levels were decreased by 20-65% depending on the type of treatment they received (patients 9, 11, 12). Importantly, the plasma apoM levels of patients 6 and 13 who were under methotrexate treatment, had physiologival levels of apoM. This was not the case for the plasma apoM levels in patients under treatment with anti-TNFa (patient 7), hydroxychoroquine (patients 10, 11) or a combination of hydroxychoroquine and prednisone (patient 12). Overall, the patients with the lowest apoM levels in the plasma (patients 5, 11 and 12) were patients with active disease suggesting that disease status is positively correlated with plasma apoM levels.

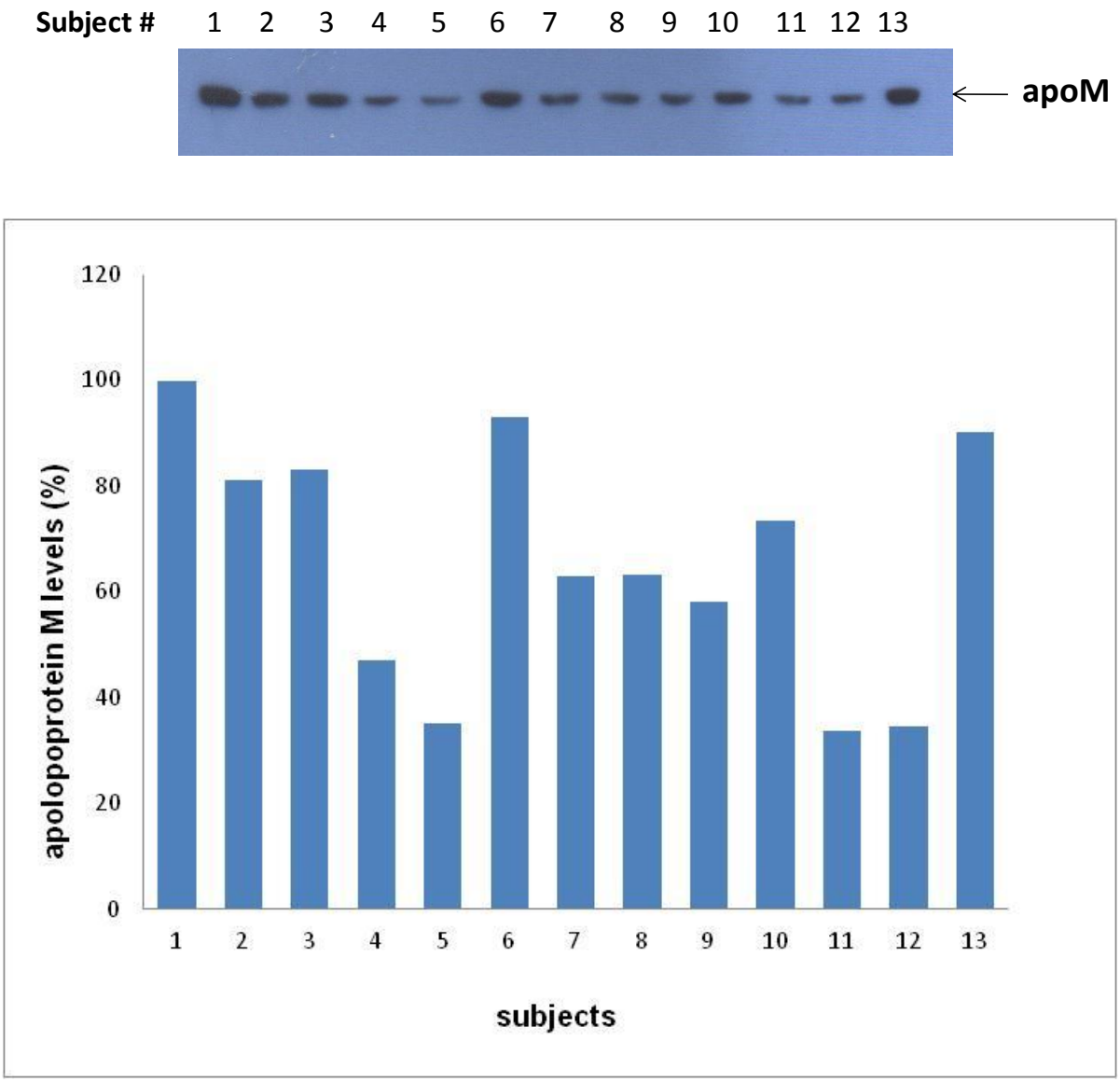


Figure 3.1. Plasma apoM levels in patients with RA or SLE. Numbering 1-13 corresponds to the subjects in Table 3.1. Bands were quantified by laser-scanning densitometry.

HDL and apolipoprotein A-I levels were normal in patients with SLE and RA

ApoM is present in a subfraction of the total HDL particles in plasma (5-10%). This observation suggested to us that a decrease in the plasma apoM levels in patients with RA or SLE should not necessarily reflect overall changes in plasma HDL and apoA-I concentration. To address this question, we measured HDL-cholesterol and total cholesterol in the plasma of the healthy subjects and the patients with RA or SLE using a cholesterol kit from Thermo Scientific. As shown in Table 3.2, total cholesterol and HDL cholesterol levels were not reduced in patients

with RA or SLE with the exception of patient 4 whose HDL cholesterol levels were approximately 50% relative to the healthy subjects. The same patients also had slighty decreased levels of apoM (Fig. 3.1). We concluded that there is no correlation between disease status and plasma HDL or total cholesterol levels in the pool of patients that were examined in the context of this study.

Table 3.2. *Levels of whole serum cholesterol and HDL cholesterol from healthy controls and patients with SLE and RA.*

	Whole serum cholesterol	HDL cholesterol
1.	205.5	77.8
2.	207.3	65.4
3.	216.5	73.6
4.	101	33.6
5.	178.2	65.4
6.	195.8	80.2
7.	145.6	43.7
8.	171.7	67.5
9.	165	61.1
10.	204.2	91.5
11.	173	64.9
12.	155.8	88.4
13.	155.7	62

Apolipoprotein A-I plasma levels in patients with RA or SLE were normal and correlated with HDL cholesterol levels

We then measured the plasma levels of apoA-I in the healthy subjects and the patients with RA or SLE. ApoA-I is a necessary protein constituent of all HDL subfractions, thus the plasma levels of apoA-I accurately reflect changes in total HDL cholesterol and vice versa. Thus, to validate the data of Table 3.2, we performed western blot analysis to identify the levels of apoA-I in the plasma of healthy controls and patients. As shown in Figure 3.2, there is no reduction in the plasma apoA-I levels from patients with RA or SLE. Overall, plasma apoA-I levels correlated well with the levels of HDL as anticipated.

