Dissertation

Transcriptional Regulation of Apolipoprotein(a)
The Role of Bile Acids

submitted by

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Preface

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, [date & signature]

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Abstract

High plasma concentrations of lipoprotein(a), [Lp(a)] which is encoded by the apo(a) gene has been implicated as a genetic risk factor for coronary artery diseases, restenosis, and stroke. Unfortunately, increased Lp(a) levels are minimally influenced by dietary changes or drug treatment. Further, the development of Lp(a)-specific medications has been hampered by limited knowledge of Lp(a) metabolism.

In this study, we found that patients suffering from biliary obstructions exhibit very low plasma Lp(a) concentrations that rise significantly after surgical intervention. Consistent with the latter phenomenon, common bile duct ligation in mice lowered plasma concentrations and hepatic expression of apo(a) drastically. To test whether the bile acid receptor FXR was responsible for low plasma Lp(a) levels in cholestatic patients and mice, we treated transgenic human apo(a) mice (tg-apo(a)) and tg-apo(a)/FXR$^{-/-}$ mice with cholic acid (CA). FXR activation resulted in a significant reduction of plasma concentrations and hepatic expression of human apo(a) in tg-apo(a) mice but not in tg-apo(a)/FXR$^{-/-}$ mice. Incubation of primary hepatocytes from tg-apo(a) mice with bile acids resulted in a dose dependent down regulation of apo(a) expression. Promoter transfection experiments, mutation analysis, gel shift assay and Chromatin immunoprecipitation (ChIP) assay demonstrated that the DR-1 element between nucleotides -826 and -814bp of the apo(a) promoter functioned as a negative FXR-response element. This motif is also occupied by hepatocyte nuclear factor 4α (HNF4α), which promotes apo(a) transcription, and FXR was shown to compete with HNF4α for binding to this motif.

In summary, our findings provide mechanistic insights in to the transcriptional regulation of human apo(a) by bile acids and its receptor FXR. These findings may have important implications in designing therapeutics for patients with elevated plasma Lp(a) levels and therefore at higher atherothrombotic risk.

Zusammenfassung

Erhöhte Plasma Konzentrationen an Lipoprotein(a) [Lp(a)] welches vom apo(a)-Gen kodiert wird, erhöht das individuelle Risiko an Koronaren Herzerkrankungen, Restenosen und Gehirnschlag zu erkranken. Erhöhtes Plasma-Lp(a) kann kaum durch Diät oder Arzneimittel beeinflusst werden und die Entwicklung Lp(a)-spezifischer Medikamente scheiterte am beschränkten Wissen über den Lp(a) Metabolismus.

In der vorliegenden Arbeit fanden wir, dass Patienten mit Gallengangsverschluss sehr geringe Plasma Lp(a) Werte aufwiesen and dass diese Werte nach chirurgischer Intervention des Gallengangsverschlusses signifikant anstiegen. Im Einklang mit diesen Befunden stand auch, dass in transgenen Apo(a) Mäusen mit Gallengangsligatur die Expression von Apo(a) in der Leber drastisch abnahm. Wir vermuteten, dass der Gallensäurerezeptor FXR dafür verantwortlich sein könnte. Um dies zu testen wurden transgene Human-Apo(a) (Tg-apo(a)) Mäuse sowie doppeltransgene Tg-apo(a)/FXR$^{-/-}$ Mäuse mit Cholsäure (CA) behandelt. Dies führte zu einer FXR Aktivierung und dadurch zu einer signifikanten Reduktion der Plasma Apo(a) Konzentration sowie der hepatischen Expression von Apo(a). Doppeltransgene Tg-apo(a)/FXR$^{-/-}$ Mäuse zeigten diese Verminderung an Apo(a) jedoch nicht. Kultivierten wir primäre Hepatozyten von Tg-apo(a) Mäusen mit Gallensäuren so führte dies zu einer dosisabhängigen down-Regulierung der Apo(a) Expression. Promoter-Reporter Experimente , Mutationsanalysen , Gel-Shift- und Chromatin Immunopräzipitation (ChIP) Assays schließlich bewiesen eindeutig, dass das DR-1 Element in der Promoterregion -828 bis -814 von apo(a) als negatives FXR-response Element fungiert. Dieses DNA-Motiv bindet den Hepatozyten-Nukleärten Faktor 4α (HNF4α), welcher die apo(a) Transkription antreibt, jedoch durch FXR kompetitiv gehemmt wird.

Zusammenfassend kann gesagt werden, dass unsere Ergebnisse mechanistischen Einblick in die transkriptionelle Regulation von humanem Apo(a) durch Gallensäuren und ihren Rezeptor FXR liefert. Wir glauben, dass unsere Ergebnisse wichtige Grundlagen für die Entwicklung von Arzneimittel

liefern, welche die Therapie von Patienten mit erhöhtem Lp(a) und damit erhöhtem athero-thrombotischen Risko ermöglichen.

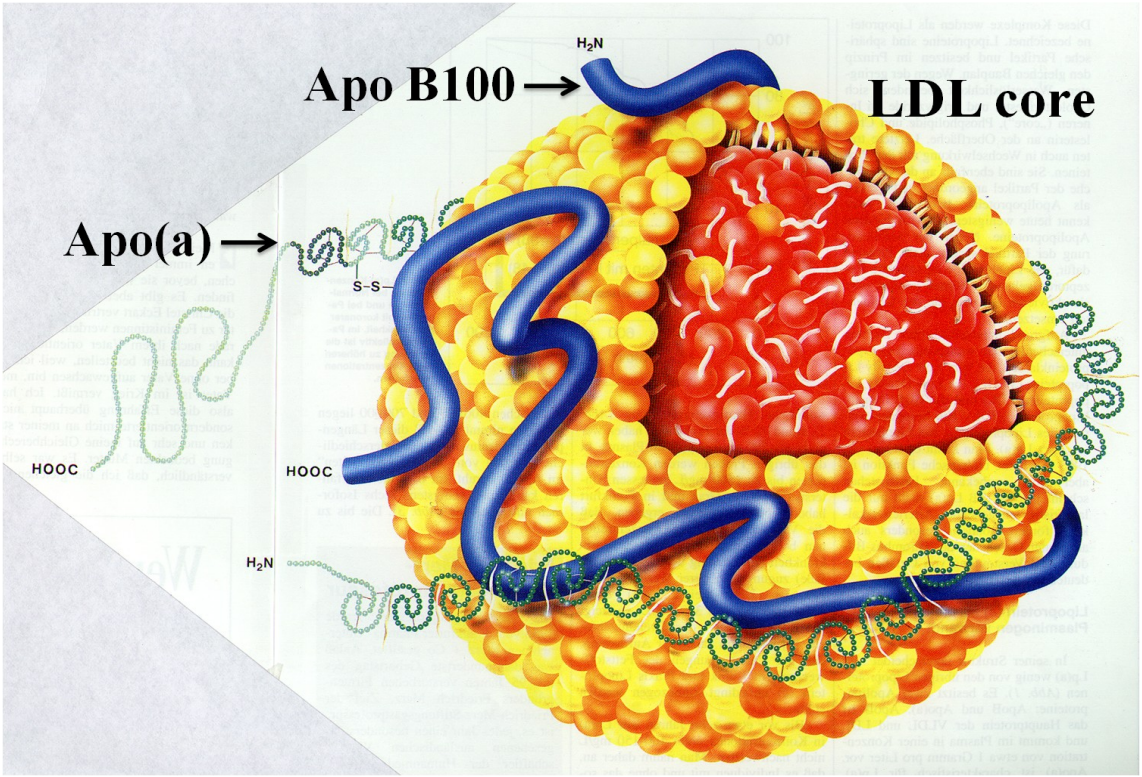
1 Introduction

Lipoprotein(a) [Lp(a)] encoded by the gene apo(a) belongs to the class of most atherogenic cholesteryl ester-rich plasma lipoproteins (Hobbs *et al.*, 1999). It consists of an LDL core covalently bound to a glycoprotein, apolipoprotein(a) [apo(a)]. This plasma lipoprotein is found in humans and old world monkeys, but is absent in conventional laboratory animals. Despite of intensive research, the physiological role of Lp(a) still remains mysterious. From numerous studies with large sample size, it became clear that the risk for developing coronary events correlates significantly with plasma Lp(a) levels (Marcovina *et al.*, 2003). Thus high plasma Lp(a) has been implicated as a quantitative genetic risk factor for coronary artery diseases, restenosis and stroke.

1.1 Lipoprotein(a): Structural and molecular properties

Lp(a) was first described as a genetic variant of β-lipoprotein in 1963 (Berg, 1963). Studying Lp(a) is a very challenging task because of its complex structure. It consists of the unique glycoprotein apolipoprotein(a) [apo(a)], which is synthesized predominantly in the liver, covalently linked to apoB-100 of low-density lipoprotein (LDL) via a disulfide bridge (Gaubatz *et al.*, 1983). A disulfide bridge links Cys-4326 of apoB-100 with the only one free Cys-4057 in apo(a) which is located in kringle IV Type-9. Chemically, Lp(a) consist of approximately 30% protein, 5% carbohydrates, 44% cholesterol + cholesteryl esters, 18% phospholipids and 3% triglycerides.

Plasma Lp(a) concentrations are under strict genetic control and range from <1 to >200 mg/dl with medians of 8-9 mg/dl reviewed in (Hobbs *et al.*, 1999; Kostner *et al.*, 2002). In healthy individuals plasma levels of Lp(a) are determined almost exclusively by the apo(a) gene (Boerwinkle *et al.*, 1992; Kraft *et al.*, 1992). Turnover studies in humans demonstrated that plasma Lp(a) levels strongly correlate with its synthetic rate, i.e. the rate of apo(a) *de novo* biosynthesis, whereas Lp(a) catabolism which is mainly by hepatic and renal pathways might have only little effect (Krempler *et al.*, 1980; Rader *et al.*, 1994).



(Adopted from Kostner, G.M et al. Forschung Frankfurt 1991)

Figure 1: General structure of Lp(a)

The apo(a) gene resides on human chromosome 6q26-q27. Apolipoprotein(a), a high molecular weight glycoprotein component of Lp(a) has a rather unique structure; apo(a) is a highly polymorphic, consisting of ten distinct plasminogen kringle IV (K-IV) domains from K-IV type 1 to K-IV type 10, one copy of a kringle V domain (K-V) and a protease domain which is catalytically inactive. Nine of the K-IV domains, K-IV type 1 and K-IV type 3-10 are present in single copies in the apo(a) gene where as the number of the K-IV type 2 repeats varies from 3-43 (Albers *et al.*, 1990; McLean *et al.*, 1987) and is responsible for the great size polymorphisms which is genetically determined (Lackner *et al.*, 1993). The number of K-IV type 2 repeats are inversely correlated with plasma Lp(a) levels. The linker region between the K-IV domains is highly glycosylated.

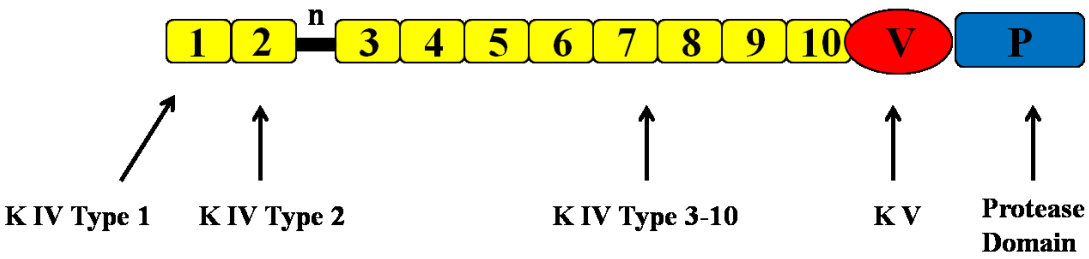


Figure 2: Schematic representation of apo(a)

Apo(a) has a striking homology with the fibrinolytic pro-enzyme plasminogen, and the size variation of apo (a) is due to the number of kringle 4 type 2 copies present in the molecule (Gaw *et al.*, 1994). Though the majority of apo(a) is complexed with LDL, a small fraction of apo(a) is present in plasma in free form which plays an important role in Lp(a) metabolism (Gries *et al.*, 1987). 90 - 95 % of plasma Lp(a) levels are inherited and the rest is caused by environmental factors.

1.2 Lp(a) in atherothrombosis

High plasma Lp(a) has been implicated as a genetic risk factor for coronary artery diseases, restenosis and stroke. Although it has been known for many years that elevated plasma Lp(a) concentrations are associated with thrombo-atherogenic diseases (Kostner *et al.*, 1981; Rhoads *et al.*, 1986; Srinivasan *et al.*, 1991), recent evidence from large cohorts has finally confirmed a causal relationship (Clarke *et al.*, 2009; Erqou *et al.*, 2010; Kamstrup *et al.*, 2009; Lanktree *et al.*, 2010; Tregouet *et al.*, 2009). Therefore in a recent consensus report, the European Atherosclerosis Society recommended screening for Lp(a) in patients at moderate to high risk of cardiovascular disease where the desirable cut-off for Lp(a) was set at <50 mg/dl (Nordestgaard *et al.*, 2010).

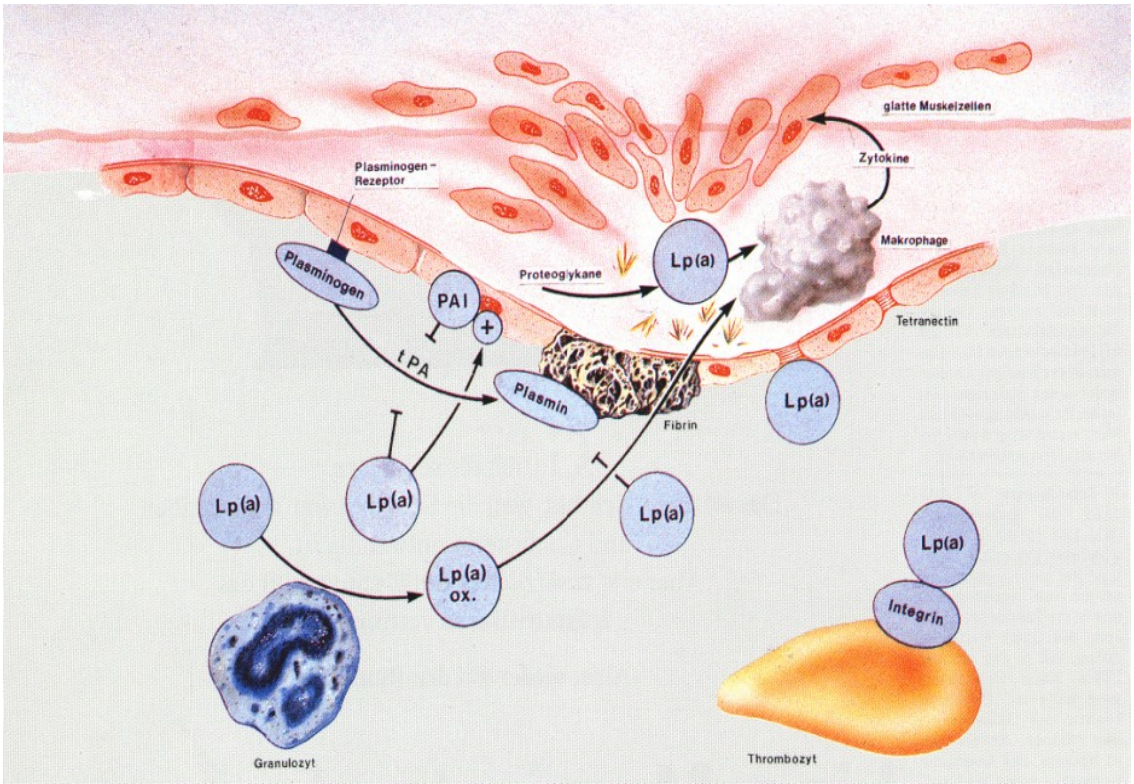
	Elevated LDL cholesterol	Elevated Lp(a)
Human epidemiology	Direct association in numerous studies	Direct association in numerous studies
Human genetic studies	Direct association in numerous studies, e.g. familial hypercholesterolaemia	Direct association in numerous studies, e.g. for kringle IV type 2 polymorphism
Mechanistic studies	Mechanism clearly demonstrated: LDL accumulate in intima and cause atherosclerosis	Mechanism similar to that for LDL cholesterol and/or prothrombotic/anti-fibrinolytic effects
Animal models	Proatherogenic effect in numerous studies	Proatherogenic effect in numerous studies
Human intervention trials	Statin trials gave final proof of causality	Niacin trials are favourable
Interpretation in 2010	Causality	Probably causal

(Adopted from Nordestgaard B G et al. Eur Heart J, 2010)

Table 1: Comparison of elevated LDL cholesterol and elevated Lp(a) levels as cardiovascular risk factors

The thrombo-atherogenic properties of Lp(a) have also been very well documented in transgenic mice (Callow *et al.*, 1995; Lawn *et al.*, 1992). Several

hemostatic pathways have been attributed to the patho-mechanisms of Lp(a) (Kostner *et al.*, 2005; Rouy *et al.*, 1991). Upon entering into the arterial intima from circulation, Lp(a) appears to be retained more avidly than LDL by binding to the extracellular matrix not only through apo(a), but also via its apoB-100 moiety, thereby contributing cholesterol to the progressing atherosclerotic plaque (Nielsen, 1999). In *in vitro* conditions, Lp(a) binds to several extracellular matrix proteins like fibrin and defensins that are released by neutrophils during severe infection and inflammation (Lundstam *et al.*, 1999). Lipoprotein(a) has also been shown to be retained more at sites of mechanical injury, thereby increasing fibrin deposits at those sites (Nielsen, 1999). Lp(a) also can interact with the β2-integrin Mac-1 by its apo(a) moiety, and promotes the adhesion and transendothelial migration of monocytes (Sotiriou *et al.*, 2006). Lp(a) was shown to bind to pro inflammatory-oxidized phospholipids through K-V by a covalent bonding, there by acting as an effective carrier lipoprotein for phospholipids (Edelstein *et al.*, 2003; Tsimikas *et al.*, 2005).

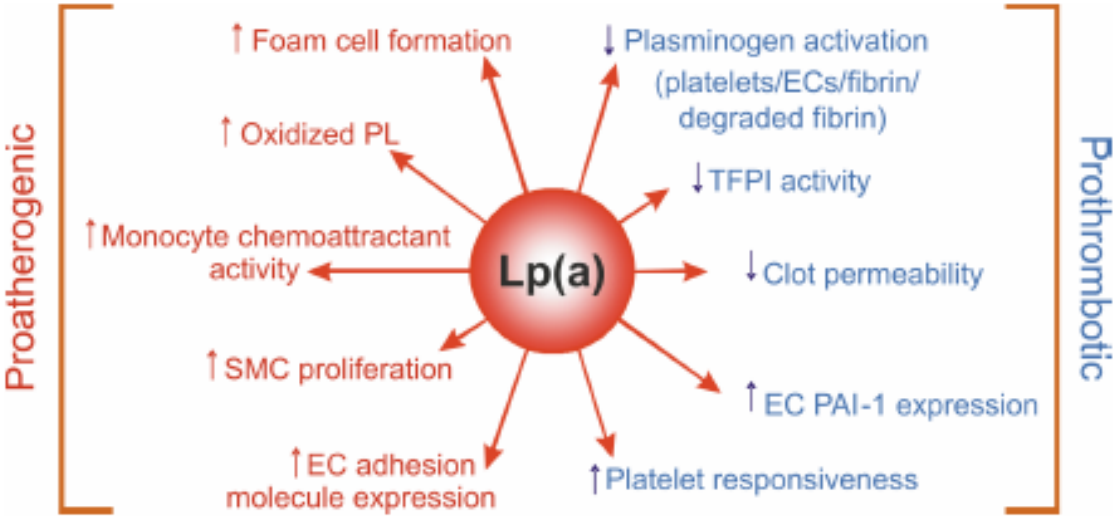


(Adopted from Kostner, G.M et al. Forschung Frankfurt 1991)

Figure 3: The role of Lp(a) in atherothrombosis

Due to its remarkable homology with the fibrinolytic pro-enzyme plasminogen, Lp(a) / apo(a) attenuate fibrinolysis and promote coagulation by interfering with the tissue-type plasminogen activator mediated activation of plasminogen on fibrin surfaces (Marcovina *et al.*, 2003; Nordestgaard *et al.*, 2010; Nowak-Gottl *et al.*, 2001; von Depka *et al.*, 2000).

Because of its similarity to LDL and plasminogen, Lp(a) plays an enticing role in the processes of atherosclerosis and thrombosis. Beyond any doubt, Lp(a) is considered as a well recognized risk factor for atherothrombotic events.



(Adopted from Taewoo Cho, 2009)

Figure 4: Proatherogenic and prothrombogenic effects *of* Lp(a)

Because of the structural entities similar to LDL and plasminogen, Lp(a) is known to possess proatherogenic as well as prothrombogenic properties. PL, phospholipids; EC, endothelial cells; SM, smooth muscle cells; PAI-1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor.