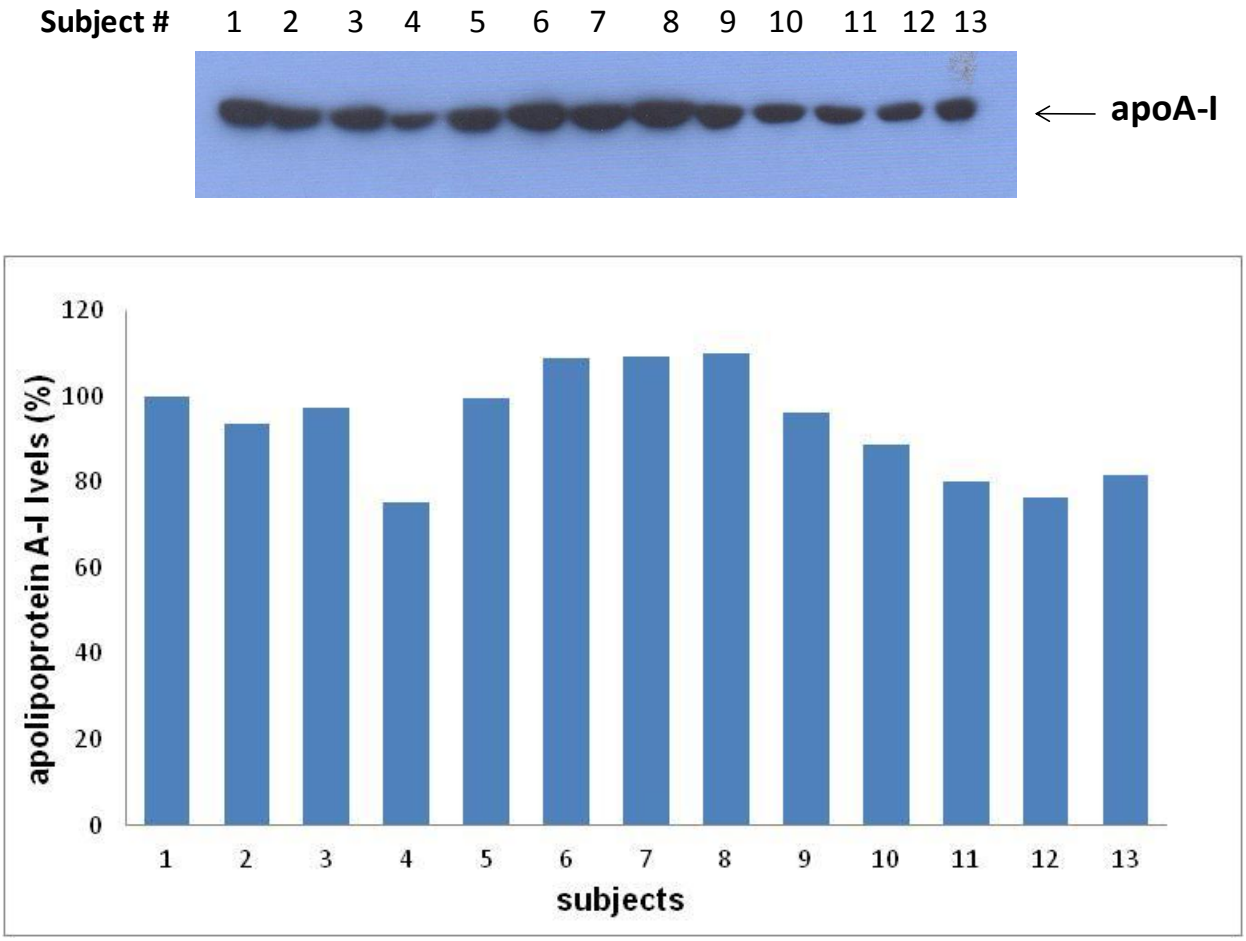


Figure 3.2. ApoA-I levels in the plasma of patients with RA or SLE and healthy controls. Numbers refer to Table 3.1. Bands were quantified by laser-scanning densitometry

Discussion

It is well known that HDL cholesterol levels are inversely correlated with the atherosclerotic risk (Miller and Miller, 1975). However, numerous studies indicated that the quality of HDL is equally important for its atheroprotective role. HDL protects against atherosclerosis by promoting cholesterol efflux from macrophages, as well as by protecting the endothelium. In addition, HDL exerts anti-atherosclerotic function via its anti-inflammatory, anti-oxidant and anti-thrombotic properties (Barter et al 2004). Recent evidence highlights the important role of apolipoprotein-M in the atheroprotective functions of HDL. Besides that apoM binds oxidized phospholipids protecting LDL against oxidation, it is also the physiological and sole carrier protein of S1P in HDL, a bioactive sphingolipid with protective role on the endothelium (Christoffersen et al., 2011; Elsoe et al., 2012).

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are autoimmune inflammatory conditions characterized by increased circulating levels of pro-inflammatory cytokines, endothelial damage and accelerated atherosclerosis (Symmons and Gabriel, 2011). We have shown recently that pro-inflammatory cytokines such as Tumor Necrosis Factor α inhibit the expression of the human apoM gene in hepatic cells via a mechanism that involves transcription factors HNF-1α and AP-1 competing for the same regulatory element on the proximal apoM promoter (Mosialou et al., 2011). Therefore, we wanted to examine apoM levels in serum from patients with SLE or RA. Our hypothesis was that if our findings in the cell cultures were correct, we should observe reduced levels of plasma apoM in patients with chronic inflammation and to identify a positive correlation between diseases status and apoM levels. Furthermore, we anticipated that treatment of patients with anti-rheumatic drugs such as anti-TNFα should normalize plasma apoM levels. We observed that in essentially all patients, apoM levels were decreased compared to healthy controls (Figure 3.1). Importantly, patients not receiving any medication had the lowest levels of apoM in their serum. This observation correlates the absence of apoM with the accelerated atherogenesis. Lower levels of apoM in the serum correspond both to a non sufficient protection of LDL phospholipids against oxidation and to lower levels of S1P bound to HDL. As a result, an atherogenic environment is created.

Moreover, we wanted to examine whether this alternation in the apoM levels was correlated with a whole reduction of HDL levels. Interestingly, we observed that both HDL levels and apoA-I levels were unchanged (Table 3.2, Figure 3.2). This indication confirms that HDL exerts its protective role only when it is bound with the relevant proteins. In fact, Besler et al showed that HDL obtained from patients with coronary artery disease (CAD) does not exert anti-inflammatory function and was not able to stimulate endothelial repair (Besler et al., 2011).

Inflammatory diseases promote the non-protective, pro-inflammatory functions of HDL, by affecting its protein components. Mosialou et al showed that TNFa, a pro-inflammatory cytokine, downregulates apoM expression (Mosialou et al., 2011).Interestingly, patient 7, who is under anti-TNFa treatment, has higher apoM levels compared to patients under no medication. This indicates that inflammation affects the expression of distinct atheroprotective proteins. Kumaraswamy et al found that apoM levels were decreased in patients with sepsis and inflammatory response syndromes (Kumaraswamy et al., 2012)

However, more clinical data are needed to elucidate the correlation between an inflammatory disease and the serum levels of important atheroprotective

apolipoproteins. In addition, more experiments should be done to clarify how inflammation affects both the expression and the function of important proteins that participate in the cholesterol homeostasis, including apoM, apoA-I, ApoA-II and LXR. As mentioned, HDL under inflammatory conditions could be non-protective. So, it is interesting to examine how HDL isolated from patients with an inflammatory disease could affect endothelial cells ex vivo.

PART 2: Mechanisms of transcriptional regulation of the human LXRa gene in hepatic cells

Both isoforms of Liver-X-receptor (LXRα and LXRβ) play key role in the regulation of both cholesterol and lipid homeostasis, by controlling the expression of important genes involved in these pathways. ABCA1, ABCG1, PLTP, ABCG5, ABCG8 and the Inducible Degrader of the Low-density lipoprotein receptor (IDOL) are some of the genes that are under the control of LXRs (Kidani and Bensinger, 2012). Importantly, LXRs have been indicated to exert anti-inflammatory properties, since they inhibit macrophage inflammatory gene expression, including iNOS, COX-2, cytokines IL-6 and IL-1β and MCP-1, through transrepression mechanisms (Ogawa et al., 2005).

However, the mechanisms that control the expression of both LXR isoforms are not completely understood. Whereas LXRβ seems to be constitutively expressed in many cell types, the regulation of LXRα expression seems to be of great importance, because it is highly expressed in metabolic tissues such as the liver, adipose tissue, intestine, kidney and tissue macrophages (Repa and Mangelsdorf, 2000). Many studies have shown that LXRα mRNA levels are downregulated by LPS, TNFα and IL-1β, while the exact mechanism that drives this regulation has not been elucidated until now (Fang et al., 2004; Kim et al., 2007; Kim et al., 2010; Lu et al., 2006; Wang et al., 2005). Thus, in the second part of this study, we wanted to identify novel regulatory elements in the human LXRa promoter, as well as to elucidate the mechanism by which LPS inhibits LXRα expression. In our studies we utilized the human LXRα promoter between nucleotides -2625/+385, that was fused with the luciferase gene (Stender et al., 2011).