Easy oxidisability of Lp(a) and formation of highly atherogenic complexes with LDL in the vessel wall
Increase of oxidation rate, uptake and retention of LDL
Enhancement of lipid uptake by macrophages
Competitive inhibition of plasminogen during the binding to cellular receptors and protein binding sites
Decreased thrombin formation and inhibition of fibrinolysis
Inhibition of t-PA and increased formation of PAT-1

Inactivation of tissue factor pathway inhibitors
Increase of thrombin synthesis
Facilitation of thrombus formation at sites of tissue lesions
Increase of proliferation and migration of smooth vascular muscle cells
Inhibition of transforming growth factor β
Increased expression of intercellular adhesion molecules
Inhibition of the formation of collateral vessels

(Adopted from Siekmeier R et al. 2008)

Table 2: Summary of mechanisms related to Lp(a) that lead to atherothrombosis.

1.3 Factors affecting Lp(a) levels:

There have been numerous factors described that influence plasma Lp(a) levels including genetic factors, diseased conditions and drugs. The most important ones are summarized in tabular form.

Determinant	Effect on Lp(a) concentration
Genetic - Polymorphisms in apo(a) gene	Variable
Polymorphisms in apoE gene	Variable
Polymorphisms in LDL receptor gene	Variable
Metabolic- Acute phase response	Increase
Chronic renal failure	Increase
Diabetes mellitus (type 1 and type 2)	Increase
Cancer	Increase
Gout	Increase
Menopause	Increase
Hypothyroidism	Increase
Antiphospholipid Antibody Syndrome	Increase
Liver failure	Decrease

Red wine consumption	Decrease
Hyperthyroidism	Decrease
Pharmacological Niacin	Decrease
N-Acetyl cysteine	Decrease
Gemfribrozil	Decrease
Estrogens	Decrease
Progesterone	Decrease
Testosterone and anabolic Steroids	Decrease
Cyclosporin	Increase

Table 3: Summary of factors influencing plasma Lp(a)

1.4 Therapy

Due to its high atherogenicity, numerous attempts were made to treat individuals with increased Lp(a) levels by either medication or diet (Kostner *et al.*, 2005), without much success. Even if nicotinic acid and its derivatives may lower Lp(a) levels by up to 30%, they are not widely used due to adverse side-effects. Therefore to date, there is no safe drug available for the treatment of individuals with elevated plasma Lp(a) levels, and the development of new drugs is hampered by a lack of detailed knowledge of both Lp(a) biosynthesis and catabolism. As plasma Lp(a) levels strongly correlate with the rate of biosynthesis of apo(a), thus any attempt to control plasma Lp(a) levels must focus on an interference with apo(a) biosynthesis. This has been supported by *in vivo* studies using anti-sense strategies in which plasma levels of an N-terminal apo(a) fragment expressed in mice under the control of the CMV promoter were reduced to almost zero (Frank *et al.*, 2001). Unfortunately this type of treatment is not yet approved for human use, and small molecule medications are not yet available.

It is therefore of importance to uncover the steps involved in Lp(a) biosynthesis in detail and in particular the mechanism controlling apo(a) expression at the

molecular level in order to develop strategies to interfere with elevated plasma Lp(a) in hyper-Lp(a) states.

1.5 Nuclear receptors

Nuclear receptors (NRs) are ligand activated transcription factors that regulate many aspects of mammalian physiology including development, reproduction and metabolism (Chawla *et al.*, 2001) (Mangelsdorf *et al.*, 1995). Because of their importance in different metabolic pathways, they have become attractive targets for drug development.

The members of the nuclear receptor superfamily share a basic structure. The nuclear receptors are characterized by a DNA-binding domain (DBD) located towards the N-terminal region which targets receptor to specific binding sequences called response elements on the target gene. DBD consists of two highly conserved zinc-fingers which distinguish nuclear receptors from other DNA binding proteins (Berg, 1989). The C-terminal region of the receptor consists of the ligand-binding domain (LBD) which is crucial for the ligand recognition and ensures specificity and selectivity to physiological response. Upon ligand binding, nuclear receptor switches to a transcriptionally active state. The DBD and LBD are joined by a poorly conserved hinge region.

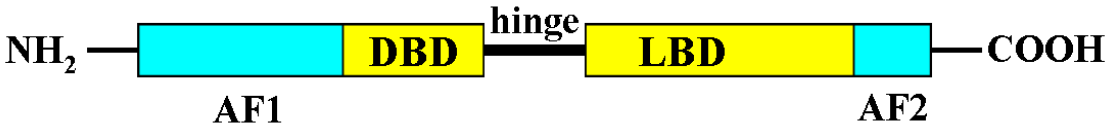


Figure 5: Structural organization of nuclear receptors.

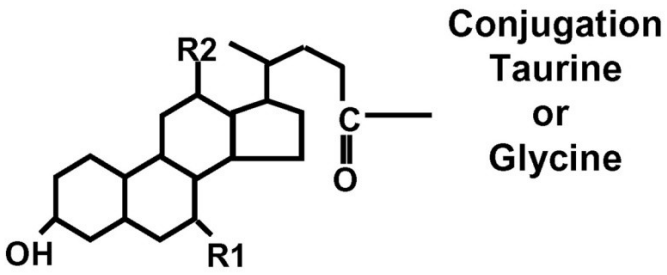
A typical nuclear receptor contains an N-terminal region harboring a transcription activation function (AF-1) followed by the DNA binding domain (DBD). The DBD is connected to the ligand binding domain (LBD).LBD allows hormone recognition and receptor dimerization and harbor activation function (AF-2).

Upon activation by ligand binding, nuclear receptor binds to specific sequences called response elements (REs) in a genes promoter, recruiting coactivator or corepressor proteins resulting in activation or repression of gene expression

(Karpen, 2002). These co-regulators act by interfering with the chromatin structure, acetylation or deacetylation of histones and RNA polymerase II activity (Lee *et al.*, 2001) (Hu *et al.*, 2000). Activated nuclear receptors bind to specific response elements as monomers, homo- or heterodimers (Glass, 1994).

1.6 FXR

The farnesoid X receptor (FXR; NR1H4) is a bile acid activated receptor and belongs to the nuclear receptor superfamily of ligand-activated transcription factors (Forman *et al.*, 1995; Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang *et al.*, 1999). FXR was first isolated in 1995 from rat liver cDNA library (Forman *et al.*, 1995). FXR plays important roles in bile acid, cholesterol, lipoprotein, and triglyceride metabolism. Activation of hepatic FXR modulates the expression of many hepatic genes involved in lipid metabolism (Kim *et al.*, 2007; Sinal *et al.*, 2000).



	R1	R2	FXR
LCA	H	H	+
CDCA	αOH	H	+++
DCA	H	αOH	++
CA	αOH	αOH	++
UDCA	βOH	H	weak

(Adopted from Claudel, T. et al. Arterioscler Thromb Vasc Biol 2005)

Figure 6: Structure and activation potency of several bile acids on human FXR

Two known FXR genes are FXRα and FXRβ. FXRβ is a pseudogene in humans but is present in rodents, rabbits and dogs, whose physiological role is still elusive (Otte *et al.*, 2003). FXRα gene is conserved from fish to humans suggesting importance of this gene in many species (Maglich *et al.*, 2003). FXR

is highly expressed in liver, intestine, kidney and adrenal gland. FXR gene is mapped at 12q23.3 on human chromosome and 10c.2 on mouse chromosome. Studies using *Fxr*$^{-/-}$ mice have illustrated the importance of this nuclear receptor in maintaining cholesterol and bile acid homeostasis.

FXR heterodimerizes with the retinoid X receptor (RXRα; NR2B1), binds to FXR response elements (FXREs) that are usually but not exclusively, inverted repeat -1 (IR-1) and regulates transcription of target genes (Laffitte *et al.*, 2000). A direct repeat (DR) with a similar core sequence is also compatible for binding of FXR either as a monomer or heterodimer (Barbier *et al.*, 2003; Claudel *et al.*, 2002; Laffitte *et al.*, 2000; Lu *et al.*, 2005). In addition, FXR can bind monomeric response elements and hence directly repress gene transcription (Barbier *et al.*, 2003; Claudel *et al.*, 2002; Laffitte *et al.*, 2000; Lu *et al.*, 2005). Recently, the location and sequence of FXRE was systematically studied in mice *via* ChIP and sequencing (Chong *et al.*, 2010). In this work, Chong *et al.,* identified 1656 binding sites, including 10% located in the proximal 2kb of the promoter. Moreover, up to 25% of these FXREs were not classical IR-1.

Figure 7: Sequences of consensus FXREs are shown.

Gene	NR1H4 12q23.3
Expression	Liver Small intestine Kidney Adrenals Vascular smooth muscle Adipose tissue Breast cancer
Natural agonists	**Primary bile acid:** CA, CDCA **Secondary bile acid:** LCA, DCA **Polyunsaturated fatty acids:** arachidonic acid; docosahexaenoic acid, and linolenic acid (endogenous and selective bile acid receptor modulators that specifically regulate expression of certain FXR targets) [21] **Bile acid metabolites:** 26- or 25-hydroxylated bile alcohols [22] **Oxysterols:** oxysterol 22(R)-hydroxysholesterol [19] **Androsterone** (very weak activity) [20] **The order of potency of these ligands:** 26- or 25-hydroxylated bile alcohols=CDCA>LCA=DCA>CA
Synthetic agonists	GW4064 (high-affinity agonist), 6ECDCA (semisynthetic bile acid), AGN29 [23], AGN31 [23] **The potency of these ligands:** GW4064 and 6ECDCA are more potent than the bile acids AGN29 and AGN31 are FXR-selective ligands and 25-fold more potent than naturally occurring ligands
Antagonists	Guggulsterone, lithocholate, AGN34 [23]
Response elements	**IR-1:** GAGTTAaTGACCT GGGTGAaTAACCT GGGACAtTGATCCT AGGTCAaGTGCCT GGGTCAgTGACCC **DR-1:** AGAGCAnAGGGGA **ER-8:** TGAACTcttaaccaAGTTCA **Monomer binding site:** GATCCTTGAACTCT TGAACT
Relevant diseases	Cholestasis Diabetes Atherosclerosis Cholesterol gallstone disease Liver regeneration Liver inflammation Hepatocarcinogenesis Breast cancer Colon cancer

(Adopted from Yan-dong Wang et al. Cell Research 2008)

Table 4: Summary of FXR information

2 Aim of the study

Lp(a) is one of the most atherogenic factors produced in the liver of primates. Turnover studies in humans clearly established that Lp(a) plasma concentrations are mainly controlled by the synthetic rate, i.e. the rate of apo(a) *de novo* biosynthesis. It is therefore of importance to uncover the steps involved in Lp(a) biosynthesis at the molecular level in detail and in particular the mechanism controlling apo(a) expression, in order to develop strategies to lower elevated plasma Lp(a) in hyper-Lp(a) states.

The general aim of this project was to elucidate the liver-specific transcriptional regulation of human apo(a) gene, that in fact was very fragmentary when I started my PhD work. Since we had some indications that bile acids lower plasma apo(a) levels, it was the main aim to explore in detail the mechanism of transcriptional regulation of apo(a) by bile acids and its receptor FXR.

The major part of the work presented in my thesis has been summarized in the manuscript "**Farnesoid X receptor represses hepatic human *APOA* gene expression in transgenic mice"** by Chennamsetty *et al*., The Journal of Clinical Investigation (in press).

3 Materials and Methods

This chapter lists all the chemicals, solutions, buffers for cell culture experiments, molecular biology techniques and biochemical methods which were used in this thesis.

3.1 Materials

3.1.1 Chemicals

Chemical	Company
Actinomycin D	Sigma
Agar	Serva
Agarose	Gibco
Albumin, bovine serum, fraction V	Sigma-Aldrich
Ampicillin	Roth
γ-^{32}P-ATP	Hartmann Analytic
Bromo phenol blue	Merck
$CaCl_2$	Merck
Chenodeoxycholic acid	Sigma
Chloroform	Fluka
Cholic acid	Sigma
Cyclohexamide	Sigma
Collagenase	Worthington
Deoxycholic acid	Sigma
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich
DMSO	Merck
Dulbecco´s modified Eagle´s medium (DMEM)	Gibco

EDTA	Roth
Ethanol	Merck
Ethidium bromide	Sigma-Aldrich
Fast green	Serva
Fetal bovine serum (FCS)	PAA
Formaldehyde	Sigma-Aldrich
FuGENE® 6 reagent	Roche
Glucose	Sigma
Glycerin	Merck
GW4064	Tocris
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Isopropanol	Merck
KBr	Roth
KCl	Merck
$K_4[Fe(CN)_6]$ x 3 H_2O	Merck
$K_3[Fe(CN)_6]$	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
KCl	Merck
KOH	Merck
β - Mercaptoethanol	Merck
Morpholinopropansulfonicacid (MOPS)	Roth
NaCl	Merck
NaN_3	Sigma

NaOH	Roth
Na- taurocholate	Fluka
Nile red	Sigma
Penicillin/streptomycin	Cambrex
Peptone	Gibco
PD98059	Calbiochem
Protease inhibitor cocktail (PIC)	Sigma
QuantiFast™ SYBR Green PCR Kit	Qiagen
RNAse Inhibitor	Qiagen
Recombinant FGF19	R&D sytems
SB203580	Calbiochem
SDS	Serva
Sodium chloride	Roth
Sodium sulfite	Merck
Sodium thioglycollate	Sigma
SP600125	Calbiochem
TEMED	Sigma Aldrich
TRI-reagent	Peqlab
Tris	Roth
Triton X-100	Merck
Trypsin	PAA
U0126	Upstate biotech
X-Gal	Roth

Table 5: List of chemicals

3.1.2 Kits

Kit	Company
Bradford protein assay	Bio-Rad
ECL chemiluminescence detection system	Thermo Scientific
Triglyceride FS	Diasys
Total Cholesterol	Greiner
Bile Acid Kit	DiaSys Diagnostic Systems
Sybr Green Kit	Quiagen
RNA Isolation	Peq Lab
Wizard R SV Genomic DNA purification	Promega
Wizard R plus SV Miniprep kit	Promega
QIAprep R Spin Miniprep kit	Quiagen
QIAquick Gel Extraction	Quiagen
Luciferase Assay System	Promega
β-Galactosidase Enzyme Assay System	Promega
Quik Change™ site-directed mutagenesis system	Stratagene
TNT® T7 Quick Coupled Transcription/Translation System	Promega
Gel shift assay system	Promega
EpiQuik™ tissue ChIP kit	Epigentek

Table 6: Kits

3.1.3 Antibodies

Standard/Antibody	Dilution	Company
Polyclonal Lp(a) (Rabbit)	1:1250	Self Made
SHP (Goat)	1:100	Santa Cruz
Phospho-p38 MAP kinase (Rabbit)	1:1500	Cell Signaling Technology
p38 MAP kinase (Rabbit)	1:1300	Cell Signaling Technology
pERK1/2 (Mouse)	1:1200	Cell Signaling Technology
ERK1 (Rabbit)	1:2000	Santa Cruz
Phospho-SAPK/JNK (Mouse)	1:150	BD Biosciences
SAPK/JNK (Rabbit)	1:1000	Cell Signaling Technology
Anti-mouse β-actin	1:2000	(Santa Cruz Biotechnology

Table 7: Antibodies

3.1.4 Enzymes

Enzymes	Company
Hotfire Pol®	Solis Biodyne Estland
Advantage®cDNA Polymerase	ClonTech
T4 DNA Ligase	Fermentas
GoTaq DNA pol	Promega
Pfu polymerase	Stratagene
Dpn1	Fermentas
Multiscribe Reverse Transcriptase	Applied biosystems
Restriction Enzymes	New England BioLabs (NEB) Fermentas

Table 8: Enzymes

3.1.5 RNA buffers

- **DEPC water:**

400 µl DEPC were added to 400 ml ddH_2O. The mixture was left open ON in the hood, autoclaved on the next day and stored at RT.

- **10 x MOPS buffer:**

MOPS	10.5 g
Na-Acetate	1.7 g
0.5M EDTA	5 ml
DEPC-water	200 ml

pH was adjusted to 7.0 with 10 N NaOH. final volume was made up to 250 ml with DEPC water.

RNA sample buffer (3 ml)

99.5 % Formamide	1.44 ml
10 x MOPS buffer	280 µl
37 % Formaldehyde	520 µl
DEPC water	400 µl
80 % sterile Glycerol	200 µl
Ethidium bromide	10 µl

Storage at 4°C.

3.1.6 DNA buffers

- **1M Tris-HCL buffer pH 6-8**

Tris base (MW.121.14)	12.1 g
ddH_20	100 ml

Adjust pH with concentrated HCL.

- **5 x TAE buffer**

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
ddH_20	900 ml

Adjust solution volume to 1000 ml. Solution can be stored at RT. Buffer has to be discarded if a precipitate has formed.

- **0.5M EDTA pH 8.0**

EDTA	186.1 g
NaOH pellets	20 g
ddH_20	900 ml

Adjust the pH to 8.0 with NaOH slowly. Make up the solution volume to 1000 ml. Autoclave and store at RT.

- **DNA sample buffer (10 ml)**

Glycin	5 ml
40 x TAE buffer	250 µl
Bromo phenol blue	1 granule
H_2O	4.75 ml

Storage at RT.

- **40 x TAE buffer**

Tris	193.6 g
Na-Acetate	65.5 g
EDTA	14.9 g
ddH_20	800 ml

pH was set to 7.2 with acetic acid and the volume was made up to 1000 ml with ddH_20. Storage at RT.

3.1.7 Protein buffers

- **RIPA buffer**

50 mM KH_2PO_4, 150 mM NaCl, 1% Triton X-100, 0,5% deoxycholate and 1: 2000 PIC (added just before use). Storage at 4°C.

- **Separating gel buffer (buffer pH 8.8)**

Tris	18.2 g
10 % SDS	4 ml
H_2O	80 ml

The pH was set to 8.8 with HCl and the final volume was made to 100 ml. Stored at RT.

- **Stacking gel buffer (buffer pH 6.8):**

6 g Tris dissolved in 90 ml ddH_2O, the pH was adjusted to 6.8 with HCl and the volume was made up to 100 ml. Stored at RT.

- **SDS sample buffer:**

SDS	2.15 g
Tris	0.76 g
H_2O	45 ml

The pH was adjusted to 6.8 with HCl. 10 ml glycerol (80%) and few granules of brome-phenol blue were added and stored at RT.

- **10 x SDS running buffer**

Tris	30.3 g
Glycin	150.1 g
SDS	10 g

Volume was made up with ddH2O to 1000 ml. Storage at RT.

- **10 x Blot buffer:**

Tris	12.1 g
Glycin	30 g
EDTA	1 mg/ml

Volume was made up to 1000 ml with H2O. Storage at RT.

- **10 x Blot washing buffer:**

Tween	5 g
NaCl	90 g
1 M Tris HCl(pH 7.4)	100 ml

Volume was adjusted to 1000 ml with ddH2O. Storage at RT.

3.1.8 LacZ staining solution

- **X-Gal:** Prepared by dissolving 40mg/ml X-Gal in dimethylformamid
- **LacZ staining:**

Glutaraldehyde 25% in water

1:50 diluted in sterile PBS (=0,5%)

20mM Potassium ferricyanide K3[Fe(CN)6] dissolved in PBS

20mM Potassium ferrocyanide K4[Fe(CN)$_6$] dissolved in PBS

No. of wells	$K_3[Fe(CN)_6]$ (µl)	$K_4[Fe(CN)_6]$ x 3 H_2O (µl)	1M $MgCl_2$ (µl)	2% X-Gal (µl)	PBS (µl)
1	250	250	1	50	449
2	500	500	2	100	898
3	750	750	3	150	1347
4	1000	1000	4	200	1796
5	1250	1250	5	250	2245
6	1500	1500	6	300	2694
12	3000	3000	12	600	5388

Table 9: Amount of reagents to prepare LacZ staining solution

3.1.9 Mouse primary hepatocytes isolation buffers

- **Buffer 1**: **Krebs-Henseleit buffer:**

NaCl	115 mmol/l
$NaHCO_3$	25 mmol/l
KCl	5,9 mmol/l
$MgCl_2$	1,18 mmol/l
NaH_2PO_4	1,23 mmol/l
Na_2SO_4	1,2 mmol/l
$CaCl_2$	1,25 mmol/l
Glucose	6 mmol/l

Before adding $CaCl_2$, pH must be adjusted to 7.4.

- **Buffer 2: Krebs-Henseleit buffer without Ca^{2+} und SO_4^{2-}:**

NaCl	115 mmol/l
$NaHCO_3$	25 mmol/l
KCl	5,9 mmol/l
$MgCl_2$	1,18 mmol/l
NaH_2PO_4	1,23 mmol/l
Glucose	6 mmol/l

Buffers were then filter sterilized using Millipore filter and saturated in CO_2 incubator for overnight. Buffer 1 was stored at 4°C, Buffer 2 was at room temperature.

Collagenase solution:

Buffer 2 saturated	150 ml
10 mmol CaCl2	1.5 ml
BSA	3 g
collagenase type CLS II	30 mg

Filter sterilize the solution.

Prior to the perfusion, buffer 2 and the collagenase solution were heated in water bath.

3.2 Methods

3.2.1 Patients

Patients suffering from obstructive jaundice due to gallstones or or malignancy from the Department of Medicine, Medical University of Graz were studied for markers were studied for markers of biliary obstruction and plasma Lp(a) concentrations. Blood of patients referred to surgery or endoscopy was analyzed immediately for plasma levels of Lp(a), bilirubin, total bile acids and LP-X. After appropriate treatment, these plasma parameters were assayed again. All human studies were approved by the ethical committee of the Medical University of Graz (Auenbruggerplatz 2, Graz, Austria) and were performed in accordance with the Helsinki Declaration. Informed consent was received from all patients or their parents respectively, for drawing some extra blood to perform lipid and lipoprotein analyses.

3.2.2 Animals and diets

All animal experiments were performed following approval of the protocol by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments, Austria. FXR null mice (FXR-/-) (Sinal *et al.*, 2000) were obtained from the laboratory of Frank Gonzalez, NIH, Bethesda MD. FXR-/- mice were backcrossed for 7 generations with transgenic apo(a) (tg-apo(a)) mice carrying intact human apo(a) gene controlled by its native flanking region in YAC construct (Frazer *et al.*, 1995). Mice were hosted regularly under standard 12 h light/12 h dark cycle and fed standard rodent chow diet and water ad libitum. Female mice between 10 and 12 week old were

used in all the experiments. For feeding studies, Tg-apo(a) (n=8) and tg-apo(a)/FXR$^{-/-}$ mice (n=8) expressing the human apo(a) gene were divided into 2 groups. Animals were randomized based on plasma apo(a) levels. One group received a normal rodent chow diet (control), whereas the other group received the same diet supplemented with 0.2% (wt/wt) cholic acid (CA) for 5 days. At sacrifice, mice were fasted for 4 h before blood samples were collected. Liver and ileum samples were harvested and stored at -80°C until further analysis. 10 to 12 week old female tg-apo(a) mice (n=3) were injected intraperitoneally with vehicle (corn oil with 5% DMSO) or the synthetic FXR agonist GW4064 (30mg/kg body wt) (Gnerre *et al.*, 2004), and sacrificed after 16 h. For the ChIP assay, female tg-apo(a) mice (n=3) were fed with either normal chow (control group) or chow with 0.2% CA for 24 h. Freshly isolated liver tissue was pooled and used to isolate chromatin for immunoprecipitation.

3.2.3 Common bile duct ligation (CBDL)

12 week old female tg-apo(a) mice (n=3-4 per group) and tg-apo(a)/FXR$^{-/-}$ mice (n=3 per group) were subjected to common bile duct ligation as described previously (Wagner *et al.*, 2003) by Anna Baghdasaryan, a PhD colleague who works in the group of M.Trauner. In brief, the common bile duct was ligated close to the liver hilus immediately below the bifurcation and dissected between the ligatures. Sham-operated animals were subjected to the same surgical procedure, but without ligation of the common bile duct. Serum and livers were collected for analysis 3 days after surgery. Liver tissue was frozen in liquid nitrogen for further RNA preparations. Serum was stored at -80C until analysis. Serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase levels and bilirubin were determined by routine testing on a Hitachi 917 analyzer (Boehringer Mannheim, Mannheim, Germany) in the central core facility at ZMF as measures of degree of cholestasis. Total serum bile acid levels were determined enzymatically using Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

3.2.4 Cell culture

Mouse primary hepatocytes were prepared and cultured as described previously (Salonpaa *et al.*, 1994). The mouse liver was perfused with

collagenase solution and liver cells were collected. After filtration and centrifugation, the isolated hepatocytes were resuspended in DMEM (Gibco, Invitrogen, Lofer, Austria) supplemented with 20% (v/v) FCS (Sigma-Aldrich Chemie GmbH, Vienna, Austria), 100 units/ml penicillin, and 100 units/ml streptomycin, and placed in 6-well collagen-coated plates (BD Biosciences, Erembodegem, Belgium) at a density of 1 x 10^5 cells/well at 37°C in an atmosphere of 5% CO_2 for 4 h. Thereafter, cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin /streptomycin for 16 h. Experiments were performed in serum-free DMEM supplemented with various concentrations of the FXR ligands CA and GW4064.

The HepG2 (**ATCC Number: HB-8065)** and COS7 **(ATCC Number: CRL-1651)** cells were obtained from the American Type Culture Collection (Rockville, Maryland). The cells were maintained in DMEM containing 10% FCS, 100 units/ml penicillin/streptomycin.

3.2.5 Isolation of mouse primary hepatocytes

- Water bath was heated to 41°C. All the perfusion tubes/connectors were first washed with ethanol and then rinsed with 1x PBS. Prior to perfusion, buffer 2 and collagenase solutions were heated in water bath.
- The mouse liver was perfused first with buffer 2 for 15 minutes and then with collagenase solution until the liver becomes fragile. The liver was taken out carefully and in to a Petri dish filled with the collagenase solution.
- The liver was cut into small pieces and pressed through a sieve. Rinsed with cold buffer 1. The cell suspension was then filtered through a 70 micron cell strainer into a 50 ml Greiner tubes.
- The cell suspension was filled with cold medium to 50 ml and centrifuged for 2 minutes at 4 ° C and 500 rpm (50 g).
- The supernatant was aspirated and the pellet was resuspended in the medium.
- The number of cells and the viability was determined by counting chamber. 5 x 10^5 cells/well were seeded in 6-well plate.

3.2.6 Adenoviral infection of primary mouse hepatocytes

Primary hepatocytes from tg-apo(a) mice were isolated and maintained for 24 h before infection with 50 and 100 MOI of adenovirus encoding LacZ or HNF4α in serum free DMEM. After a 4 h infection, the cells were incubated in DMEM supplemented with 10% FCS, for 24 h, followed by cell harvesting for RNA analysis.

3.2.7 RNA isolation from cell lines with Peqlab total RNA Kit

In order to isolate RNA from cells, medium was aspirated and cells were washed once with PBS. Cells were incubated for 1 min with RNA Lysis Buffer T. Then the cells were scraped and the whole cell lysate was applied directly into a DNA Removing Column placed in a 2.0 ml collection tube, centrifuged at 12.000 x g for 1 min at room temperature. An equal volume of 70 % Ethanol was added to the flow-through lysate and mixed thoroughly. The flow-through was then added to the membrane of perfect bind RNA Column in a new 2.0 ml collection tube and centrifuged the assembly at 10.000 x g for 1 min. The flow-through was discarded and the column was washed with 500 µl wash buffer 1 and then with wash buffer 2 twice. Thereafter, the column was dried by centrifugation at 10.000 x g for 1 min. For elution of RNA, the columns were placed into a 1.5 ml RNase- free tube and RNase-free water was applied (usually about 20 µl). After 5 min incubation RNA was eluted by centrifugation at 10.000 x g for 1 min.

3.2.8 RNA isolation from mouse tissues with TRI-reagent

Mice were killed by cervical dislocation and tissues were removed surgically as quickly as possible. All tissues were frozen immediately in liquid nitrogen and stored at -70°C until use.

For RNA isolation frozen tissues were weighed and put into homogenization tubes. 1 ml TRI-reagent/100 mg tissue was added. The tissues were homogenized with a Precellys homogenizer (Peqlab, Austria) at 5000 rpm for 20 s. After the addition of 0.2 ml chloroform/ml TRI-reagent, the mixture was vortexed and incubated for 10 min at RT, the samples were centrifuged at 8500 rpm for 15 min at 4°C. The aqueous phase containing RNA was transferred into a new centrifugation tube. For RNA precipitation 0.5 ml isopropanol per 1 ml TRI-reagent was added and the samples were incubated for 10 min at RT. After

centrifugation at 8500 rpm, 10 min, 4°C, the supernatant was carefully discarded and the RNA pellet was washed twice with 1 ml 75 % EtOH per ml TRI-reagent. The samples were centrifuged again for 5 min at 8500 rpm and 4°C. Finally, the supernatant was discarded carefully and the pellet was air-dried. RNA was dissolved in 20-200 µl DEPC water (depending on the pellet size) and stored immediately at -70°C.

3.2.9 RNA quantification

2 µl of RNA was quantified on a spectrophotometer (NanoDrop) at a wavelength of 260 nm. To determine the RNA quality the absorption ratio 260 nm/280 nm was checked. This ratio should be between 1.8 and 2. Higher ratios indicate impurities of EtOH or isopropanol, while lower ratios indicate protein impurities.

3.2.10 RNA gel electrophoresis

The integrity of RNA was checked by gel electrophoresis. RNA gel was prepared by boiling 1.1 g Agarose, 11 ml 10 x MOPS, 81.9 ml DEPC-water in a microwave. After cooling down to approximately 60°C, 17.1 ml formaldehyde was added. 1 x MOPS buffer was used as running buffer. Two µg of RNA were mixed with RNA sample buffer (5 x). The gel was run at 150 V for 45 min.

3.2.11 Reverse transcription

Two µg RNA (in 10 µl ddH_2O) were reverse transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Austria) according to manufacturer's protocol. The composition of the master mix is shown in Table 10 the program of the thermal cycling conditions in Table 11.

Component	Volume (µl)
10 x RT Buffer	2
25 x dNTP Mix (100 mM)	0.8
10 x RT Random Primers	2
Multiscribe Reverse Transcriptase	1
RNase Inhibitor	0.7

Nuclease-free H_2O	3.5
1 µg RNA/10 µl ddH_2O	10
Total volume per reaction	**20**

Table 10: Master Mix for reverse transcription using the High-Capacity cDNA Reverse Transcription Kit

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 s	∞

Table 11: The programme for reverse transcription

3.2.12 Real-time quantitative PCR

Quantitative real time PCR is a very sensitive method and allows quantification of gene expression. Synthesized PCR products are detected by SYBR Green I fluorescence dye that binds specifically to the minor groove of double stranded DNA.

	temperature	**time**	**cycles**
Denaturation	95°C	5 min	1
Amplification	95°C 60°C	10 s 30 s	35
Melting curve	95°C 60°C 95°C	10 s 20 s continuous	1 1
Cool down	40°C	20 s	1

Table 12: PCR programme- real time quantitative PCR

Primer sequences for real time PCR (Table 13) were taken from the Harvard Primer Bank (http://pga.mgh.harvard.edu/primerbank/index.html), all having an annealing temperature of 60°C. All primers were obtained from Invitrogen.

First, the primer efficiency of each primer pair had to be determined. A pool of cDNAs, containing 200 ng of each cDNA, was mixed and diluted 1:5, 1:25, 1:125, 1:625 with ddH_2O. Then a master mix was prepared with 5 µl

QuantiFast™ SYBR® Green PCR Kit and 1 µl forward (10 pmol/µl) and 1 µl revers primer (10 pmol/µl) per sample. Seven µl of the master mix and 3 µl of cDNA pool were transferred to a Light Cycler 480 Multi well Plate 96. Afterwards, this plate was centrifuged for 1 min at 800 rpm and analyzed on a ROCHE Light Cycler 480. After calculating the primer efficiency (between 1.8 and 2), the single cDNAs were used to determine the relative gene expression. Cyclophilin A was used as internal normalization standard. For analyzing the expression profiles and associated statistical parameters the public domain program Relative Expression Software Tool - REST (http://www.gene-quantification.com/download.html) was used.

Gene Name	**Forward primer (5'→3')**	**Reverse primer (5'→3')**
Apoa1(Mouse)	GGCACGTATGGCAGCAAGAT	CCAAGGAGGAGGATTCAAACTG
Bsep(Mouse)	ACAGAAGCAAAGGGTAGCCATC	GGTAGCCATGTCCAGAAGCAG
Cyp7a1(Mouse)	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
Cyp8b1(Mouse)	CCTCTGGACAAGGGTTTTGTG	GCACCGTGAAGACATCCCC
Ppia(Mouse)	TTCCAGGATTCATGTGCCAG	CCATCCAGCCATTCAGTCTT
Cyp3a11(Mouse)	AGCAGGGATGGACCTGG	CGGTAGAGGAGCACCAA
Fgf15(Mouse)	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG
Hnf4a(Mouse)	CACGCGGAGGTCAAGCTAC	CCCAGAGATGGGAGAGGTGAT
*Il*b(Mouse)	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il6(Mouse)	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
APOA(Human)	CAGCACGTTCATTCCAGGGA	CACCAGGGACCATTGATGTCA
*Lrh*1(Mouse)	GAACTGTCCAAAACCAAAAAAGG	CGTTTTCTCTGCGTTTTGTCA
Shp(Mouse)	TGGGTCCCAAGGAGTATGC	GCTCCAAGACTTCACACAGTG
Tnfa(Mouse)	GCCACCACGCTCTTCTGTCT	GGCTACAGGCTTGTCACTCG

Table 13: Primer Sequences used for real-time quantitative PCR

3.2.13 DNA quantification

2 µl DNA were quantified on a spectrophotometer (NanoDrop) at a wavelength of 260 nm. DNA concentration was calculated with the following formula:

$$C_{(ngDNA/\mu l)} = \lambda_{260nm} \times 50$$

3.2.14 DNA agarose gel electrophoresis

DNA size and purity were analyzed by agarose gel electrophoresis. The concentration of agarose used for analysis is inversely proportional to the size of DNA analyzed.

Agarose concentration (%)	DNA size (kb)
0.7	20-1
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2	3-0.1

Table 14: Concentration of agarose used to resolve different DNA sizes

Agarose was weighed and dissolved in 1 x TAE buffer, boiled in a microwave. The solution was allowed to cool down to 60°C or lower before ethidium bromide was added to yield a final concentration of 0.5 µg/ml. The gel solution was poured into a gel casette and was allowed to polymerize completely. Afterwards the gel was transfered into a gel chamber with 0.5 x TAE buffer, the sample DNA was mixed with loading buffer and loaded into the well. Gel electrophoresis was carried out at 80 V for 30-40 min.

3.2.15 Lipid parameter determination in plasma

Blood was collected by retro-orbital bleeding and EDTA-plasma was prepared by centrifuging at 4°C, 8000 rpm for 5 min. (1 µl 0.5 M EDTA/ 100 µl blood). Plasma concentrations of apo(a) were measured enzymatically by an in-house DELFIA method by A. Ibovnik. Lipid parameters were quantified using standard kits according to the manufacturer's protocols. The kits for FC and TG determination were obtained from DiaSys (Holzheim, Germany), for TC measurements from Greiner Diagnostics (Langenthal, Switzerland) and for FFA detection from WAKO Chemicals GmbH (Neuss, Germany). CE was calculated from TC content minus FC content and multiplied with the factor 1.7253 for esters.

3.2.16 Lipoprotein profiling

Plasma samples of overnight fasted mice were pooled and lipoproteins were isolated by fast protein liquid chromatography (FPLC) on a Pharmacia FPLC system (Karlsruhe, Germany) with a Superose 6 column (Amersham Biosciences, Piscataway, NJ). For each run 200 µl pooled plasma samples were diluted, subjected to FPLC analysis and lipoproteins were eluted. Sixty fractions were collected and TG and TC concentrations were measured enzymatically using above mentioned kits (3.2.15). Sensitivity can be enhanced by the addition of sodium 3.5-dichloro-2-hydroxy-benzenesulfonate to the reaction buffers.

3.2.17 Protein extraction and immunoblotting

Livers were homogenized or cells were lysed in an ice-cold RIPA buffer added with PIC in 1:2000 dilution. The lysates were centrifuged (12000 *g)* at 4°C for 10 min, and the supernatant was collected. Protein was quantitated using the Bradford protein assay (Bio-Rad, Munich, Germany). Equivalent amounts of protein homogenates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antibodies to human apo(a) (1:1250) and a monoclonal anti-mouse β-actin (1:2000) (Santa Cruz Biotechnology). The Immunoblots were visualized by Pierce^{R} ECL chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA). Densitometric analysis of the gels was carried out using ImageJ software.

3.2.18 SDS-PAGE gel preparation for immunoblotting

Reagent	**3.75 % SDS-gel**	**12.5 % SDS-gel**
acrylamid	1075 µl	3583 µl
buffer pH 8.8	2170 µl	2170 µl
ddH_2O	5355 µl	2847 µl
10 % SDS	100 µl	100 µl
TEMED	4.4 µl	4.4 µl
10 % APS	76 µl	76 µl

Table 15: Components of the separating gel

Reagent	Volume
acrylamid	250µl
buffer pH 6.8	385 µl
50 % glycerin	885 µl
10 % SDS	21.5 µl
TEMED	3.8 µl
10 % APS	38 µl

Table 16: Components of the stacking gel

3.2.19 Plasmids

Expression plasmids encoding hFXR (pcDNA3-FXR), hRXRα (pSG5-RXR), SHP (pCDM8-SHP) and HNF4α (pSG5-HNF4α) were kindly provided by Dr Peter Young, Dr Philippe Lefebvre, Dr David D. Moore and Dr Mary C. Weiss, respectively. The human apo(a) promoter construct (hapo(a) -1952/+52) was obtained by PCR amplification using human genomic DNA as a template. The PCR product was cloned into the pGL3 basic vector (Promega) as a *MluI/BglII* fragment to generate h*APOA* -Luc. (Primers for forward reaction 5'-ACGCGTTCTGAGAGGGAGGTCAAAGTTTTC-3' and reverse reaction 5'-AGATCTCTTGAGAAAGCCAGCCCCAAAGGT-3'). All constructs were verified by DNA sequencing (LGC Genomics, Berlin, Germany).

3.2.20 Transient transfection and reporter gene assays

HepG2 cells were plated in 24-well plates over night before transfection. Cells at 60-70% confluency were transiently transfected with indicated reporter plasmid (150 ng) with or without receptor expression plasmids (150 ng) using FuGENE® 6 reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. β-galactosidase expression plasmid was co-transfected to assess the transfection efficiency. After 12 h of transfection, medium was changed and cells were exposed to the ligands (CDCA (100 µmol/L), GW4064 (500 nmol/L) or vehicle. After 36 h, cell extracts were prepared using passive lysis buffer (Promega, Madison, Wisconsin, USA) and assayed for luciferase and β-galactosidase activities using the Luciferase

Assay System and the β-Galactosidase Enzyme Assay System respectively (Promega, Madison, Wisconsin, USA). Luciferase activities were measured using Lumat LB9501 (Berthold) and normalized to β-galactosidase activities for each transfected well. For each experimental trial, wells were transfected in triplicate and each well was assayed in duplicate. Data are presented as means ± SD.

3.2.21 Site-directed mutagenesis

Mutagenesis was performed using the Quik Change™ site-directed mutagenesis system (Stratagene, La Jolla, CA), according to the manufacturer's manual. The mutants were verified by sequencing. The oligonucleotides

M1 (5'-GAGGGTTGGAAGCAAGAGGGG**at**CCAACGCGCACGGGGAGGAAGC-3') and M2 (5'-GAAGCAAGAGGGGGGCCAAC**at**GCACGGGGAGGAAGCATTTGGGCAG-3') were used to introduce mutations into the full length hapo(a) -1952/+52. Mutated bases are in bold, small case.

3.2.22 Electrophoretic mobility shift assays (EMSA)

Human FXR, RXR and HNF4α proteins were synthesized *in vitro* using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, Wisconsin, USA). The sense and antisense oligonucleotide probes of DR-1 Wt (5'-AGGGGGGCCAACGCGCACGGG-3'), DR-1 M2 (5'-AGGGGGGCCAAC**at**GCACGGG-3') and a FXR IR-1 consensus response element-containing oligonucleotide (IR-1: 5'-GATCTCAAGAGGTCATTGACCTTTTTG-3') were annealed and radioactively labeled at the 5' end using T4 polynucleotide kinase and γ-^{32}P-ATP (Hartmann Analytic GmbH, Braunschweig, Germany). Unincorporated nucleotides were removed by using Micro Bio-Spin 6 Columns (Bio-Rad Laboratories, Vienna, Austria). *In vitro* translated proteins (2.0 µl) were incubated for 20 minutes at room temperature in a total volume of 10 µl with binding buffer (Gel shift assay system, Promega, Madison, Wisconsin, USA) before the labeled probe was added. Binding reactions were further incubated for 30 minutes and resolved by 6% nondenaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-

EDTA (TBE) buffer at room temperature and 120 V for 3.5 h. The gel was dried and exposed to an X-ray film. For competition experiments, unlabeled probes were included in the binding reaction at the indicated excess concentrations.

3.2.23 Chromatin immunoprecipitation (ChIP) assay

The *in vivo* ChIP assay was performed with freshly isolated mouse liver tissue using EpiQuik™ tissue ChIP kit (Epigentek, Brooklyn, NY) according to the manufacturer's instructions with minor modifications. Liver tissue was fixed in formaldehyde for 12 min. and then quenched for 5 min with glycine. The nuclei were extracted and sonicated to yield 500-1,000 basepair (bp) DNA fragments. Aliquots of sheared chromatin were then immunoprecipitated using 4 µg anti-FXR (sc-13063; Santa Cruz), anti-RXR (sc-553; Santa Cruz), 2 µg anti-HNF4α antibody (sc-6556; Santa Cruz), or 1 µg anti-IgG antibody. Non-precipitated chromatin (input) was used as a positive control. DNA extractions were PCR amplified using the following flanking primers and the PCR products were analyzed by agarose gel electrophoresis. DR-1 element in the apo(a) promoter (DR-1 ChIP FWD 5' TTGGCAGTGTTATTGGGAGAC 3'; DR-1 ChIP REV 5' ACAGGCAGTTCCATCACTCC 3'), distal region of the apo(a) promoter (Distal ChIP FWD 5' TCTCCCCTTCATGTTTCCAG 3'; Distal ChIP REV 5' CCAGTGGCCGACATAGAGAT 3') and SHP promoter (SHP ChIP FWD 5'GCCTGAGACCTTGGTGCCCTG 3'; SHP ChIP REV 5' CTGCCCACTGCCTGGATGC 3').