Bioinformatic analysis of human LXRa promoter

Initially, to identify putative regulatory elements in the hLXRa promoter, we performed bioinformatic analysis in the region -2625/+385 of the hLXRα gene using the UCSC genome bioinformatics (http://genome.ucsc.edu/). Our analysis focused on the identification of DNase I hypersensitive sites, transcription factor binding sites, conserved trasncription factor binding sites, as well as the sequence conservation of this region among a variety of species. Firstly, we observed that our region contains two DNase I hypersensitive sites which are located on the proximal and the distal sites of the promoter, confirming that this promoter is transcriptionally active (Figure 3.3A). Moreover, we observed that many transcription factors bind to this region.

Figure 3.3B presents the transcription factors that have been found to bind to this region after Chip-seq analysis from Encode. It seems that the specifity protein 1 (SP1) and the Retinoid X Receptor α (RXRα) bind on the distal sites of the promoter, while a variety of transcription factors, including activating proteins 2α and 2γ (AP-2α /γ), c-Myc and the transcriptional repressor CCCTC-binding factor (CTCF) bind on the proximal sites of the promoter. Interestingly, HNF4α was identified to bind to both proximal and distal sites on the promoter indicating the existence of multiple hormone response elements in this region (Figure 3.3B). In addition to this finding, the proximal HNF4 site on the promoter is a conserved site of chicken ovalbumin upstream promoter-transcription factor I (COUP1) (Figure 3.3C). The EAR3/COUP1 transcription factor is an orphan member of the nuclear hormone receptor superfamily and binds to hormone response elements in the promoter of genes that are involved in the cholesterol homeostasis including apoA-II, apoA-IV, apoB and apoC-III (Ktistaki et al., 1994; Ladias et al., 1992). EAR/COUP1 exerts opposing transcription effects compared to HNF4α, downregulating the expression of apoA-II, apoA-IV, apoB and apoC-II genes (Ktistaki et al., 1994; Ladias et al., 1992). Finally, we wanted to investigate the conservation of our DNA sequence among human, rhesus, mouse, dog, elephant, opossum, chicken X-tropicalis and zebrafish. Figure 3.3D depicts that the proximal site of the promoter is highly conserved among species even opossum indicating the existence of important regulatory elementsin this region.

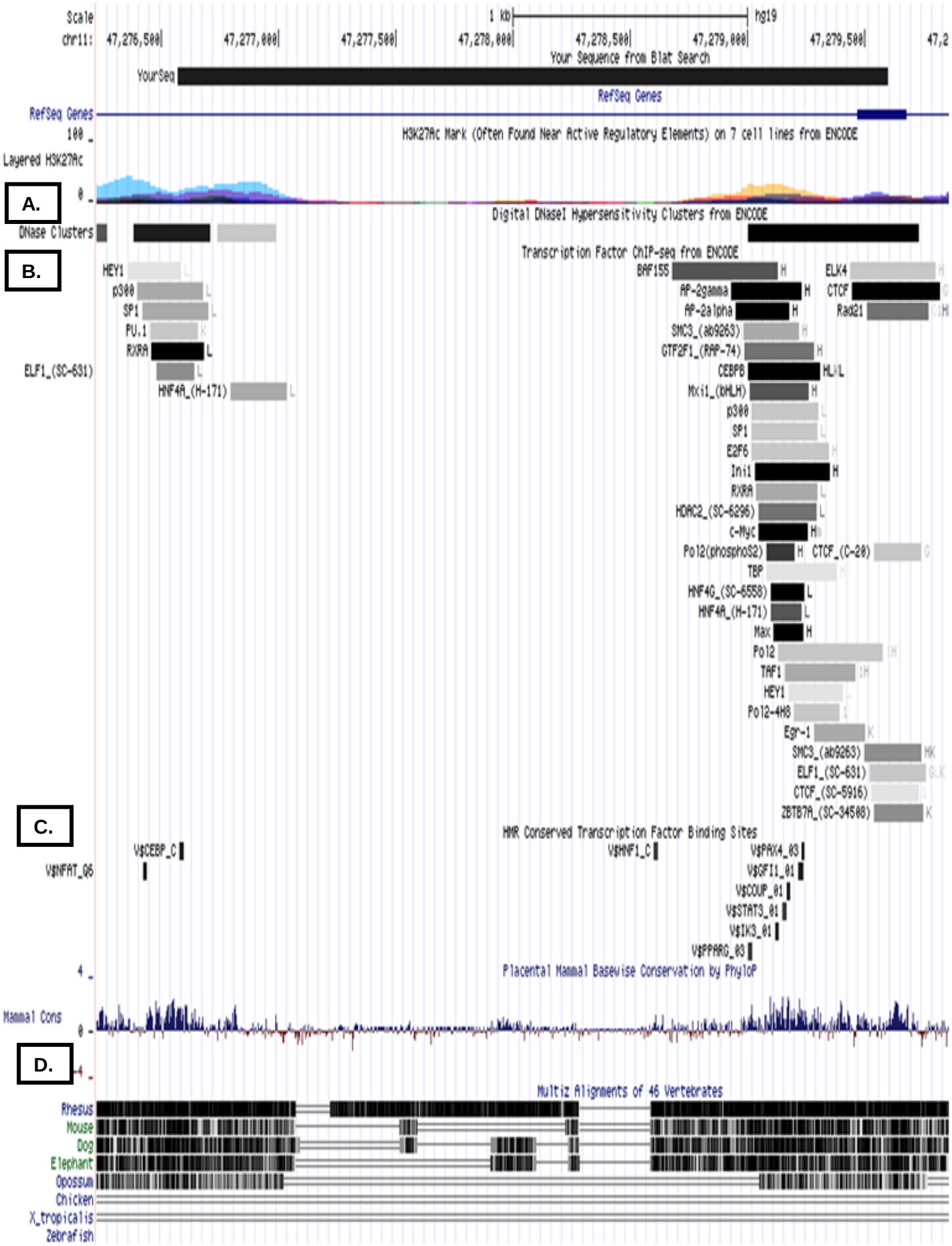


Figure 3.3. Bioinformatic analysis of the -2625/ +384 region of hLXRa gene utilizing the UCSC genome Bioinformatics. **A.** DNase I hypersensitivity sites **B.** Transcription factors binding sited after Chip-seq from Encode. **C.** Conserved transcription factor binding sites. **D.** DNA sequence conservation among many species (rhesous,, mouse, dog, elephant, opossum, chicken, X-tropicalis and zebrafish).

Regulatory elements in the human LXRα promoter

Next, we sought to identify the hormone response element in the promoter of human LXRα that could mediate its induction by Hepatocyte Nuclear Factor 4 (HNF-4). For this purpose, we performed transient transfection assays utilizing the luciferase reporter plasmids containing the truncated forms of the hLXRa promoter in the presence or absence of an expression vector for HNF4 in HEK293 cells that do not produce endogenously HNF4. As shown in Figure 3.4 HNF4 transactivated the LXR promoter fragments -2762, -844, -457, -300, -176 and -111 to various levels (1,5-3,5 fold). Importantly, HNF-4 could not transactivate the -42 LXR promoter fragment suggesting that the putative HNF-4 binding site in the human LXR promoter is located within the -111 to -42 regions. As a control, we used the promoter of the human apoC-III gene shown previously to be strongly responsive to HNF-4 (Nikolaidou-Neokosmidou et al., 2006).