3.2.24 Statistics

Statistical analyses of the experiments were performed with GraphPad Prism 5.0. Two-tailed, unpaired Student's *t*-test was applied to determine statistical significance. *** $P \leq 0.001$; ** $P \leq 0.01$; * $P < 0.05$.

4 Results

4.1 Tissue specific distribution and expression of human apo(a) gene in transgenic apo(a) mice

The apo(a) gene is present only in primates and thus expressed only in humans and in Old World monkeys. Thus there exists no conventional experimental animal model system to study the Lp(a) metabolism and the regulation of apo(a) biosynthesis. However, the development of YAC-apo(a) transgenic mice (tg-apo(a) mice) expressing complete human apo(a) gene along with the intact apo(a) promoter has proved to be a suitable model system to study the regulation of apo(a) metabolism.

In our first experiments we investigated the tissue distribution of apo(a) transgene in tg-apo(a) mice. Various tissues from the tg-apo(a) mice ((liver (L), brain (B), heart (H), gut (G), kidney (K) and testes (T)) were surgically removed, weighed and subsequently frozen in liquid nitrogen until use. Total RNA was isolated with Tri-Reagent following the recommendations of the company. The samples were subjected to reverse transcription polymerase chain reaction (RT-PCR) for the determination of transgene expression using primers specific for apo(a) gene. Of the various tissues examined, apo(a) mRNA transcript was detected only in the liver (Figure 8).

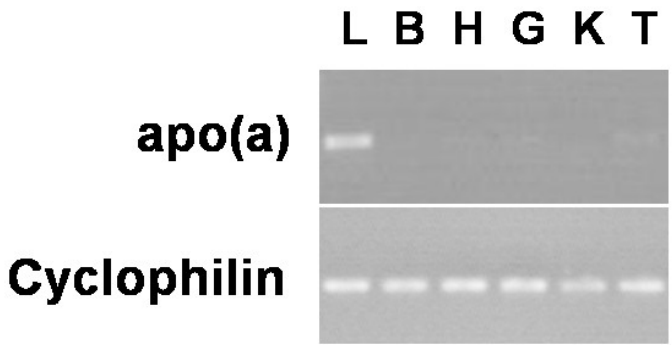


Figure 8: Tissue distribution of human apo(a) gene in tg-apo(a) mice. Apo(a) expression was analysed in various tissues by RT-PCR, normalized to cyclophilin.

Apo(a) expression levels were checked in different human hepatoma cell lines (HepG2, Huh7D), Immortalized human hepatocytes (IHH) and in primary hepatocytes isolated from tg-apo(a) mice. Only primary hepatocytes from tg-apo(a) mice showed the expression of transgene (Figure 9).

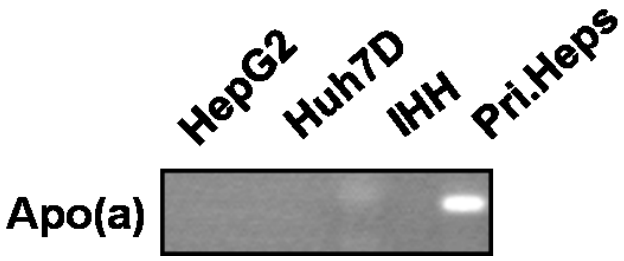


Figure 9: Apo(a) expression in various cell lines.

Agarose gel electrophoresis to check the expression of apo(a) gene in different cell lines –HepG2, Huh7D, Immortalized human hepatocytes (IHH) and mouse primary hepatocytes (Pri.Heps) isolated from tg-apo(a) mice.

We therefore decided to perform all subsequent experiments in tg-apo(a) mice and in primary hepatocytes from tg-apo(a) mice.

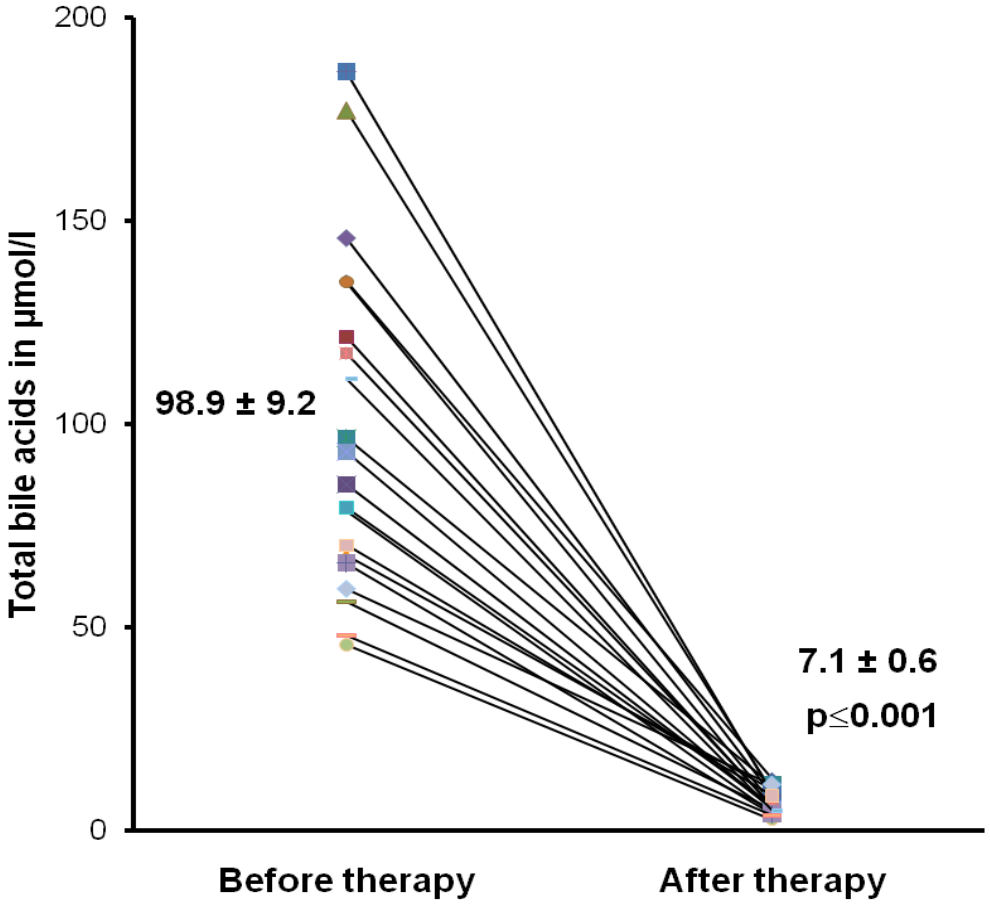
4.2 Elevated bile acid levels drastically reduce plasma Lp(a) levels in humans

We consistently noticed in our clinical service that patients suffering from obstructive jaundice exhibited very low or even undetectable levels of plasma Lp(a). To study this in a more systematic way, patients with obstructive jaundice were analyzed for markers of cholestasis, such as bilirubin, LP-X and plasma bile acid concentrations and the results were correlated with Lp(a) levels. Table 17 lists the results from 20 patients suffering from biliary obstruction due to pancreatic, gallbladder or bile duct cancer. In addition one patient with congenital biliary atresia and five patients with choledocholithiasis were included. All patients had elevated plasma bilirubin concentrations (316±48 µmol/l) and were positive for plasma LP-X (370±47.7 mg/dl). Notably, the patients had plasma total bile acid levels of 98.9±9.2 µmol/l that were more than 10-fold higher when compared to healthy individuals (Figure 10A). The plasma Lp(a) concentrations before therapy in 13 out of 20 of these patients were <1 mg/dl, which is the detection limit of the particular assay. The remaining 7 patients had very low Lp(a) levels in relation to their apo(a) isoform (K-IV repeats).

After successful surgical or endoscopic treatment of biliary obstruction, bilirubin, LP-X and total bile acid levels were normalized and Lp(a) concentrations rose

significantly to levels that correspond to those of healthy controls with the corresponding apo(a) isoforms. Mean plasma Lp(a) levels before therapy were 2.7±1.1 mg/dl and 20.3±4.4 mg/dl after therapy (Figure 10B).

A



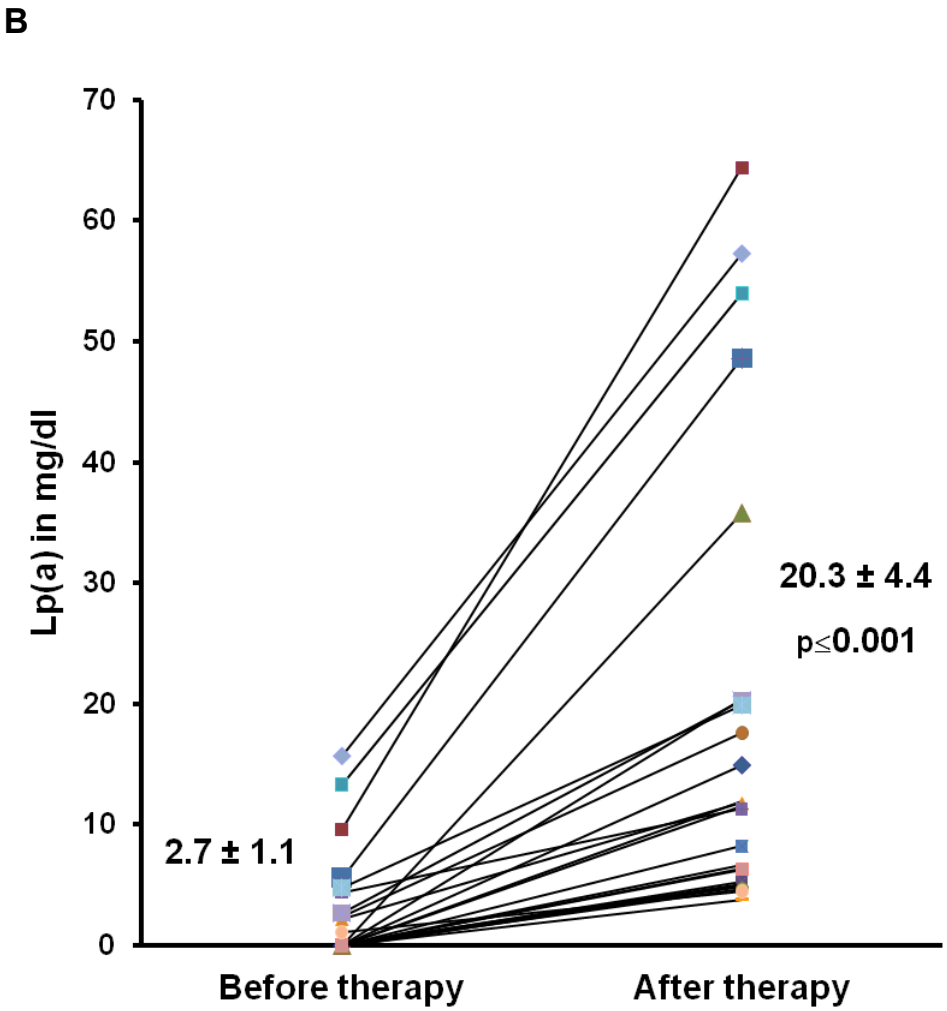


Figure 10: Low plasma Lp(a) levels in patients with obstructive jaundice.

*Plasma samples of 20 patients suffering from obstructive jaundice of different etiology were assayed for (**A**) total bile acids and (**B**) Lp(a) before and after surgical or endoscopic treatment. Values are expressed as the mean ± SEM.*

Patient	sex	age	Diagnosis	Bilirubin	[7]TBA (µmol/l)		LP-X (mg/dl) [§]		Lp(a) number	Lp(a) (mg/dl) [†]	
	m/f	(y)		µmol/l	before therapy	after therapy	before therapy	after therapy	of K-IV repeats	before therapy	after therapy
1	m	52	PC[2]	634	135,4	12,8	744	5,8	26	0	14,9
2	m	66	PC	488	121,6	5,7	268	0	16/22	9,6	64,4
3	f	49	PC	701	177,3	9,6	135	0	20	0	35,8
4	m	61	PC	285	85,3	5,5	449	0	22/26	0	5,3
5	m	55	PC	322	96,7	11,4	220	4,2	25	0	11,5
6	f	69	PC	680	135,1	6,6	376	5,5	27	2,4	17,6
7	m	72	PC	726	186,8	8,9	821	0	17/19	5,6	48,6
8	f	58	GBC[3]	189	78,6	4,3	209	0	17/30	0	5
9	m	52	GBC	112	56,4	5,2	283	0	22/28	0	11,9
10	m	70	GBC	398	145,9	8,7	645	5,1	28/37	0	6,4
11	m	39	GBC	188	79,5	6,3	489	0	17	13,3	54
12	f	73	BDC[4]	95	67,8	10	377	0	29/40	2,2	11,8
13	f	62	BDC	174	93,3	5,3	245	0	23/28	0	8,2
14	m	67	BDC	229	117,5	6,1	513	4,7	24	0	20,4
15	f	4w[1]	CBA[5]	183	45,8	2,8	75	8,6	22	0	4,7
16	m	42	CD[6]	198	65,9	4,2	281	0	20/29	4,4	11,3
17	m	39	CD	331	111,3	5	657	0	20	0	6,7
18	f	51	CD	117	48,2	3,9	125	0	26	0	3,8
19	m	48	CD	98	59,6	11,4	268	4,9	18/28	15,7	57,3
20	m	44	CD	176	70,3	8,7	224	0	22	0	6,3
Mean				**316,20**	**98,915**	**7,1‡**	**370,2**	**1,9‡**		**2,7**	**20,3‡**
SEM				**48,0**	**9,2**	**0,6**	**47,7**	**0,6**		**1,1**	**4,4**

Table 17: Patients and plasma parameters.

Plasma samples of 20 patients suffering from obstructive jaundice of different etiology were assayed for Lp(a), total bile acids and other variables of cholestasis before and after treatment of biliary obstruction.

[1] Newborn child at 4 weeks of age; [2]PC: pancreas carcinoma; [3]GBC: gallbladder carcinoma; [4]BDC: bile duct carcinoma; [5]CBA: congenital biliary atresia; [6]CD: choledocholithiasis; [7]TBA: total plasma bile acids. [§]Values below the accuracy limit of

the LP-X assay (3 mg/dl) were set zero; †Values below 1 mg/dl were set zero; ‡significantly different from values before therapy ($p<0.001$).

4.3 A cholestatic mouse model with elevated bile acid levels exhibits very low hepatic expression and plasma concentrations of apo(a)

To determine the effects of obstructive cholestasis on plasma levels and hepatic apo(a) expression, tg-apo(a) and tg-apo(a)/FXR$^{-/-}$ mice were subjected to biliary obstruction by common bile duct ligation (CBDL) for 3 days. CBDL in tg-apo(a) mice resulted in significantly elevated serum liver enzymes (Table 18), total bile acids and bilirubin (Figure 11A & B). The accumulation of endogenous bile acids in tg-apo(a) mice led to dramatic reduction of plasma apo(a) levels by 87% (Figure 11C) and of hepatic apo(a) mRNA expression by 98% (Figure 11D).

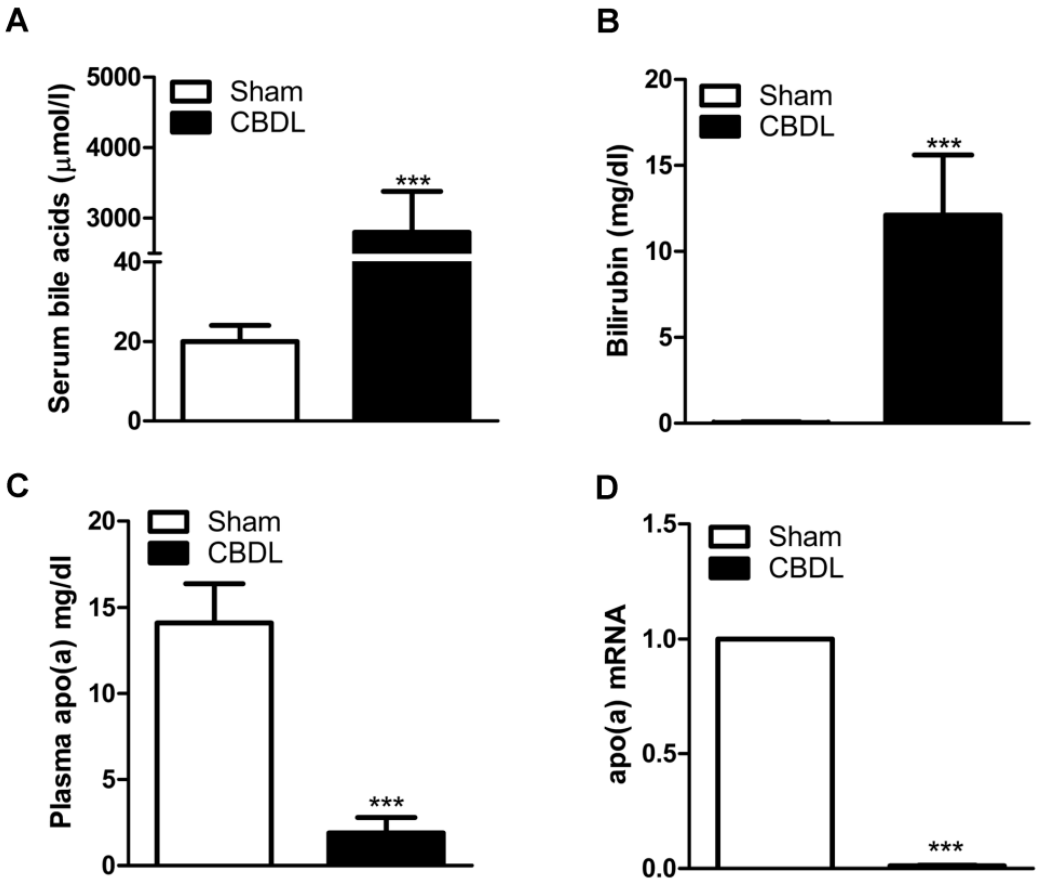


Figure 11: Drastic reduction in plasma levels and hepatic mRNA expression of apo(a) in tg-apo(a) mice-mouse model of cholestasis.

*Tg-apo(a) mice were subjected to biliary obstruction by common bile duct ligation (CBDL) (n=3 per group) or sham operation (n=4 per group) for 3 days. (**A** and **B**) Total bile acids and bilirubin were measured in serum. Data are presented as mean ± SD. *** p≤0.001 when compared to sham operated mice. (**C**) Plasma levels of apo(a) were measured by DELFIA and expressed as mean ± SD. (**D**) Liver apo(a) mRNA levels were analyzed by real-time quantitative PCR normalized to cyclophilin and expressed relative to sham operated mice. Results represent the mean ± SEM (*** p≤0.001).*

Variable	Sham	CBDL
Alanine aminotransferase (U/L)	31 ± 1.7	2230 ± 560[a]
Alkaline phosphatase (U/L)	73.5 ± 9	2858 ± 781[a]
aspartate aminotransferase (U/L)	90.7 ± 49	3289 ± 396[a]

Table 18: Effect of common bile duct ligation (CBDL) on liver enzymes in tg-apo(a) mice.

Tg-apo(a) mice were subjected to biliary obstruction by common bile duct ligation (CBDL) (n=3 per group) or sham operation (n=4 per group) for 3 days. Serum levels of liver enzymes were measured as indicators of cholestasis. Values are expressed as means ± SD . [a] *P<0.0001, compared to sham operated mice*

CBDL in tg-apo(a)/FXR$^{-/-}$ mice showed a small but measurable reduction of plasma apo(a) by 15% and hepatic mRNA by 19% (Figure 13) which might be due to inflammation and hepatic injury.

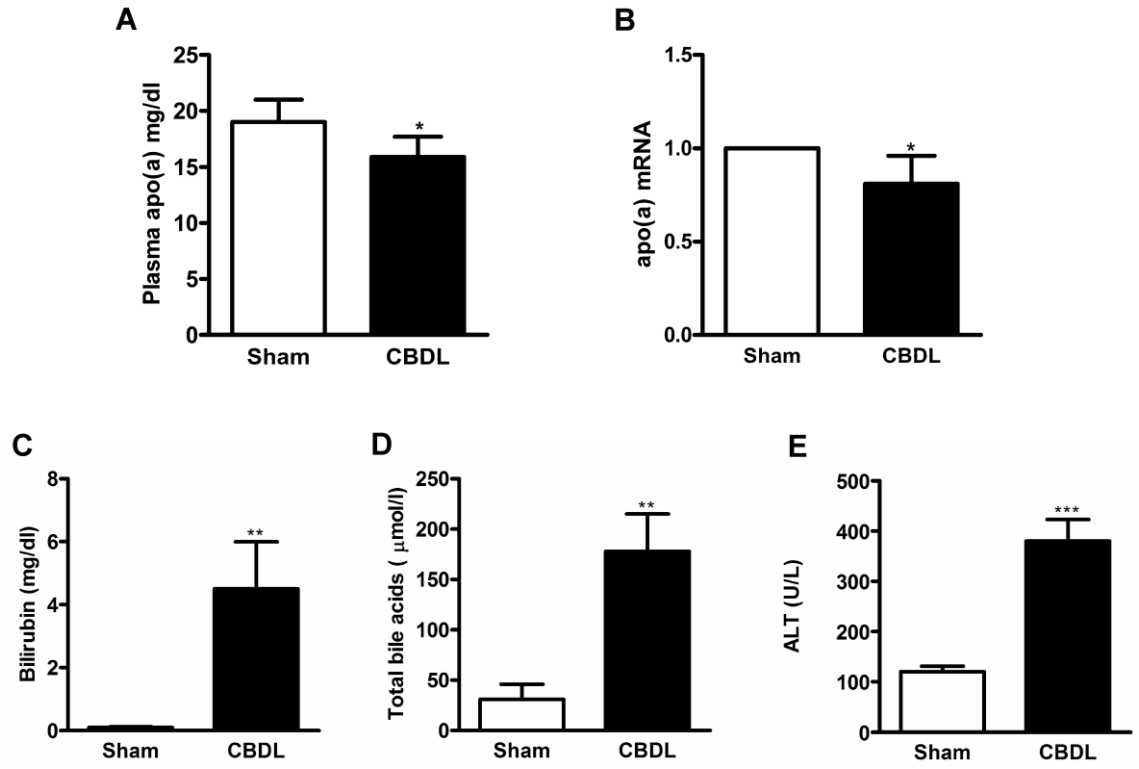


Figure 12: Common bile duct ligation in tg-apo(a)/FXR$^{-/-}$ mice.

*Tg-apo(a)/FXR$^{-/-}$ mice were subjected to biliary obstruction by common bile duct ligation (CBDL) (n=3 per group) or sham operation (n=3 per group) for 3 days. (**A**) Plasma levels of apo(a) were measured by DELFIA and expressed as mean ± SD (* p≤0.05). (**B**) Liver apo(a) mRNA levels were analyzed by real-time quantitative PCR normalized to cyclophilin and expressed relative to sham operated mice. Results represent the mean ± SEM (* p≤0.05). Serum levels of bilirubin (**C**), total bile acids (**D**), liver enzyme alanine aminotransferase (ALT) (**E**) were measured. Data are presented as mean ± SD. *** p≤0.001, ** p ≤0.01 when compared to sham operated mice.*

Taken together low apo(a) levels found in mouse and human cholestasis suggested that apo(a) expression is regulated by bile acids *in vivo*.

4.4 Cholic acid feeding reduces plasma apo(a) concentrations and hepatic apo(a) expression in transgenic apo(a) mice.

In order to support our assumption that human apo(a) gene expression is indeed regulated *in vivo* by bile acids, tg-apo(a) and tg-apo(a)/FXR$^{-/-}$ mice expressing human apo(a) gene were fed for 5 days with either a normal chow diet (control) or the chow diet supplemented with 0.2% cholic acid (CA) (wt/wt).

No changes in body weight or food intake were observed between control and treated groups (data not shown).

A 0.2% CA supplementation led to a significant decrease in plasma apo(a) levels by 72% in tg-apo(a) mice (Figure 13A). To evaluate whether the reduction of plasma apo(a) levels correlated with decreased apo(a) mRNA levels in liver, real-time quantitative PCR analysis was performed. Apo(a) mRNA levels were significantly decreased in the livers of the CA-fed tg-apo(a) mice (Figure 13B). Western blot analysis of liver homogenates confirmed that this repression also occurs at the protein level upon CA feeding in tg-apo(a) mice (Figure 13C). In tg-apo(a)/FXR$^{-/-}$ mice, however, plasma apo(a) concentrations (Figure 13D), hepatic apo(a) mRNA (Figure 13E) and protein levels (Figure 13F) were comparable in control and CA treated mice.

Taken together, these data indicate that both plasma levels and hepatic expression of human apo(a) are down-regulated by CA feeding in tg-apo(a) mice in an FXR-dependent manner.

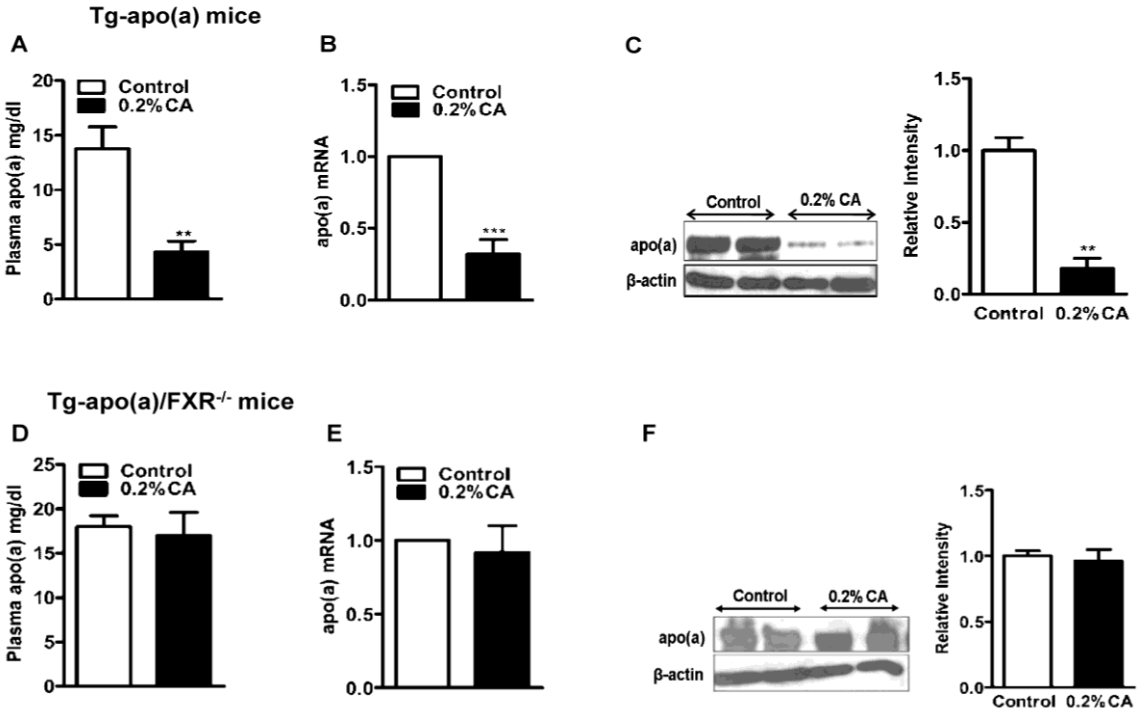


Figure 13: Cholic acid (CA) decreases plasma levels and hepatic expression of apo(a) in tg-apo(a) mice but not in tg-apo(a)/FXR$^{-/-}$ mice.

Tg-apo(a) mice (n=8 per group) and tg-apo(a)/FXR$^{-/-}$ mice (n=8 per group) expressing human apo(a) were fed with 0.2% CA (wt/wt) mixed in normal chow for 5 days. Control

*mice received normal rodent chow. (**A** and **D**) Plasma levels of apo(a) were measured by DELFIA and expressed as mean ± SD. (**B** and **E**) Mouse liver apo(a) mRNA levels were analyzed by real-time quantitative PCR and expressed relative to control cyclophilin. Values are expressed as the mean ± SEM (*** p≤0.001). (**C** and **F**) Western blot analysis (left) and densitometric quantification (right) of apo(a) levels in the protein extracts from liver tissue normalized to ß-actin controls and expressed as mean ± SD.*

We also followed plasma lipid values in CA fed mice. A 0.2% CA supplementation led to significant reduction in the plasma total cholesterol and triglyceride levels in tg-apo(a) mice, but these parameters remained unchanged in tg-apo(a)/FXR$^{-/-}$mice.

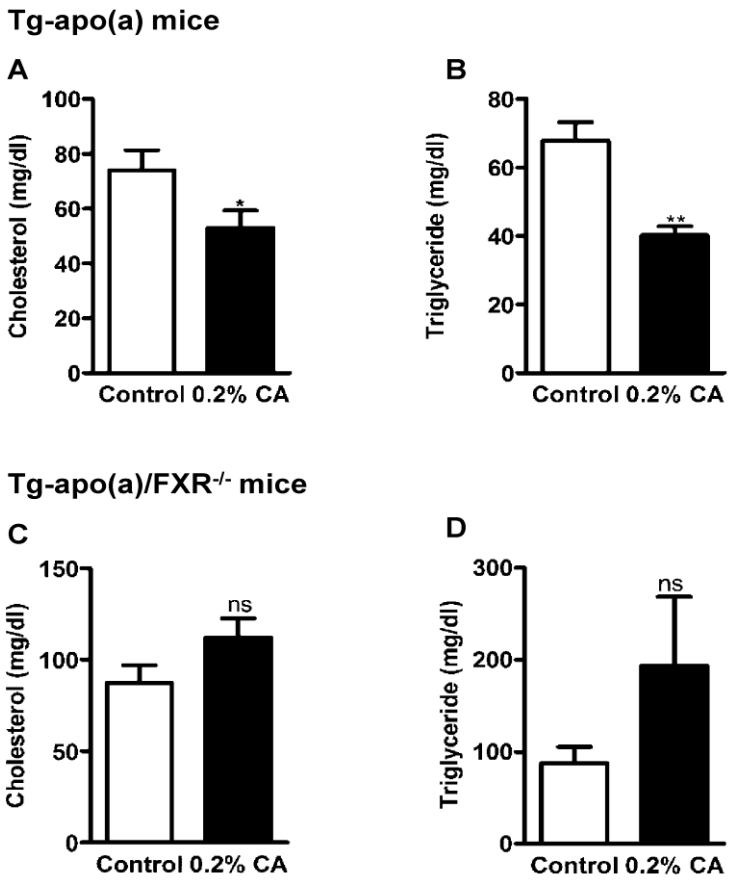


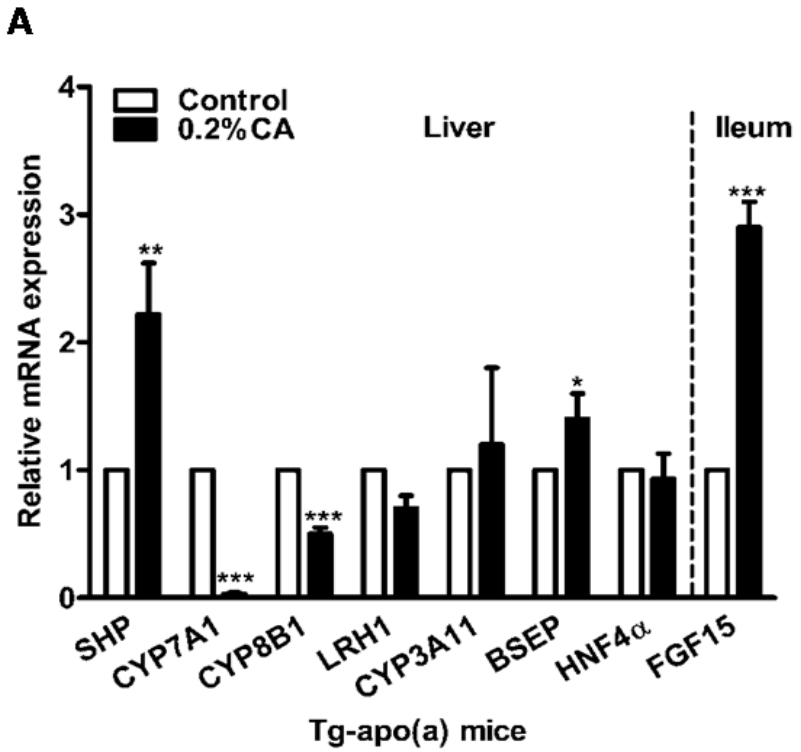
Figure 14: 0.2% CA feeding decreases plasma total cholesterol and triglyceride levels in apo(a) transgenic mice.

*Tg-apo(a) and tg-apo(a)/FXR$^{-/-}$ mice were fed a diet containing 0.2% CA (wt/wt) mixed in standard rodent chow for 5 days. (**A** and **C**) Total cholesterol, (**B** and **D**) triglycerides levels were measured in plasma after 4 h fasting period as described in Methods. Data*

*are presented as mean ± S.D. (n = 8). ** p ≤0.01, * p<0.05, ns, statistically not significant when compared with chow fed control group.*

Subsequently, we profiled hepatic expression of known FXR target genes involved in bile acid and cholesterol metabolism following 5-day feeding of tg-apo(a) and tg-apo(a)/FXR$^{-/-}$ mice with CA (Figure 15). As expected, CA treatment of tg-apo(a) mice led to a strong inhibition of both CYP7A1 and CYP8B1 gene expression (del Castillo-Olivares *et al.*, 2001; Goodwin *et al.*, 2000; Lu *et al.*, 2000), 2.3-fold up-regulation of SHP (Brendel *et al.*, 2002) and induction of BSEP. No changes were observed in the hepatic mRNA expression of LRH1 and HNF4α. FGF15 mRNA in the ileum was up-regulated by 2.8 fold (Inagaki *et al.*, 2005). CA feeding did not change hepatic expression of CYP3A11, a target gene of pregnane X receptor (PXR; NR1I2). Unchanged CYP3A11 revealed that PXR was not activated by 0.2% CA in the diet.

In contrast, CA treatment had no impact on mRNA levels of FXR target genes in tg-apo(a)/FXR$^{-/-}$ mice. However, CYP3A11 expression was induced in this context, probably as a consequence of PXR activation in tg-apo(a)/FXR$^{-/-}$ mice fed with bile acids (Schuetz *et al.*, 2001; Zollner *et al.*, 2003).



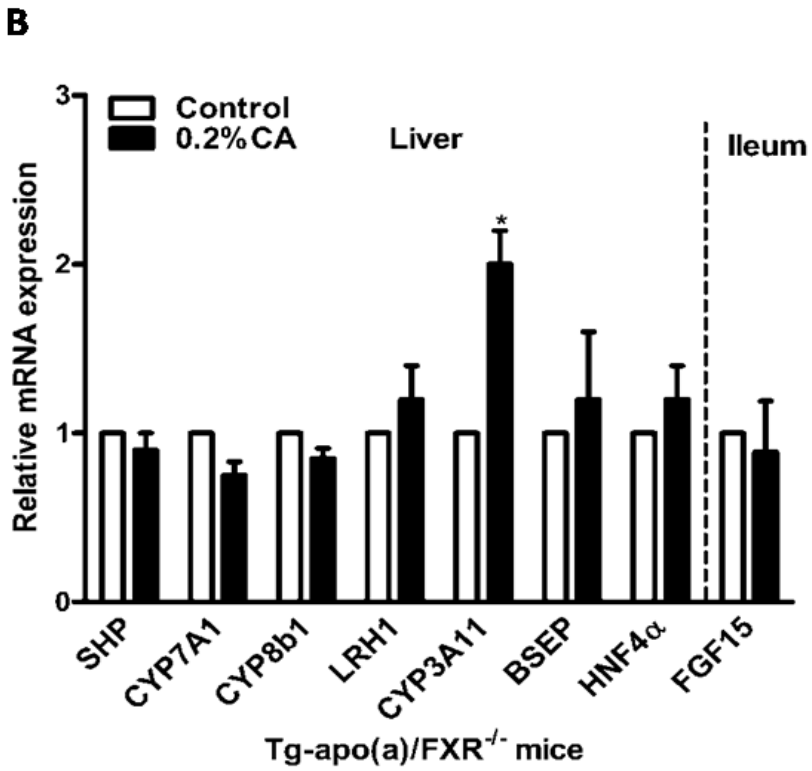


*Figure 15: Gene expression profiling in (**A**) tg-apo(a) mice (n=8) and (**B**) tg-apo(a)/FXR$^{-/-}$ (n=8).*

*mRNA expression of the FXR target genes like SHP, CYP7A1, CYP8B1, APOA1, LRH1, CYP3A11, BSEP in the liver and FGF15 in ileum were analyzed by real-time quantitative PCR and normalized to cyclophilin. Results represent the mean ± SEM (*** p≤0.001, ** p ≤0.01, * p<0.05).*

Bile acids can induce inflammation in the liver and cause liver damage (Davis *et al.*, 2002). Moreover, cholestasis in humans and mice is characterized by high inflammation (Claudel *et al.*, 2010). Therefore we studied the hepatic expression of several pro-inflammatory genes upon a 0.2% CA feeding. As shown in Figure 16A CA did not change the expression levels of pro-inflammatory cytokines such as IL6, IL1β and TNFα in tg-apo(a) mice, however there were 2.6-fold increased IL6 and slightly increased TNFα levels in tg-apo(a)/FXR$^{-/-}$ mice (Figure 16B).

Taken together, these results indicate that bile acids repress apo(a) expression directly *via* FXR and independent of any inflammatory stimulus.

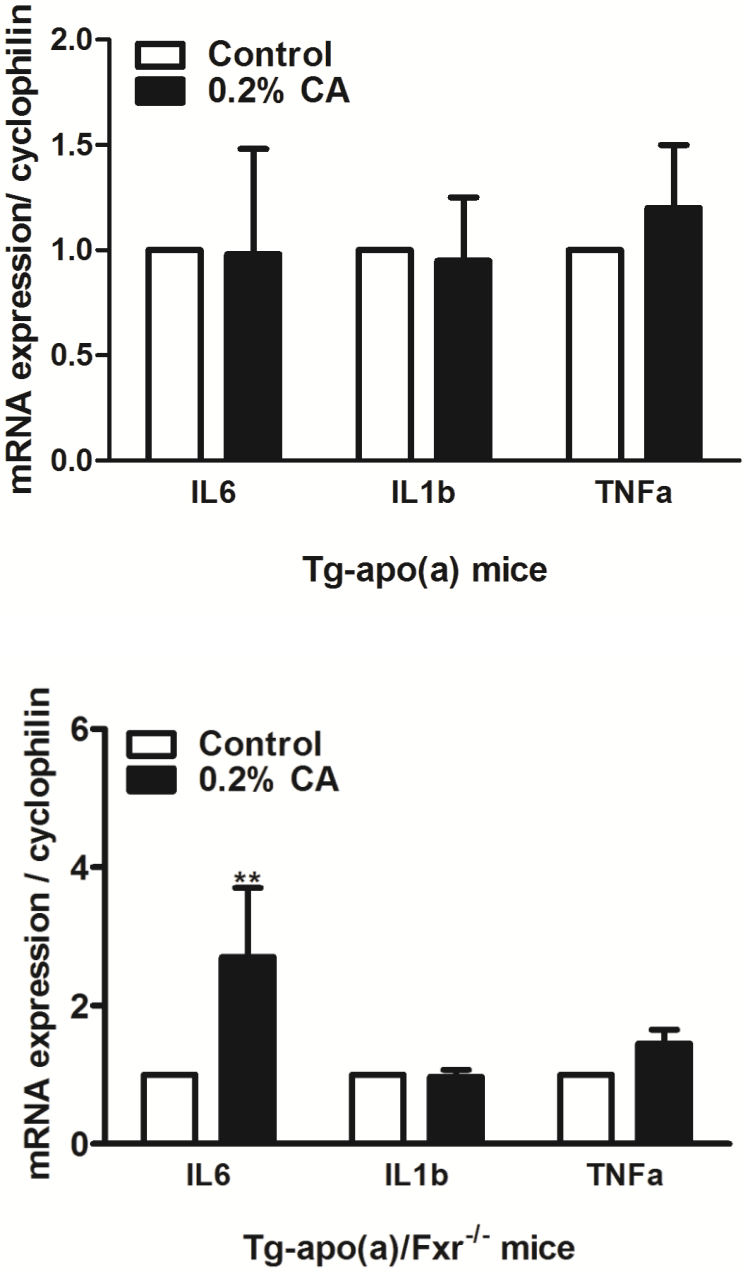


Figure 16: Influence of CA feeding on the expression of inflammatory genes.

*The mRNA levels of IL6, IL1β and TNFα were analyzed in tg-apo(a) and tg-apo(a)/FXR$^{-/-}$ mice (**A** and **B**) upon 0.2% CA supplementation by real-time quantitative PCR and normalized to cyclophilin. Values are reported as mean ± SEM. Data indicate the relative mRNA expression in comparison with chow fed control mice (** p ≤0.01, * p<0.05).*

4.5 The selective non steroidal FXR agonist GW4064 decreases apo(a) expression in vivo in transgenic apo(a) mice

Since bile acids are known to exhibit pleiotropic effects, and to further verify whether the observed effects of CA were mediated through hepatic or intestinal FXR activation, the tg-apo(a) mice were injected intraperitoneally with either vehicle or the non-steroidal FXR agonist GW4064 30 mg/kg. 16h treatment with GW4064 resulted in significantly decreased plasma apo(a) levels by 67% and hepatic apo(a) mRNA by 59%. Western blot analysis of liver homogenates demonstrate a strong reduction in apo(a) protein levels when compared to vehicle-treated controls (Figure 17).