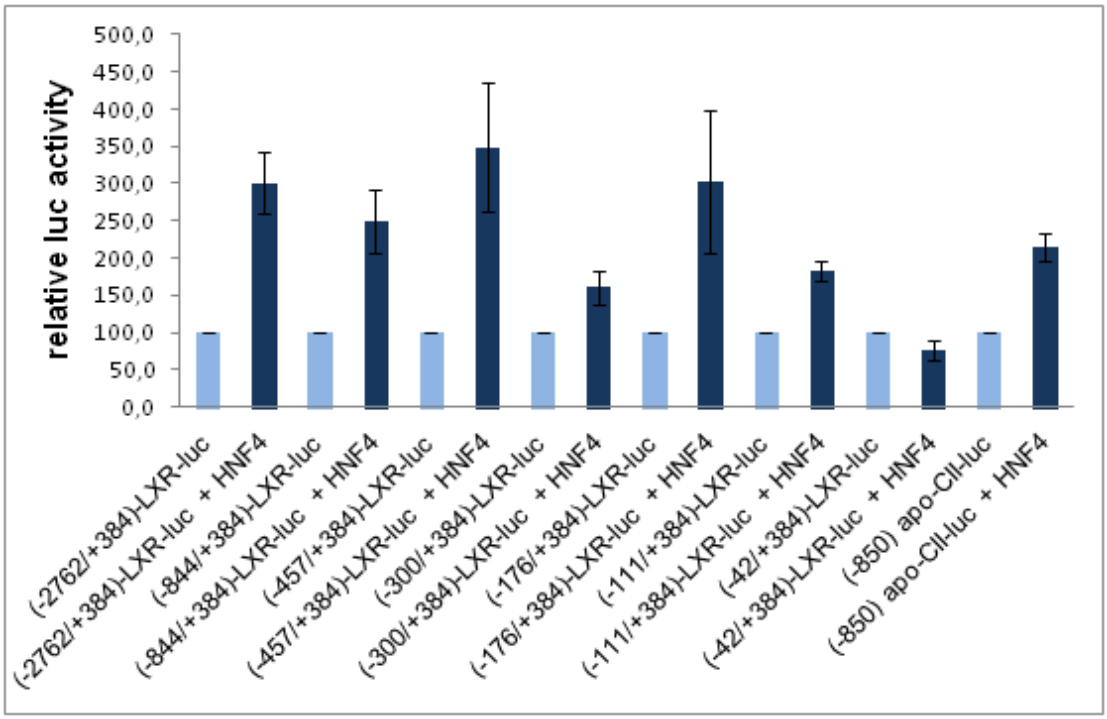


Figure 3.4. HNF4 overexpression upregulates hLXRa promoter activity. HEK cells were transiently co-transfected with the luciferase reported plasmids indicated at the bottom of the graph (0.5 μg) along with a HNF4 expression vector (1.0 μg) and a β-galactosidase expression vector (1.0μg).

Next we performed transient transfection assays in HepG2 cells, utilizing the luciferase reporter plasmids containing the hLXRa promoter fragments in the presence or in the absence of a vector expressing a short hairpin RNA for HNF-4 (shHNF4). In agreement with the data of Fig. 3.4 we observed that in the presence of this shRNA, the activity hLXRa promoter fragments -300, -177 and -111 was downregulated. In contrast, the LXR promoter fragment -45/+384 was not downregulated by shHNF-4 (Figure 3.5). The promoter of apoM was utilized as a positive control (Mosialou et al., 2010).

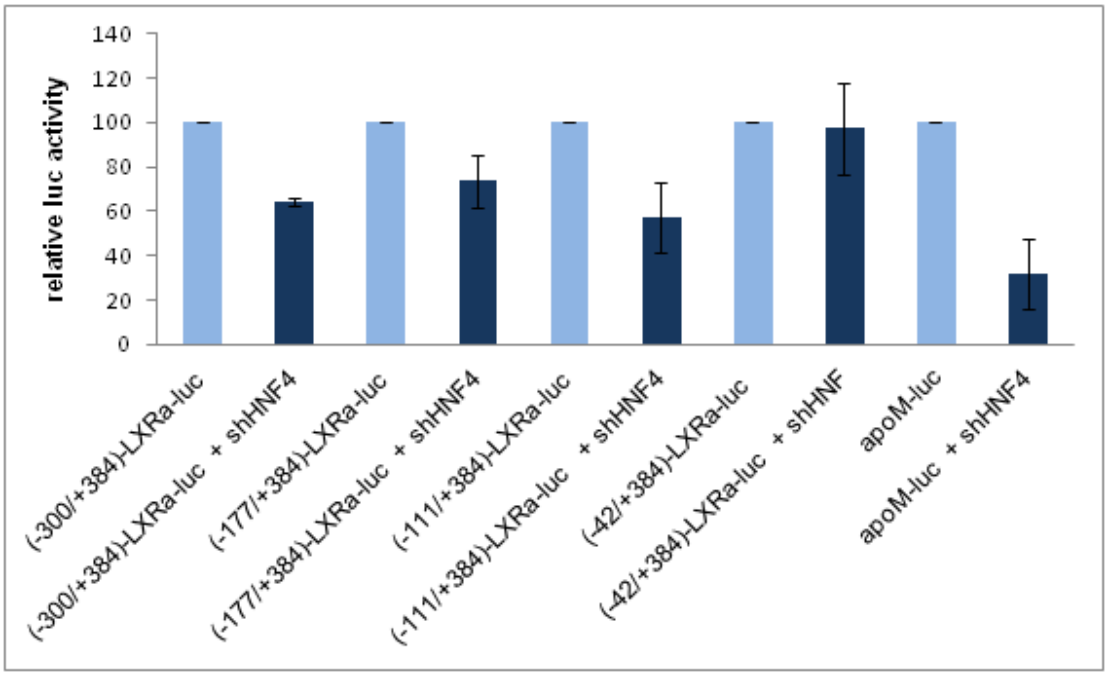


Figure 3.5. Silencing of HNF4 by shRNA caused downregulation of hLXRa promoter activity. HepG2 cells were transiently co-transfected with the luciferase reported plasmids indicated at the bottom of the graph (0.5 μg) along with a shRNA for HNF4 expression vector (1.0 μg) and a β-galactosidase expression vector (1.0μg).

Regulation of LXR by inflammatory signals

Previous results from our laboratory indicated that lipopolysaccharide (LPS), an inflammatory stimuli, downregulates the expression of the hLXRa gene and the activity of the hLXRa promoter in hepatic cells and in macrophages but the mechanism remained unknown (Aristidis Anestis, MSc thesis, University of Crete). In the present study, we sought to investigate in more detail the regulation of the hLXRa promoter by LPS.

As mentioned in the “Introduction”, LPS is a specific activator of the Toll Like Receptor 4 (TLR4) receptor. LBP (LPS binding protein), CD14 and MD-2 are important co-receptors for the LPS dependent TLR4 signaling (Guo and Friedman, 2010). Therefore, to uncover the mechanism of hLXRa gene regulation by LPS, we overexpressed TLR4 protein in HEK293T cells and we monitored the activity of the various truncated LXR promoters. As shown in Figure 3.6, overexpression of TLR4 in HEK293T cells caused a significant downregulation of all LXR promoter fragments tested (-2625, -844, -457, -300, -176, -111 and -42) suggesting that the putative LPS responsive element is located in the proximal region defined by nucleotides -42 and +384.