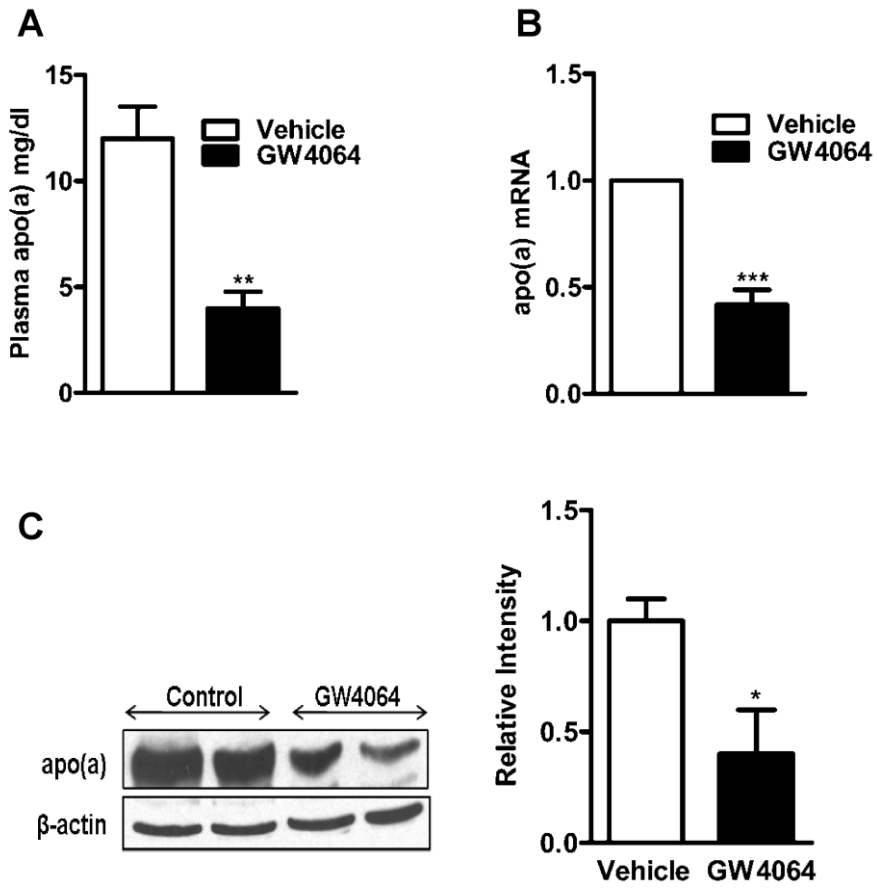


Figure 17: The synthetic FXR ligand GW4064 down-regulates apo(a) expression in tg-apo(a) mice.

Tg-apo(a) mice (n=3 per group) were injected i.p, 30mg/kg (wt/wt) GW4064 in 5% DMSO + corn oil. After 16h, (A) plasma levels of apo(a) was measured by DELFIA, expressed as mean ± SD. (B) Liver mRNA levels of apo(a) were analyzed by real-time

*quantitative PCR, normalized to cyclophilin and expressed relative to vehicle treated controls. Values are expressed as the mean ± SEM. (*** p≤0.001, ** p ≤0.01, * p<0.05). (C) Apo(a) protein levels were quantified in liver extracts by Western blotting, normalized to ß-actin and expressed as mean ± SD relative to controls.*

We next investigated the hepatic expression levels of well-established FXR target genes (Figure 18). Administration of GW4064 led to up-regulation of FXR and SHP in the liver. CYP7A1, CYP8B1 were significantly reduced. FGF15 mRNA in the ileum was up-regulated by 1.4 fold.

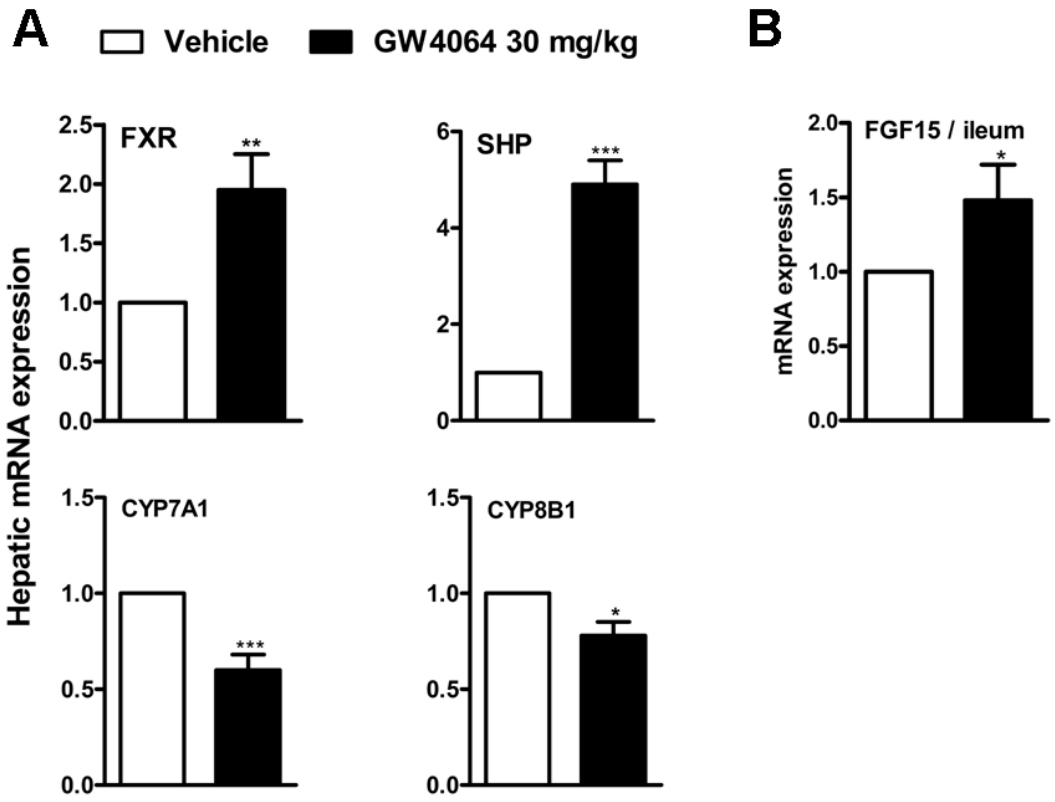
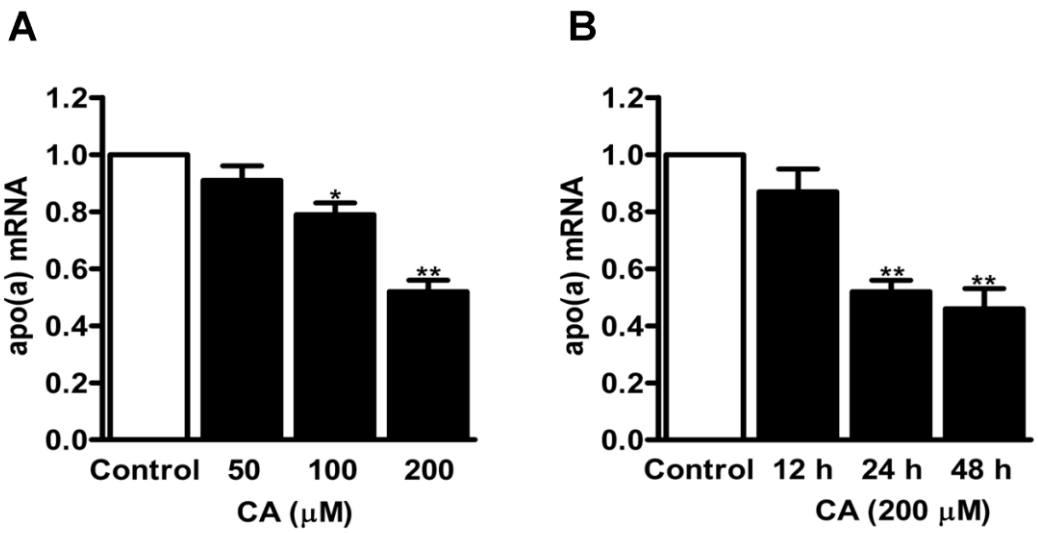


Figure 18: Gene expression profile in tg-apo(a) mice upon GW4064 treatment.

*Tg-apo(a) mice (n=3 per group) were injected i.p, 30mg/kg (wt/wt) GW4064 in 5% DMSO + corn oil. After 16h, (A) liver mRNA levels of FXR and its target genes like SHP, CYP7A1, CYP8B1 and (B) FGF15 in the ileum were analyzed by real-time quantitative PCR, normalized to cyclophilin and expressed relative to vehicle treated control mice. Values are expressed as the mean ± SEM. (*** p≤0.001, ** p ≤0.01, * p<0.05).*

4.6 Cholic acid and GW4064 decrease human apo(a) gene expression in primary hepatocytes

To further study the mechanism of the inhibitory effect of FXR on hepatic apo(a) expression, we then studied the influence of FXR agonists on *apo(a)* expression in mouse primary hepatocytes. For this purpose, primary hepatocytes were isolated from tg-apo(a) mice and incubated with different concentrations of the natural FXR ligand CA. Analysis of mRNA levels by real-time quantitative PCR revealed a significant dose-and time-dependent decrease in apo(a) transcript levels suggesting a transcriptional effect. Western blot analysis confirmed that this CA-mediated repression also occurs at the protein level (Figure 19). Cell viability assay was performed with trypan blue exclusion test and all concentrations of CA were well tolerated by the cells (data not shown).



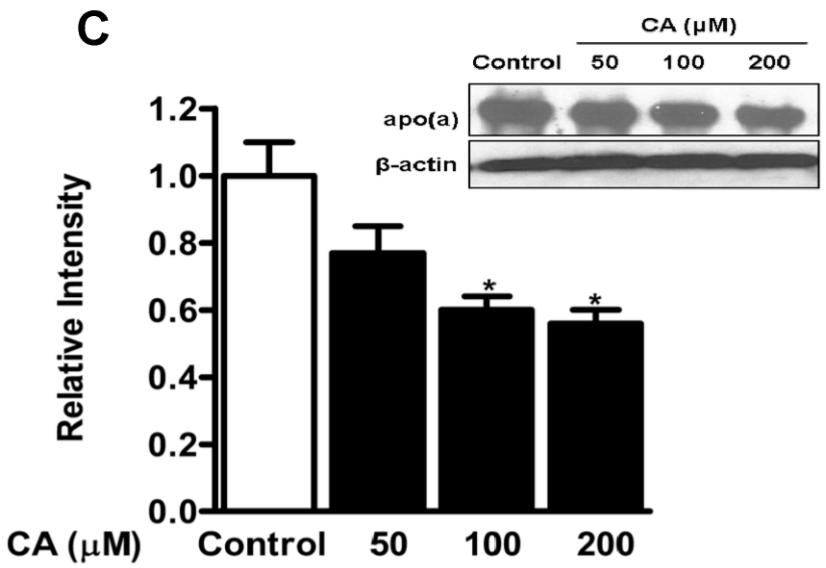


Figure 19: FXR natural agonist CA downregulate apo(a) gene expression in a dose and time-dependent manner in primary mouse hepatocytes.

*(**A**) Primary mouse hepatocytes from tg-apo(a) mice were incubated with increasing concentrations of CA (50,100 and 200 μM) or vehicle (control) for 24 h. mRNA levels of apo(a) were analyzed by real-time quantitative PCR. (B) Primary mouse hepatocytes were incubated with CA (200 μM) or vehicle for 12, 24, and 48 h. Apo(a) mRNA levels were measured by real-time quantitative PCR. Results represent the mean ± SEM of three independent experiments (** p ≤0.01, * p<0.05). (C) Western blotting and densitometric analyses of apo(a) and β-actin expression in whole cell lysates.*

Since bile acids may exert FXR-independent effects by activating other signal transduction pathways (Staudinger *et al.*, 2001),(Gupta *et al.*, 2001), we additionally tested the influence of the synthetic non-steroidal FXR agonist GW4064 on apo(a) gene expression. Treatment of primary hepatocytes with 5 μmol/L of GW4064 for 24 h resulted in a significant decrease of apo(a) mRNA and protein levels when compared with vehicle-treated control cells (Figure 20).

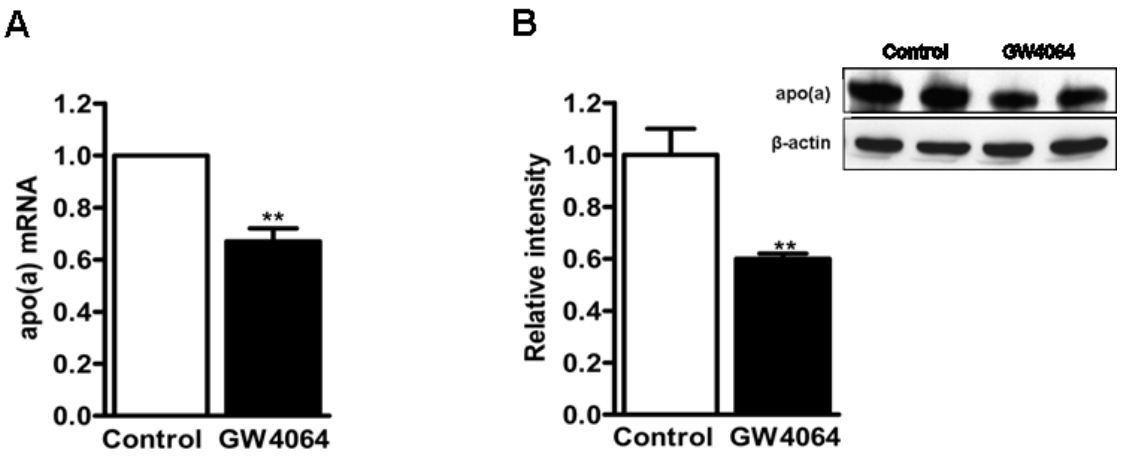


Figure 20: FXR synthetic agonist GW4064 downregulate apo(a) gene expression in primary mouse hepatocytes.

(A and B) Primary hepatocytes were treated with GW4064 (5 µM) for 24 h and analyzed for mRNA and protein expression levels of apo(a).

In addition, we measured the expression levels of control FXR target genes after treatment with CA and GW4064. We found increased SHP, markedly decreased CYP7A1 and APOA1 mRNA levels by both ligands (Figure 21).

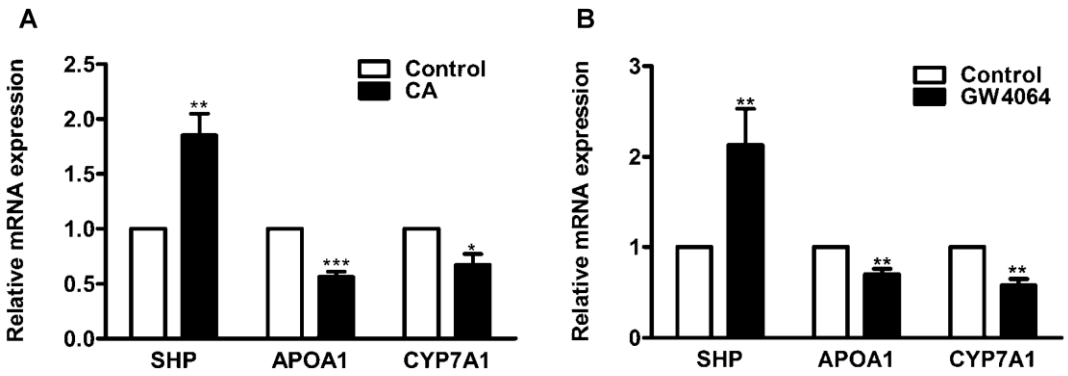


Figure 21: Gene expression profiling in primary mouse hepatocytes treated with natural and synthetic FXR agonists.

*Primary mouse hepatocytes from tg-apo(a) mice were incubated with (**A**) CA (200 µM) or (**B**) GW4064 (5 µmol/L) or vehicle (control) for 24 h. mRNA levels of well known FXR control genes were analyzed by real-time quantitative PCR normalized to cyclophilin and expressed relative to control treated cells. Values are expressed as the mean ± SEM. (*** p≤0.001, ** p ≤0.01, * p<0.05).*

Overall, these results demonstrate that both natural and synthetic FXR agonists down-regulate *via* a transcriptional mechanism human apo(a) expression in cultured mouse primary hepatocytes. In addition, the direct repression of apo(a) by FXR agonists in mouse primary hepatocytes confirmed that intestinal FGF signaling played a minor regulatory role.

4.7 Mapping of an FXR response element in the human apo(a) promoter

To provide direct evidence for the FXR-mediated inhibitory effect on apo(a) promoter and to further identify relevant promoter element(s), 2kb fragment of human apo(a) promoter (hapo(a) -1952/+52) was cloned in to pGL3-luciferase reporter plasmid (Figure 22A). In addition we generated a series of 5′ deletion constructs as shown in Figure 22B.

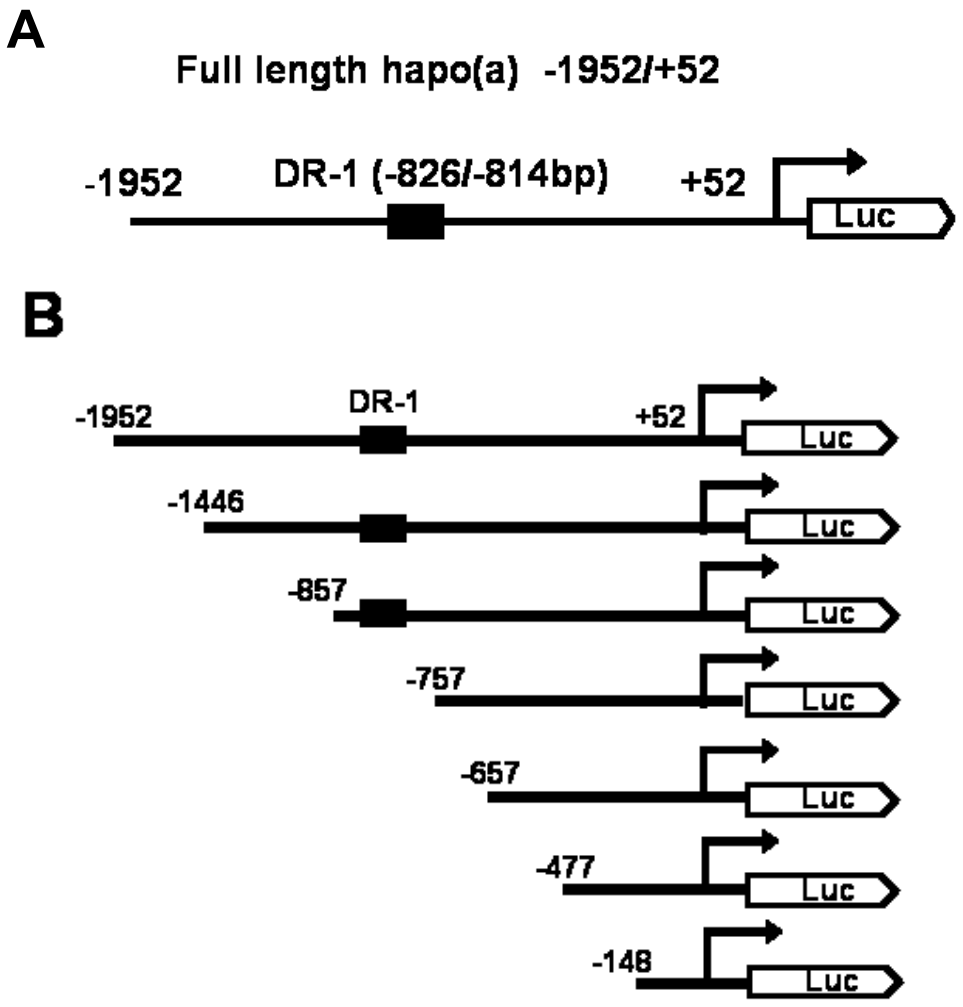
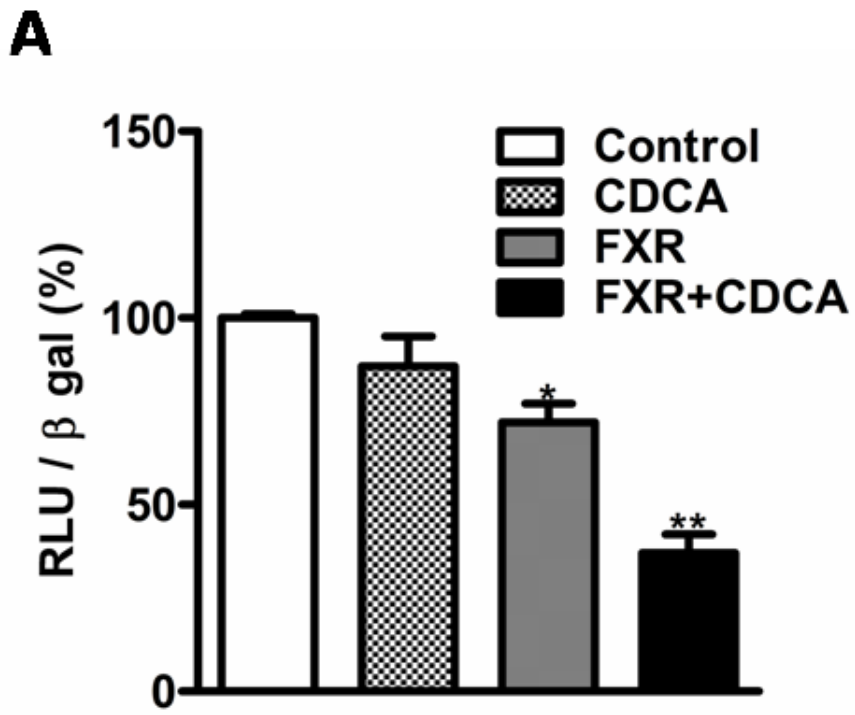


Figure 22: Scheme of full length hapo(a) -1952/+52 promoter and deletion constructs of the hapo(a) promoter used in luciferase reporter assays.

Transient transfections were performed in HepG2 cells with the hapo(a) -1952/+52 promoter construct in the absence or presence of FXR and FXR agonists. FXR alone resulted in a decreased promoter activity by 29% and this effect was further enhanced by the addition of CDCA (63%) (Figure 23A). Likewise, incubation with FXR and GW4064 also strongly repressed the activity of the hapo(a) -1952/+52 promoter by 57% (Figure 23B).

In the absence of FXR, the hapo(a) -1952/+52 promoter was inhibited ≤25% by CDCA or GW4064 alone. This decrease likely resulted from the activation of the endogenous FXR that is expressed in HepG2 cells (Claudel *et al.*, 2002).



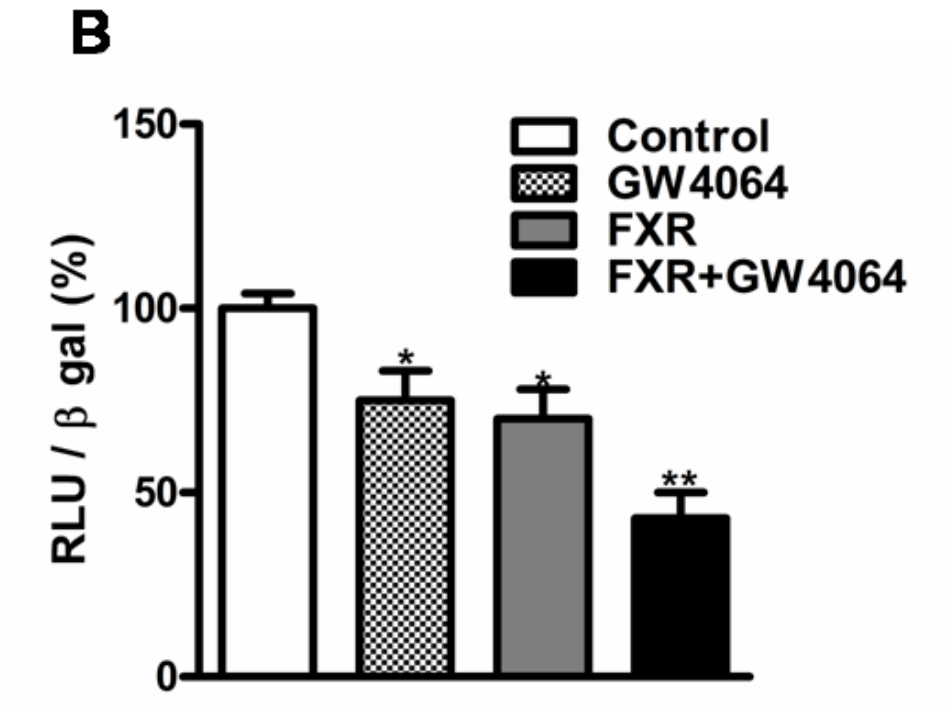


Figure 23: Bile acids and the nonsteroidal FXR agonist GW4064 down-regulate human apo(a) promoter activity via FXR.

*(**A** and **B**) HepG2 cells were transfected with the hapo(a) -1952/+52 promoter reporter plasmid (150 ng) in the presence of either the pcDNA3 (control) or FXR expression vector (150 ng). Cells were subsequently treated with CDCA (100 µM), GW4064 (500 nM) or vehicle for 36 h. Values are expressed as percentage of control, normalized to internal control β-galactosidase activity. Transfections were performed in triplicates, and each experiment was repeated at least 3 times. Data are presented as mean ± SD (** p ≤0.01, * p<0.05).*

To avoid endogenous FXR-mediated feedback inhibition, transient transfection experiments were performed in COS-7 cells, a non-hepatic cell line. Transfection of COS-7 cells in the absence or presence of FXR repressed the hapo(a) -1952/+52 promoter activity by 24% an effect that was significantly enhanced by CDCA (43%) (Figure 24).

These experiments demonstrated that ectopic expression of FXR and a physiological concentration of CDCA are required to repress the apo(a) promoter activity in non-hepatic cells.

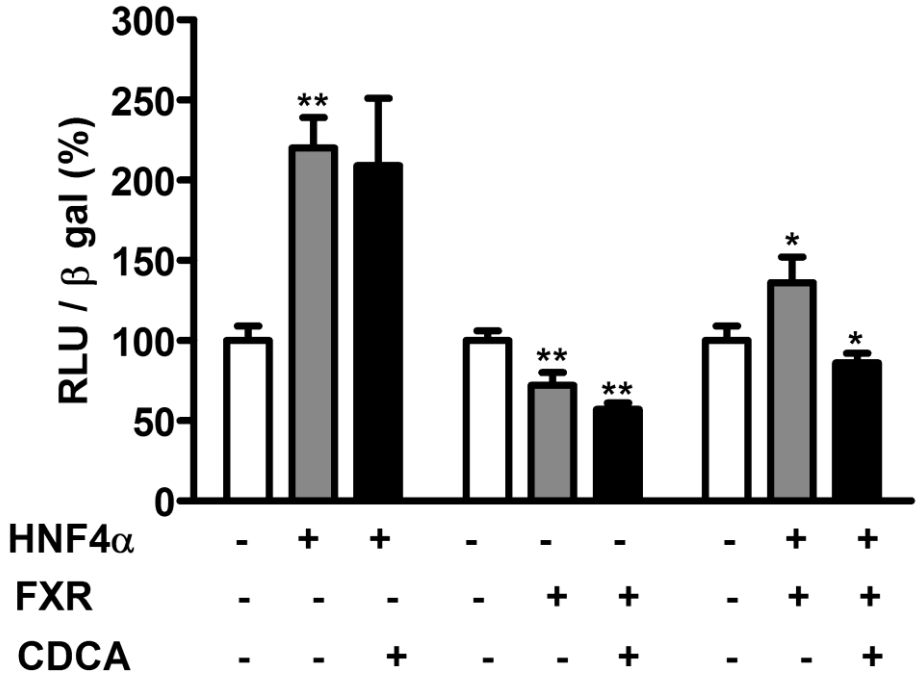


Figure 24: Effects of FXR and HNF4α overexpression on hapo(a) promoter activity in COS-7 cells.

*HNF4α mediated transactivation of the human apo(a) promoter is inhibited by cotransfection with FXR in a non hepatic cell line COS-7. COS-7 cells were co-transfected with the hapo(a)-1952/+52 promoter reporter plasmid (150 ng), expression vectors for HNF4α, FXR or HNF4α and FXR in the absence or presence of CDCA. Luciferase activity was measured, the values were normalized to internal control β-galactosidase and expressed in percentage. Values shown are mean ± SD of 3 replicates (** p ≤0.01, * p<0.05).*

Since SHP was induced by CA treatment *in vivo* and *in vitro*, we subsequently studied the apo(a) promoter activity upon co-transfection of cells with increasing concentrations of a SHP expression plasmid. Surprisingly, SHP did not lower apo(a) promoter activity but further enhanced it (Figure 25).

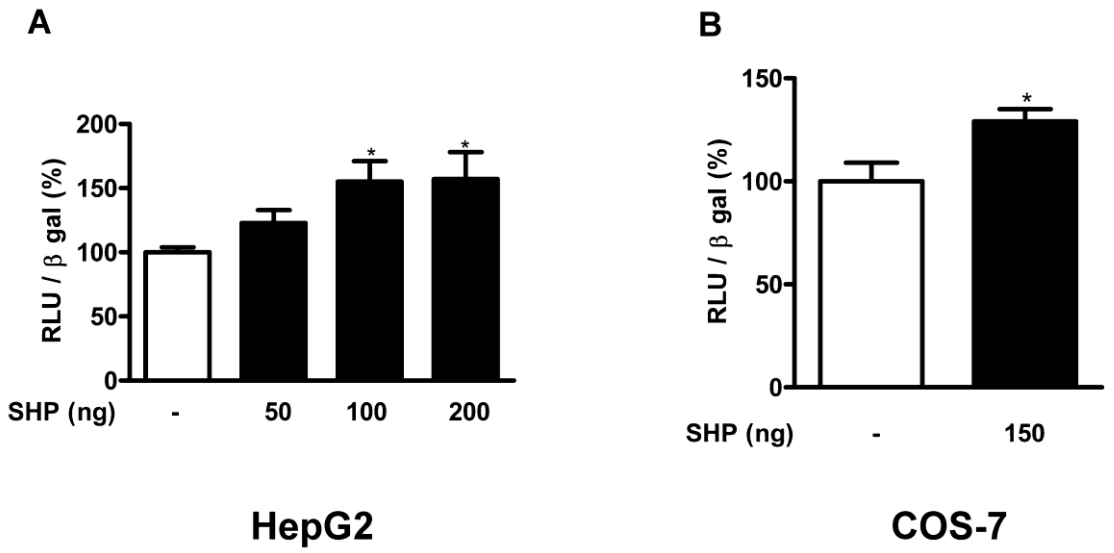


Figure 25: Effect of SHP on human apo(a) promoter activity.

HepG2 and COS-7 cells (A and B) were transfected with the full length hapo(a) -1952/+52 promoter reporter plasmid (150 ng) in the presence of either empty pcDNA3 or SHP expression vector Values are expressed as percentage of control, normalized to internal control β-galactosidase activity. Data are presented as mean ± SD (p<0.05).*

Taken together, these results showed that FXR can regulate apo(a) promoter activity in a direct and SHP independent manner.

Next, to identify promoter elements responsible for the observed effects of FXR, HepG2 cells were transfected with 5' deletion constructs of the hapo(a) promoter in the absence or presence of FXR and/or CDCA. Reduced promoter activities were noted for both the -1446 and -857 constructs (Figure 26). However, the repression was relieved for -757, -657, -477 and -148 promoter constructs indicating that the region between –857 to –757bp of the human apo(a) promoter contains a potential negative FXRE which might be responsible for the observed bile acid response.

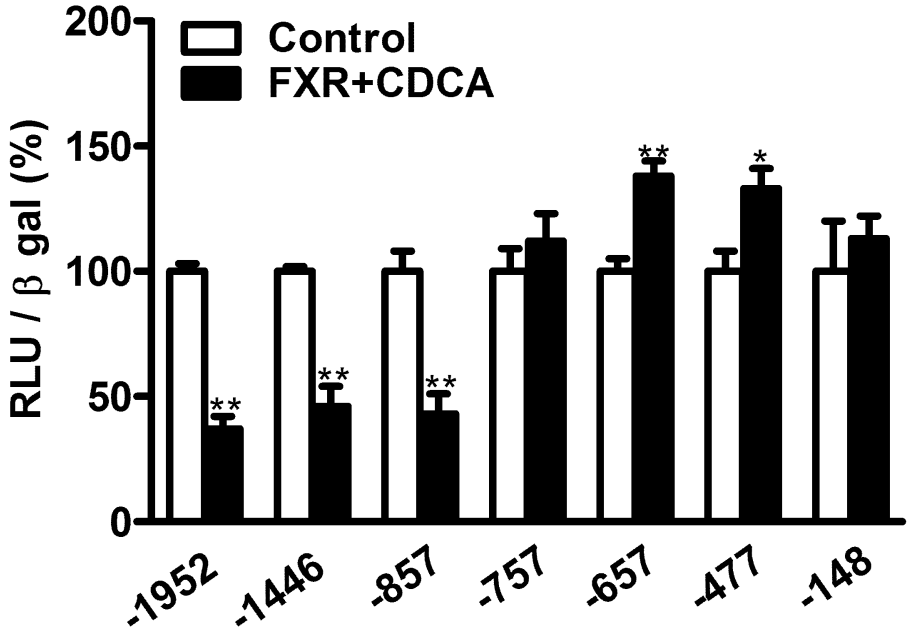


Figure 26: promoter elements responsive to FXR

*HepG2 cells were transfected with the indicated hapo(a) promoter reporter plasmids (150 ng) in the presence of either the pcDNA3 empty or FXR containing expression vector (150 ng). Cells were then treated with CDCA (100 µM), or vehicle for 36 h. Values are expressed as percentage of control, normalized to internal control β-galactosidase activity. Data are presented as mean ± SD (** p ≤0.01).*

Notably, *in silico* Matinspector promoter analysis (Cartharius *et al.*, 2005) and NUBIScan algorithm (Podvinec *et al.*, 2002) suggested the presence of a DR-1 element located between nucleotides -826 and -814. Previous studies have already shown that DR-1 element can function as FXRE (Anisfeld *et al.*, 2003; Claudel *et al.*, 2003; Laffitte *et al.*, 2000).

To test whether this DR-1 site could mediate FXR dependent repression of apo(a) promoter, we introduced mutations in the context of the full length hapo(a) -1952/+52 promoter (Wt) and generated two mutant constructs (M1 and M2) as shown in Figure 27. Mutation (M2) of this site completely abolished the FXR mediated repression of apo(a) promoter activity (Figure 28), indicating the binding of FXR to the second half site of the DR-1 element.

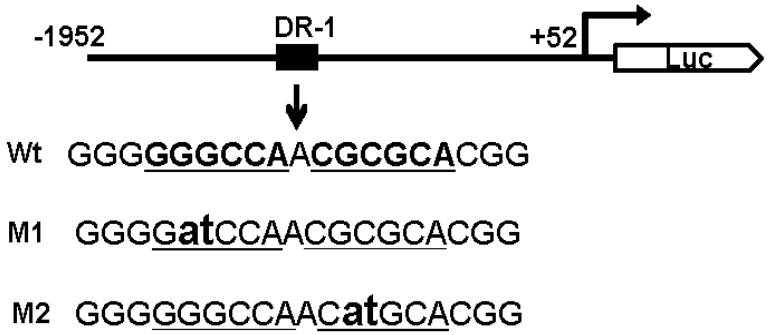


Figure 27: Scheme showing wild type and mutant sequences.

Mutations are indicated in bold small letters.

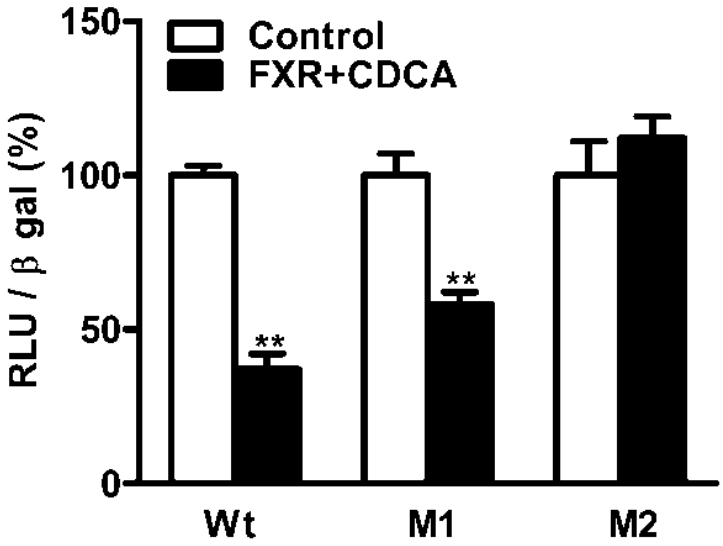


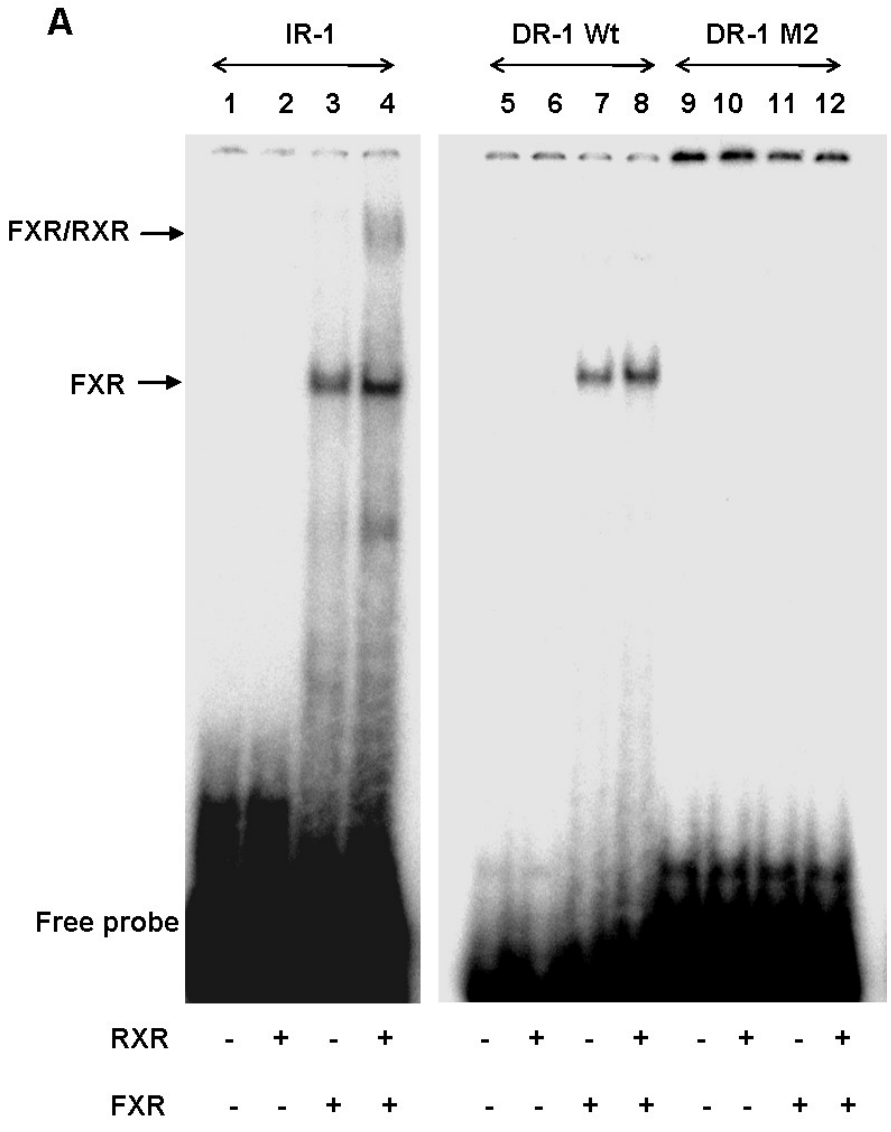
Figure 28: Mutational analysis of hapo(a) promoter.

*HepG2 cells were transfected with the wild type and mutant (M1, M2) hapo(a) promoter reporter plasmids in the presence of either the pcDNA3 or FXR expression vector (150 ng). Cells were then treated with CDCA (100 µM), or vehicle for 36 h. Values are normalized to β-galactosidase activity. Data are presented as mean ± SD (** p ≤0.01, * p<0.05).*

Taken together, these results suggest that the DR-1 site, located between nucleotides -826 and -814 is a negative response element *via* which FXR repress human apo(a) promoter activity.

4.8 FXR binds to the DR-1 site of the apo(a) promoter in electrophoretic mobility shift assay

To provide additional evidence that the DR-1 element at -826bp region of the human apo(a) promoter can function as an FXRE, gel shift assays were performed. Consensus IR-1 probe was used as a positive control. FXR bound the labeled IR-1 probe both in the absence (**Error! Reference source not found.**A, lane 3) and presence (**Error! Reference source not found.**A, lane 4) of RXR. In contrast, FXR bound as a monomer to the radiolabeled probe containing a wild type DR-1 element (DR-1 Wt) (**Error! Reference source not found.**A, *lanes 7 and 8*), but not to the probe carrying the mutated DR-1 element (DR-1 M2) (**Error! Reference source not found.**A, *lanes 11 and 12*).