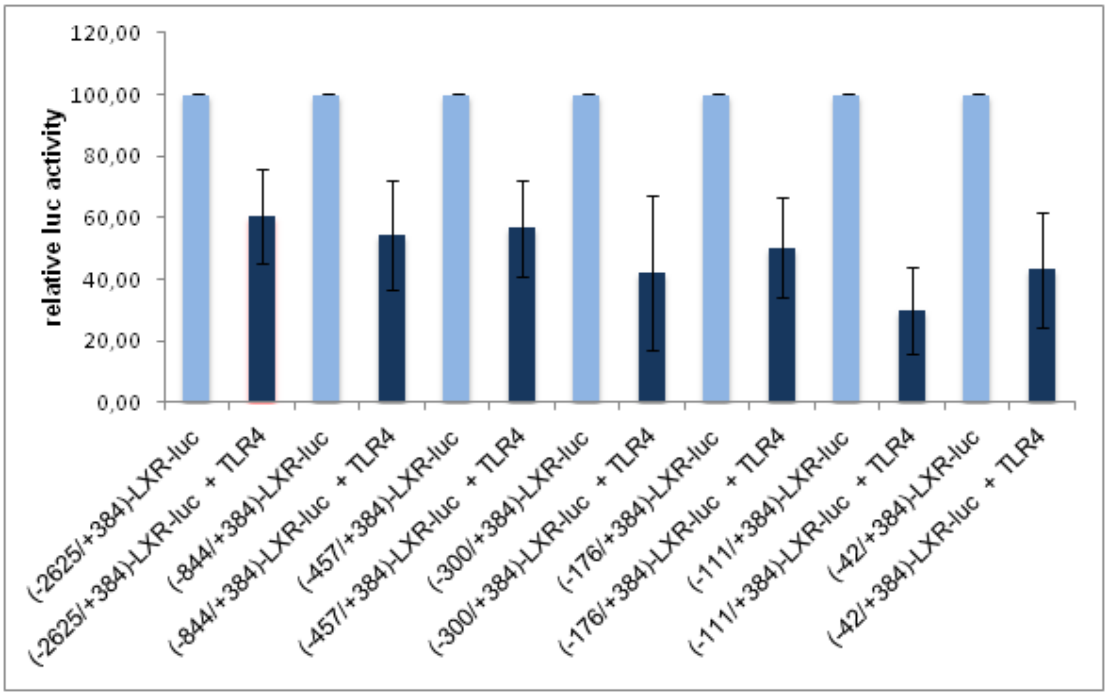


Figure 3.6. *TLR4 overexpression regulates negatively the promoter of hLXRa. HEK293T cells were transiently co-transfected with the luciferase reported plasmids indicated at the bottom of the graph (0.5 μg) along with a TLR4 expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg).*

Inhibition of hLXRa promoter by TLR4 does not involve Interferon Regulatory Factor 3 (IRF3)

Next, we wanted to investigate the implication of Interferon Regulatory Factor 3 (IRF3), shown previously to be activated by TLR4 signaling, in hLXRa regulation. First, we performed control experiments in HepG2 cells using a luciferase reporter plasmid bearing the promoter of INFβ, a known substrate of IRF3, in the presence or in the absence of LPS and expression vectors for IRF3 or a dominant negative form

of IRF3 (DN-IRF3). As shown in Figure 3.7 both LPS and IRF3 upregulated the activity of INFβ promoter 2 and 4.5-fold respectively. Importantly, overexpression of the dominant negative form of IRF3 abolished INFβ promoter induction by LPS indicating that IRF3 functions downstream the TLR4 signaling in HepG2 cells.

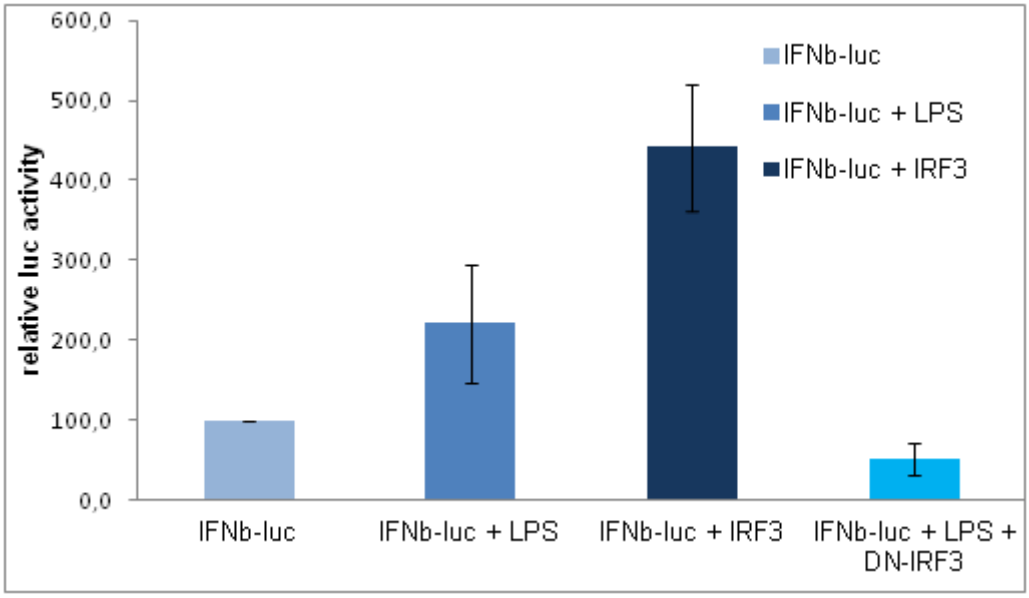


Figure 3.7 LPS induces INF-β promoter activity through IRF-3 in HepG2 cells. HepG2 cells were transiently co-transfected with the luciferase reported plasmid expressing the promoter of INF-β along with an IRF3 expression vector (1.0 μg) or a DN-IRF3 expression vector and a β-galactosidase expression vector (1.0μg) in the presence or the absence of LPS (1μg/ml). LPS treatment was performed for 18 hours.

Next, we wanted to investigate whether IRF3 is activated by TLR4 in a different cell model, the HEK293T fibroblasts which lack the TLR4 coreceptor MD-2. As shown in Figure 3.8, upon TLR4 overexpression in HEK293 cells, IFNβ promoter activity remained unaffected. These results indicateδ that in the absence of the coreceptor MD-2, TLR4 receptor is unable to activate IRF3 and thus the downregulation of hLXRa promoter activity by LPS is not mediated by IRF3 transcription factor.

For further confirmation of the finding, we overexpressed IRF3 in HEK293 cells. As expected, we observed no regulation of the (-457/+384)-hLXRa promoter activity by IRF3 (Figure 3.9A). However, in a control experiment, INFβ promoter activity was upregulated by IRF3 overexpression (Figure 3.9B). Thus, it seems that IRF3 does not regulate the hLXRa promoter.

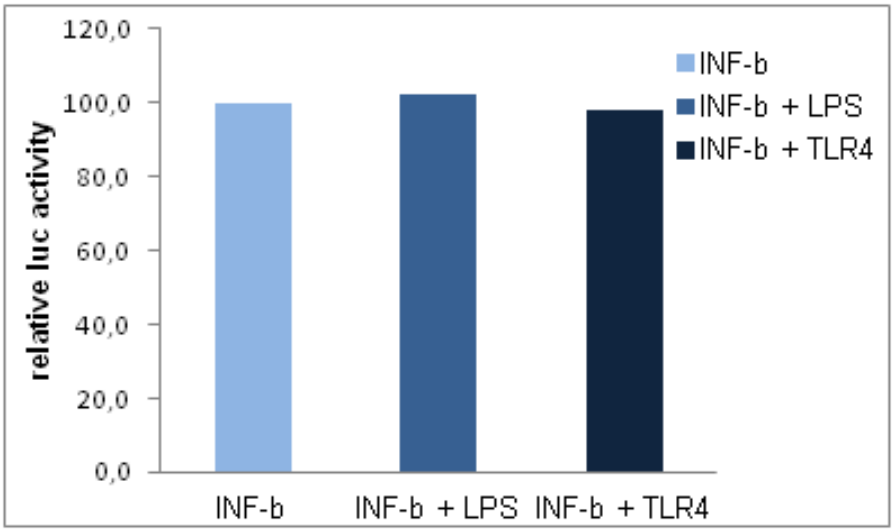
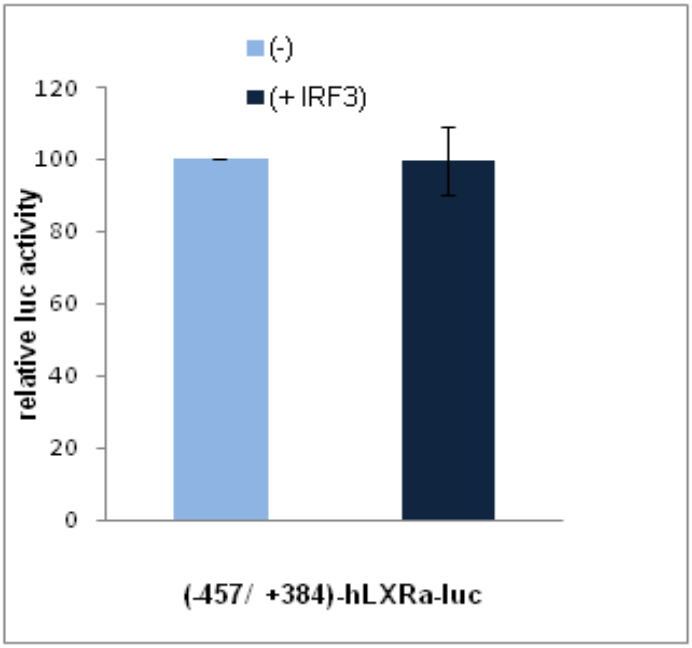


Figure 3.8 LPS treatment or TLR4 overexpression do not regulate INF-β promoter in HEK cells. HEK cells were transiently co-transfected with the luciferase reported plasmid expressing the promoter of INF-β along with a β-galactosidase expression vector (1.0μg) and with or without a TLR4 expression vector in the presence or the absence of LPS (1μg/ml). LPS treatment was performed for 18 hours.

A.



B.

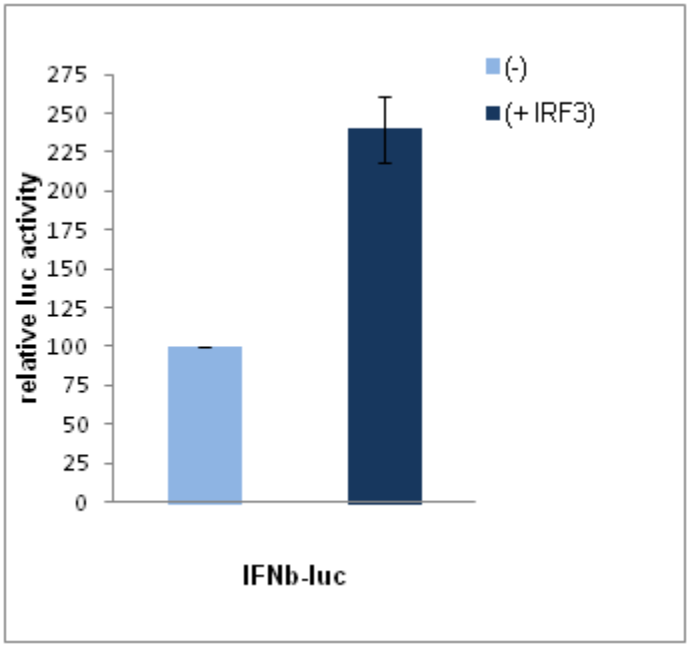


Figure 3.9, IRF3 overexpression does not affect hLXRa promoter activity. **A.** HEK293 cells were transiently co-transfected with the luciferase reported plasmid expressing the (-457/+384) fragment of hLXRa promoter (0.5 µg) along with a β-galactosidase expression vector (1.0µg) and with an IRF3 expression vector (1.0µg). **B**.HEK cells were transiently co-transfected with the luciferase reported plasmid expressing INFb (0.5 µg) along with a β-galactosidase expression vector (1.0µg) and with an IRF3 expression vector (1.0 µg).

TLR4 downregulates hLXRa promoter activity through NF-κB.

Since we confirmed that IRF3 is not implicated in the downregulation of hLXRa promoter by TLR4, we examined the role of another transcription factor that is activated by TLR4 signaling, the NF-κB. For this purpose, we performed overexpression of TLR4 in HEK293 cells in the presence of a luciferase reported plasmid expressing a promoter with 3 binding sites of NF-κB (3*-kB). As anticipated, TLR4 transactivated the 3*-kB promoter 2-fold, indicating that TLR4 overexpression causes the downstream activation of NF-KB in HEK293 cells (Figure 3.10).

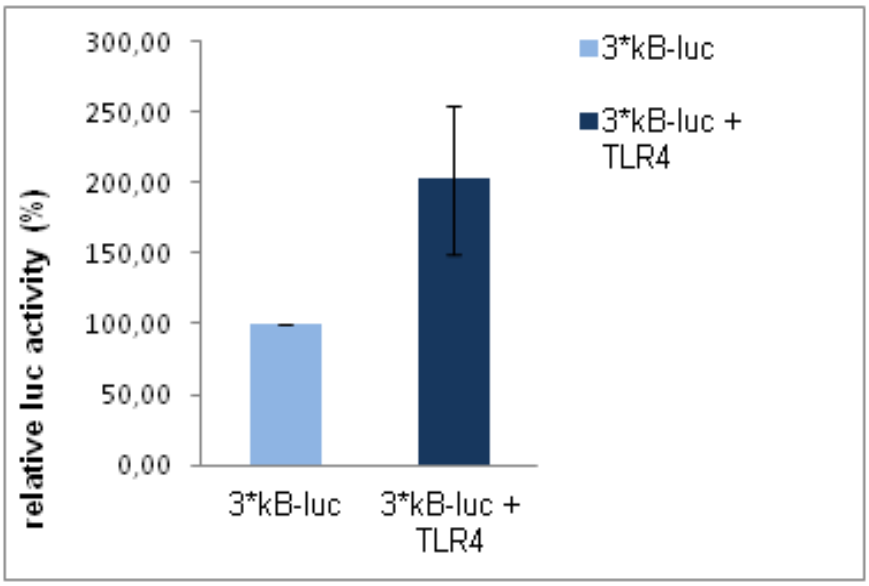


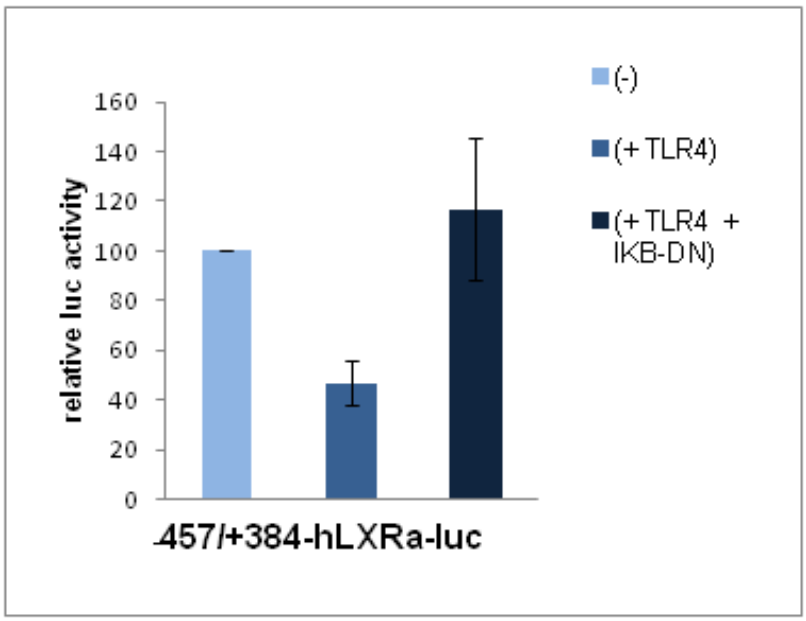
Figure 3.10, TLR4 overexpression induces NF-κB activation in HEK292T cells. HEK293T cells were transiently co-transfected with the luciferase reported plasmid expressing a promoter with 3 sites binding for NF-κB (0.5 μg) along with a β-galactosidase expression vector (1.0μg) and with a TLR4 expression vector.

To confirm that TLR4 inhibits hLXRa promoter activity through NF-κB, we performed overexpression of TLR4 in the presence NF-κB inhibitors. Figure 3.11A shows that in the presence of a dominant negative isoform of the Inhibitor of NF-κB (IkB-non degradable), TLR4 lost its ability to downregulate the hLXRa promoter. As a positive control, we utilized the 3*-kB promoter and we observed that in the presence of IKB-DN, NF-κB is not activated by TLR4(Figure 3.11B). These findings indicate that TLR4 regulates negatively the promoter of hLXRa through NF-κB.

In addition, when we used a dominant negative form of the IkB kinase b (IKKb Δ39), hLXRa promoter activity was again downregulated in the presence of both TLR4 and IKK (Δ39) (Figure 3.12A). However, in the presence of IKK (Δ39), TLR4

did not activate 3*-kB promoter (Figure 3.12B). This finding indicates an alternative pathway of NF-κB activation by TLR4, independent of IKK.

A.



B.

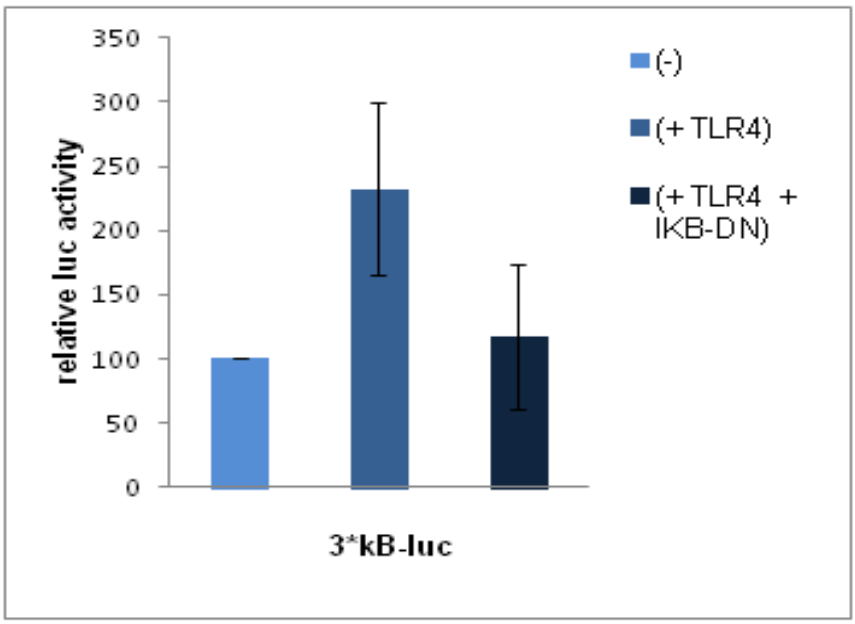
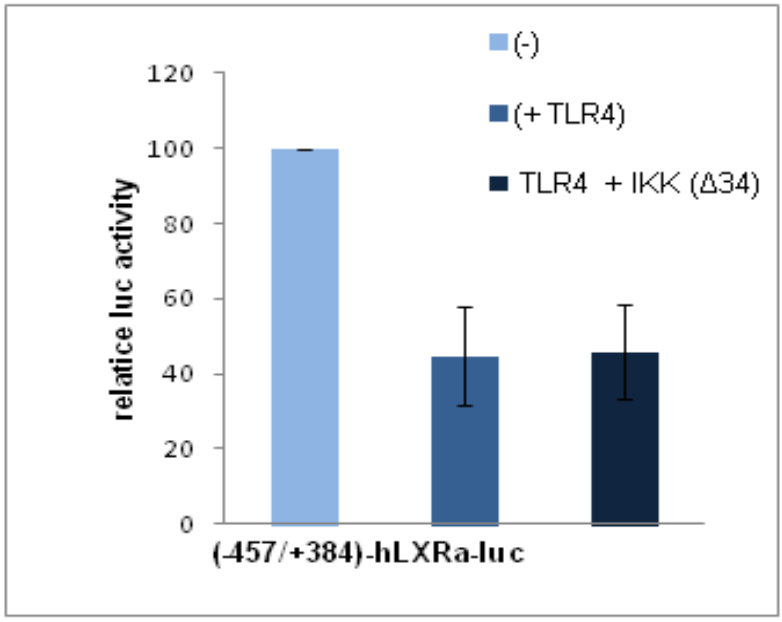


Figure 3.11 TLR4 overexpression downregulates hLXRa activity through NF-κB in HEK293T cells. **A.** HEK293T cells were transiently co-transfected with the luciferase reported plasmid expressing the -457/+384 fragment of hLXRa promoter (0.5 μg) along with a β-galactosidase expression vector (1.0μg), with TLR4 expression vector and with a vector expressing a dominant negative isoform of IKB (IKB-DN) (1.0 μg). B) HEK293T cells were transiently co-transfected with the luciferase reported plasmid expressing a promoter with 3 sites binding for NF-κB (0.5 μg) along with a β-galactosidase expression vector (1.0μg), with a TLR4 expression vector (1.0 μg) and with a vector expressing a dominant negative isoform of IKB (IKB-DN) (1.0 μg).

A.



B.

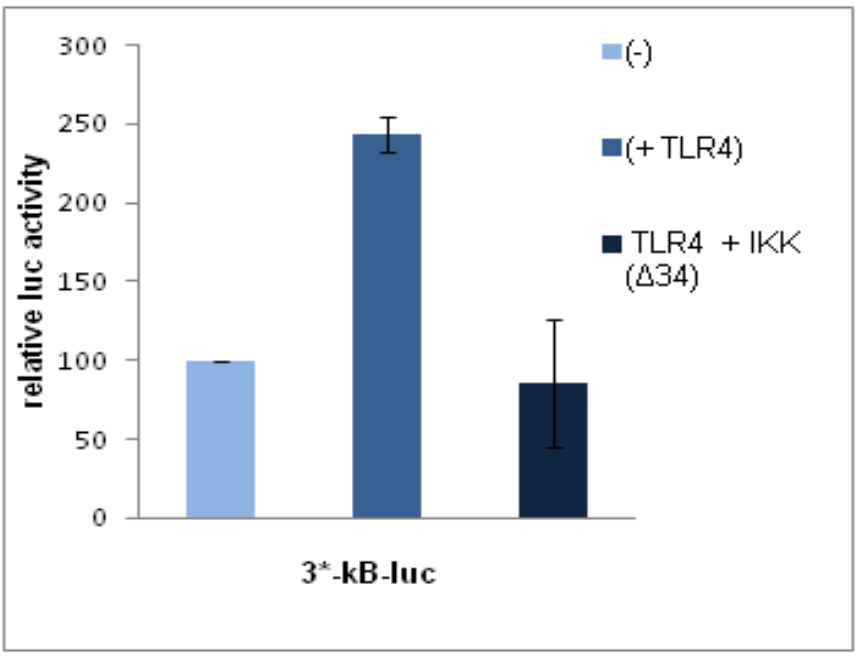


Figure 3.12, TLR4 overexpression downregulates hLXRa activity through an IKK-independent pathway. **A.** HEK cells were transiently co-transfected with the luciferase reported plasmid expressing the -457/+384 fragment of hLXRa promoter (0.5 μg) along with a β-galactosidase expression vector (1.0μg), with a TLR4 expression vector (1.0μg) and with a vector expressing a dominant negative of IKK (IKK-Δ34) (1.0μg) B) HEK cells were transiently co-transfected with the luciferase reported plasmid expressing 3*-kB (0.5 μg) along with a β-galactosidase expression vector (1.0μg),with TLR4 expression vector (1.0 μg) and with a vector expressing a dominant negative of IKK (IKK-Δ34) (1.0μg).

Discussion

LXRα is an oxysterol-activated transcription factor with crucial role not only in cholesterol and fatty acid metabolism, but also in innate and adaptive immunity (Bensinger and Tontonoz, 2008). Specifically, LXRα induces the expression of target genes which participate in the intestinal absorption and biliary excretion of cholesterol, in the reverse cholesterol transport and in the liver lipogenesis (Kidani and Bensinger, 2012). Also, by interacting with co-repressors, LXRα inhibits the function of pro-inflammatory transcription factors, especially by inhibiting the pro-inflammatory function of NF-κB (Ogawa et al., 2005).

However, despite the progress in research of LXRα function, very little is known about the regulation of its own gene. Some studies show that in humans the LXRa gene is auto-regulated and others have shown that peroxisome proliferator activated receptor gamma (PPARγ) ligands induce LXRα gene expression (Chawla et al., 2001a; Laffitte et al., 2001). So in the second part of this study, we wanted to identify putative regulatory elements in the hLXRa promoter. For this reason we utilized a luciferase reporter plasmid expressing the -2672/+384 hLXRa promoter and also plasmids containing consecutive 5´ deletions of this promoter.

First, we performed a bioinformatic analysis in the -2672/+384 region of hLXRa gene. We observed that this region is transcriptionally active (Fiure 3.3A) and many transcription factors bind to it (Figure 3.3B). Interestingly, an HNF4 binding site was identified in the proximal region which coincides with a conserved binding site for EAR3/COUP1 transcription factor. It has been reported that EAR/COUP1 and HNF4 bind on the same hormone response elements and exert opposite effects (Figure 3.3B,3.3C) (Ktistaki et al., 1994; Ladias et al., 1992). Thus, this observation strongly suggests the existence of a hormone response element in this site, but this needs to be validated by protein-DNA interaction experiments which are under way.

We wanted to examine whether HNF4 regulates hLXRα promoter activity. We observed that HNF4α overxpression upregulates hLXRα promoter activity in HEK293 cells (Figure 3.4), which do not produce HNF4 endogenously. On the contrary, expression of a shRNA for HNF4 in HepG2 cells led to downregulation of hLXRα promoter activity (Figure 3.5). Interestingly, this regulatory effect was abolished in the -45/+384 fragment of the hLXRa promoter indicating that an HNF4 binding site is located in the region -111 to -45 of the hLXRα promoter.

Further experiments should be performed in order to functionally characterize this proximal hormone response element in the hLXRα promoter. As we observed that HNF4 exerts an opposite effect on hLXRa gene expression than TLR4 mediated signaling, a possible cross talk between these two pathways should be examined. It

seems that the LXRa levels in the cells are dependent on the balance between these two signaling pathways.

Moreover, we wanted to investigate how the hLXRα promoter is regulated under inflammatory conditions. Castrillo et al showed that LPS, through IRF3 activation, inhibits the ability of LXRα to induce the expression of its target genes, including ABCA1 (Castrillo and Tontonoz, 2004). To examine whether TLR4 signalling regulates also the hLXRα gene expression, we used a series of luciferase reporter plasmids containing consecutive 5´ deletions of the hLXRa promoter. We performed co-transfection of HEK293 cells with these plasmids along with plasmids expressing mediators of TLR4 signalling (TLR4, IRF3, NF-κB, IKKb and IkB). The HEK293 cell line was utilized because it does not express endogenously the TLR4 receptor and the MD-2 and CD-14 co-receptors. We observed that TLR4 overexpression in HEK293 cells inhibit the hLXRa promoter activity (Figure 3.6). Ryan et al also showed that TLR4 activation by another factor, the surface layer protein produced by *C. difficile*, could be mediated again in the absence of CD-14 (Ryan et al., 2011).

It is known that TLR4 triggers a signal cascade resulting in the activation of NF-κB and IRF3 transcription factors (Figure 1.5) (Guo and Friedman, 2010). . So, we wanted to identify the transcription factor that is implicated in the inhibition of hLXRa promoter activity by TLR4. We observed that IRF3 could not be activated following TLR4 overexpression in HEK293 cells, indicating that it cannot be the responsible transcription factor for the downregulation of hLXRa promoter activity (Figure 3.8). Also, IRF3 overexpression in HEK293 cells did not affect hLXRa promoter activity (Figure 3.9). On the other hand, NF-κB was activated following TLR4 overexpression in HEK293 cells (Figure 3.10). Additionally, in the presence of a negative regulator of NF-κB (a non degradable form of IkB), TLR4 abolished its function to inhibit hLXRa promoter activity (Figure 3.11). Interestingly, we observed that in the presence of a dominant negative form of IKKb (an activator of NF-κB), TLR4 did not abolish its function to inhibit hLXRa activity (Figure 3.12).

IKKb plays crucial role in the activation of NF-κB. Despite that IKKb phosphorylates IkB causing its degradation, it also phosphorylates the p65 subunit NF-κB on the serine 536 residue (Ser536) increasing the transcriptional activity of p65 (Yang et al., 2003). Aye et al. also observed a disability of the TPCA-1 inhibitor of IKKb to inhibit the phosphorylation of NF-κB after oxysterols treatment. They showed that oxysterols activate NF-κB causing proinflammatory cytokine production by binding to TLR4 in trophoblasts. In the presence of the TPCA-1, p65 phosphorylation by oxysterols was not attenuated, while this effect was abolished

after LPS treatment. In addition, after TPCA-1 treatment the production of pro-inflammatory cytokines was decreased. They proposed that oxysterols phosphorylate other NF-κB residues, in addition to Ser536, to produce their inflammatory effects (Aye et al., 2012). In the case of hLXRα, it seems that the same mechanism accounts for the downregulation of hLXRα promoter activity by TLR4.

TLR4 and its accessory proteins CD14 and MD-2 are recruited into lipid microdomains (lipid rafts) along with MyD88. Hence modulation of membrane cholesterol levels can diminish the inflammatory response by reducing TLR4 trafficking into lipid microdomains (Triantafilou et al., 2002; Wong et al., 2009). Aye et al showed that LXRα activation or membrane cholesterol depletion inhibits oxysterol-stimulated proinflammatory cytokine production (Aye et al., 2012). Thus, we can hypothesize that TLR4 inhibits LXRα expression in order to prevent cholesterol efflux from the cell thus protecting the lipid raft integrity and consequently the activity of TLR4 signaling cascade.

Besides LPS, endogenous or dietary factors could act as TLR4 agonists (Erridge, 2011) including free fatty acids, oxysterols and modified LDL (Milanski et al., 2009; Mogilenko et al., 2012; Shi et al., 2006). Taking into consideration that LXRα has been shown to activate basal lipolysis in adipocytes (Stenson et al., 2011), a putative inhibition of hLXRα expression by TLR4 may serve as a negative feedback control for free fatty acids release, a characteristic of adipose tissue in obesity.

The mechanism by which TLR4 signaling inhibits LXRα gene expression requires further investigation. TLR4 signaling may directly regulate LXRα gene expression through a TLR4 element in the LXRα promoter, by inhibiting the binding of other transcription factors, or by promoting histone modifications in this promoter. Since inflammation is a trigger for many metabolic disorders including atherogenesis, obesity and diabetes, it is crucial to understand the physiological role of this inhibitory effect.

Conclusions

Part I

- We identified low levels of apolipoprotein M in the plasma of patients with Rheumatoid arthritis and Systemic Lupus Erythematosus from the Rheumatology Clinic of the University Hospital of Crete.
- Both HDL cholesterol and apolipoprotein A-I levels were in the physiological range in these patients.
- Anti-TNFα treatment partially restored apoipoprotein M levels
- Our finding verify the inhibitory role of inflammation in the expression of distinct atheroprotective proteins of HDL

Part II

- Bioinformatic analysis indicates that the region -2.672/+384 of the human LXRα promoter is transcriptionally active and contains putative hormone response elements including an HNF4/-COUP TF1 binding site.
- HNF4α overxpression upregulated hLXRα promoter activity in HEK293 cells while, expression of a shRNA for HNF4 in HepG2 cells led to downregulation of hLXRα promoter activity. This regulatory effect is mediated through a site in the -111/-42 region of the promoter.
- Overexpression of Toll Like receptor 4 (TLR4), which is the natural receptor of lipopolysaccharide (LPS) in HEK293 cells inhibited the hLXRa promoter activity indicating the negative role of inflammation in LXRα gene expression.
- Overexpression of TLR4 in HEK293 cells was followed by a potent activation of NF-κB in an IKKβ and IRF3 independent manner.
- Our findings provide new insights into the mechanisms of LXR gene regulation by the pro-inflammatory NF-κB pathway as well as by hormone nuclear receptors and their agonists including oxysterols (autoregulation).

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