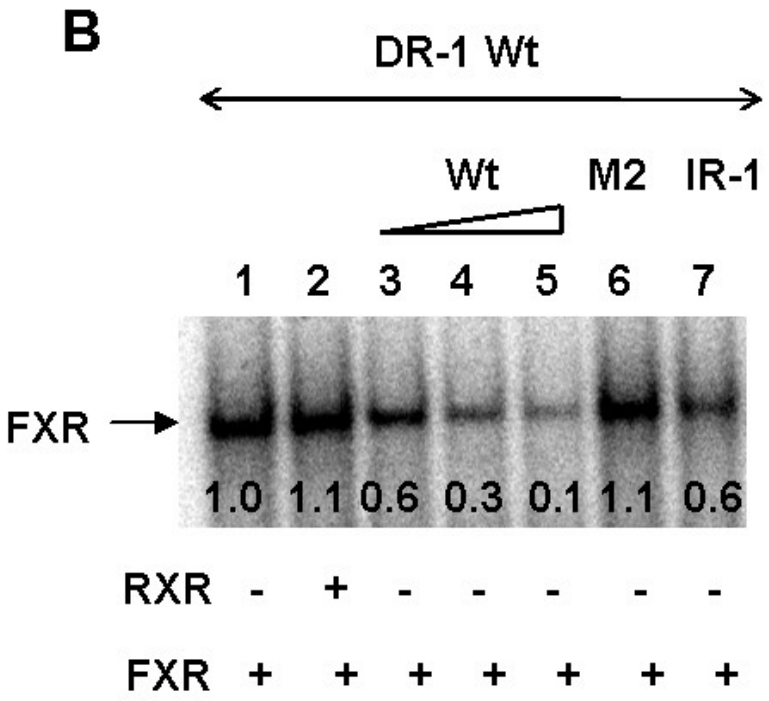


Figure 29: FXR binds to the DR-1 element of human apo(a) *promoter as a monomer.*

*(**A**) Electrophoretic mobility shift assays (EMSA) were performed with radiolabeled IR-1 consensus FXRE (lanes 1-4), DR-1 Wt (lanes 5-8) and DR-1 M2 (lanes 9-12) probes using in vitro transcribed/translated RXR (lanes 2, 6 and 10), FXR (lanes 3, 7 and 11), both RXR and FXR (lanes 4, 8 and 12), or unprogrammed reticulocyte lysate (lanes 1, 5 and 9) as indicated. (**B**) Competition EMSAs on radiolabeled DR-1 Wt probe were performed by adding 50- fold, 100- fold, 200- fold molar excess of the indicated cold DR-1 Wt (lanes 3, 4 and 5) and 50- fold molar excess of cold DR-1 M2 (lane 6) and IR-1 (lane 7) probes.*

Formation of FXR-DNA complex was specifically competed by cold DR-1 Wt probe, whereas the DR-1 M2 probe did not compete. Binding of FXR to the DR-1 Wt probe was also competed by a cold IR-1 probe (**Error! Reference source not found.**B, *lane* 7), notably the cold IR-1 and cold DR-1 Wt probes competed with a similar efficiency for the labeled DR-1 Wt oligo.

These results indicated that FXR binds specifically to the DR-1 site of human apo(a) promoter.

4.9 FXR Compete for HNF4α Binding to the DR-1 element

DR-1 elements have been shown to function as HNF4α response elements (Fraser *et al.*, 1998). In order to investigate whether HNF4α could regulate apo(a) gene expression, we examined whether over-expression of HNF4α induces endogenous expression of the hapo(a) gene in cultured primary hepatocytes. As shown in Figure 30A, adenovirus-mediated over-expression of HNF4α in mouse primary hepatocytes from tg-apo(a) mice dose-dependently induced the expression of apo(a) mRNA levels compared to LacZ-transfected cells.

Next, we studied the effect of HNF4α overexpression on the activity of the hapo(a) -1952/+52 promoter. As shown in Figure 30B, overexpression of HNF4α in HepG2 cells dose-dependently transactivated hapo(a) promoter. However, additional co-transfection with FXR and/or CDCA treatment abolished the HNF4α mediated transactivation (Figure 30C). This effect might be due to the occupancy of HNF4α response element (DR-1) by FXR.

HNF4α mediated trans-activation of hapo(a) -1952/+52 promoter was also observed in the non-hepatic cell line COS-7 that does not express FXR nor HNF4α. Co-transfection with FXR alone, FXR and CDCA significantly inhibited HNF4α transactivation (Figure 24) suggesting that FXR competes with HNF4α for the DR-1 binding motif.

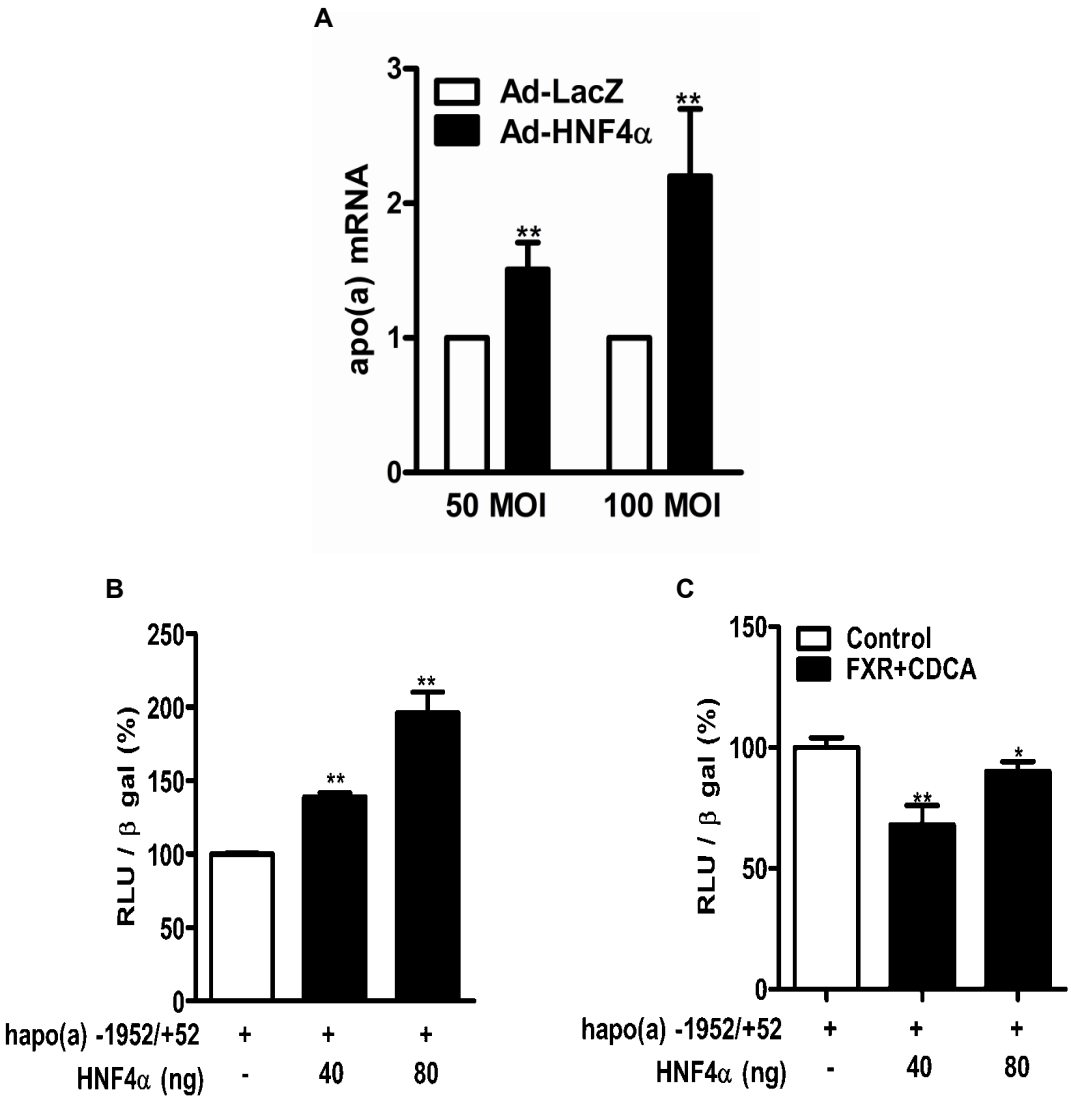


Figure 30: Effects of hepatocyte nuclear factor HNF4α overexpression on human apo(a).

*(**A**) Primary Hepatocytes from transgenic apo(a) mice were infected with either adenovirus coding for ß-galactosidase (Ad-LacZ) or human HNF4α (Ad-HNF4α) for 4 h and then changed the medium. After 24 h, total RNA was extracted, and gene expression was measured by real-time quantitative PCR. These data are presented as mean ± SEM. (** p ≤0.01). (**B**) HepG2 cells were transfected with the hapo(a) -1952/+52 promoter reporter plasmid (150 ng) in the presence of either the pSG5 empty or increasing amounts of HNF4α expression vector (40, 80 ng). (**C**) Competition assay between FXR and HNF4α. HepG2 cells were transfected with the human apo(a) -1952/+52 promoter reporter plasmid in the presence or absence of FXR and HNF4α. Cells were then treated with CDCA (100 µM), or vehicle for 36 h. Values are*

*normalized to internal control β-galactosidase and expressed in percentage. Values represent mean ± SD (** p ≤0.01, * p<0.05).*

We then performed a mobility shift assay to check whether HNF4α binds to the DR-1 element at -826bp region of the human apo(a) promoter. HNF4α bound to the radiolabeled probe containing wild type DR-1 element (DR-1 Wt) and the protein-DNA complex was specifically competed by cold unlabelled Wt probe (Figure 31).

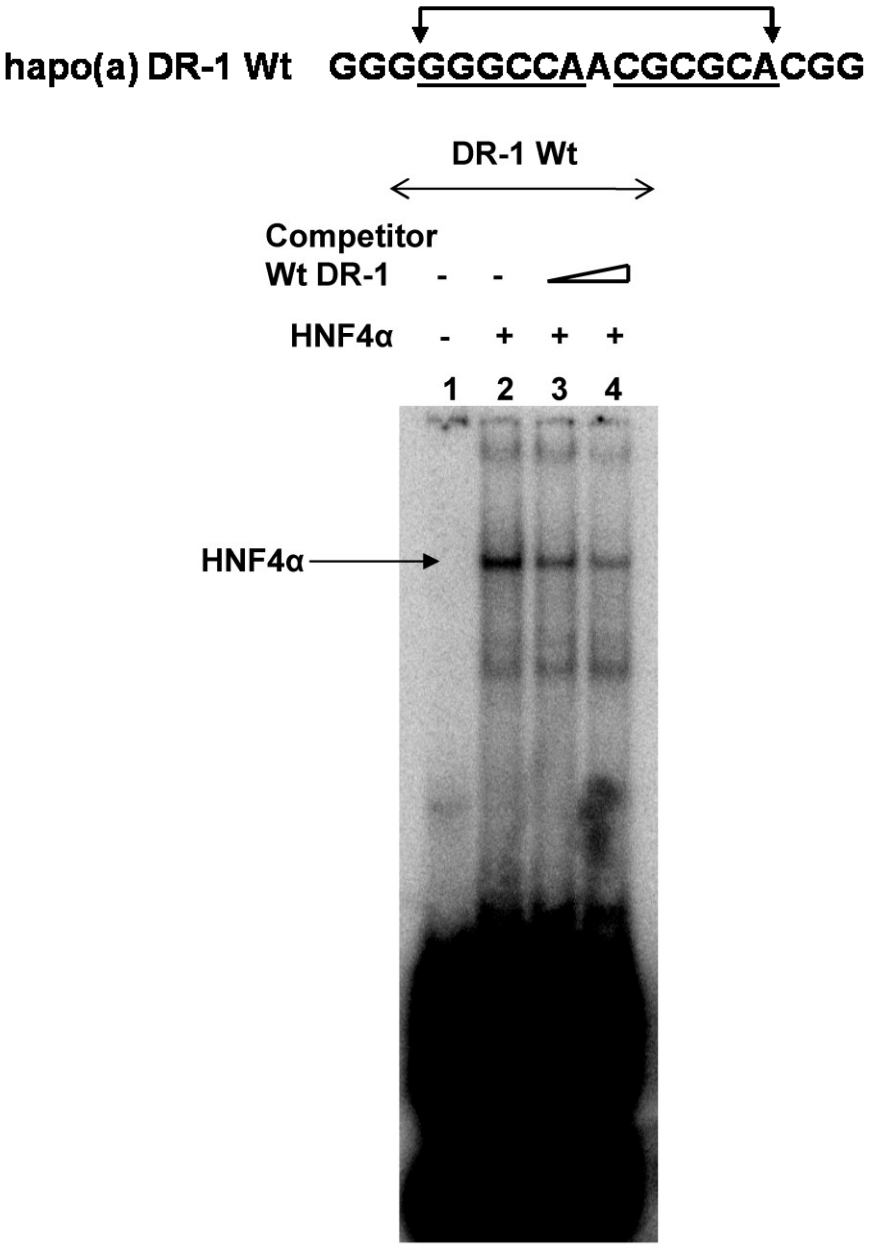


Figure 31: HNF4α binds to the DR-1 motif in the human apo(a) promoter.

EMSAs were performed with end labeled DR-1 Wt probe using in vitro transcribed/translated HNF4α (lanes 2). Competition analysis was performed by adding 50- fold (lane 3) and 100- fold (lane 4) molar excess of the indicated cold DR-1 Wt probe.

Taken together, these results suggest that this response element at -826bp might be occupied by HNF4α at the basal level, whereas bile acid activation leads to a switch of occupancy of this site by FXR.

To further confirm the interaction of FXR with the DR-1 element in the apo(a) promoter, *in vivo* chromatin immunoprecipitation experiments were performed with liver tissue isolated from tg-apo(a) mice fed for 24 h with normal chow or with chow containing 0.2%CA (Figure 32).

In the control group, antibodies against HNF4α precipitated DNA encompassing the DR-1 element (-826/-814bp region) in the apo(a) promoter. By contrast, 0.2% CA feeding led to occupancy of this response element by FXR alone without RXR. As a negative control, an equivalent amount of chromatin precipitated with a non-relevant anti-IgG antibody did not result in any signal. The same DNA samples were PCR amplified by using primers covering the distal region of the apo(a) promoter, but no signal was observed, where as 0.2% CA feeding increased the occupancy of both FXR and RXR to the SHP promoter.

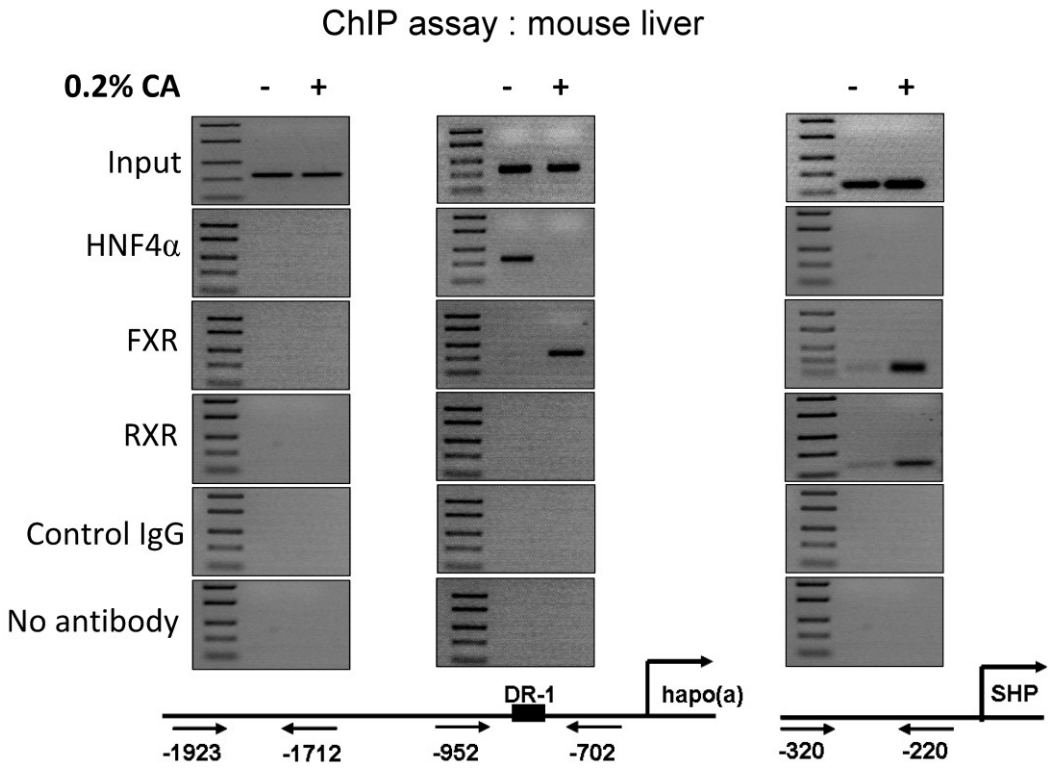


Figure 32: Competitive binding between HNF4α and FXR to the DR-1 element on the apo(a) promoter.

Tg-apo(a) mice were fed normal chow or 0.2% CA chow for 24 h, and livers were collected for ChIP analyses. For ChIP assay, sheared chromatin was immunoprecipitated with the indicated antibodies. The final DNA extractions were amplified by PCR using primer pairs covering the distal region and the DR-1 motif of the apo(a) gene promoter. As a positive control for FXR/RXR binding SHP gene promoter was amplified by PCR. DNA from samples before immunoprecipitation was used as input.

Taken together, all these results proved that the DR-1 element at the -826/-814bp region of human apo(a) promoter could mediate the FXR repression of apo(a) transcription by allowing a competition between FXR and HNF4α (Figure 33).

4.10 Model for HNF4α and FXR counter regulation of apo(a)

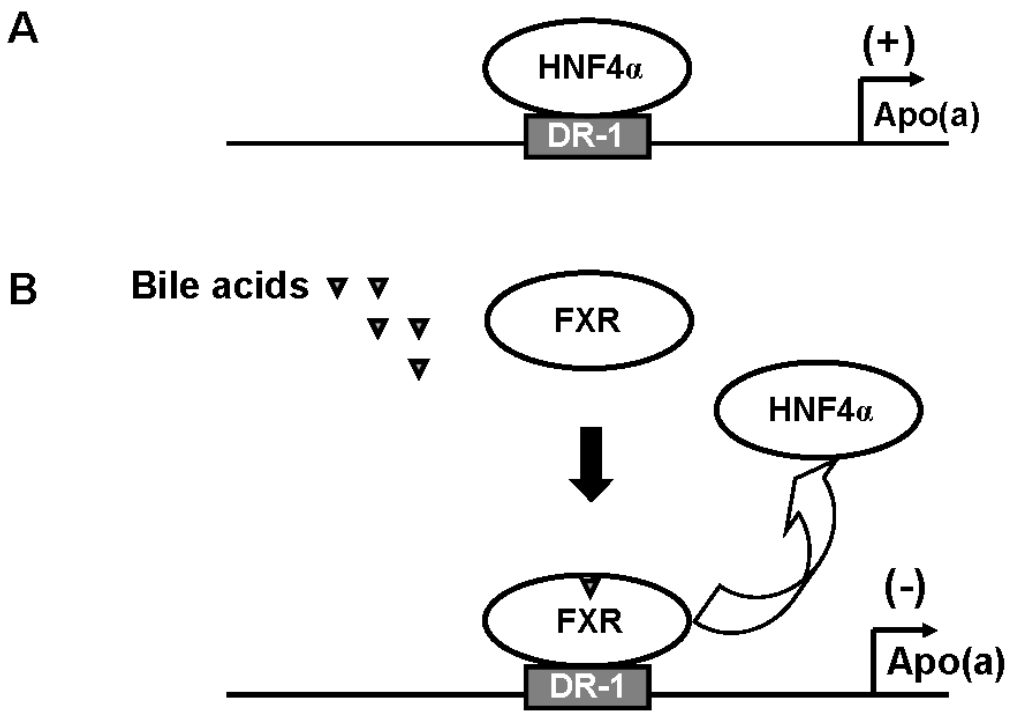


Figure 33: Proposed model for HNF4α and FXR counter regulation of apo(a) promoter activity.

*(**A**) In basal conditions, HNF4α is bound to the DR-1 site and promotes apo(a) transcription by interacting with co-factors enhancing the polymerase II activity.*

*(**B**) During cholestasis or FXR activation, FXR displaced HNF4α from the DR-1, thereby lowering the polymerase II activity and in turn apo(a) gene expression.*

5 Discussion

Meta-analyses from prospective and epidemiological studies demonstrated an association of elevated plasma Lp(a) levels with an increased risk for ischemic heart diseases and stroke (Clarke *et al.*, 2009; Erqou *et al.*, 2010; Kamstrup *et al.*, 2009; Lanktree *et al.*, 2010; Tregouet *et al.*, 2009). Thus Lp(a) is causally associated with an increased risk for myocardial infarction and reported to increase 2.3 fold the likelihood for major adverse cardiovascular events when plasma Lp(a) levels exceed 30 mg/dl (Kamstrup *et al.*, 2009),(Nicholls *et al.*, 2010). Therefore, unraveling molecular and pharmacological factors reducing Lp(a) constitute a novel goal with important therapeutic and pharmacological implications for treatment of humans at increased athero-thrombotic risk.

In this study, we identified the bile acid activated receptor FXR as a major repressor of Lp(a) levels in patients and mice with elevated bile acid levels. Notably, therapeutic normalization of bile acid concentrations, lead to increased plasma Lp(a) levels. Similarly, bile duct ligation in transgenic apo(a) mice virtually abrogated apo(a) expression. Therefore, we hypothesized that high intra hepatic bile acid could suppress apo(a) expression. In order to firmly demonstrate that bile acid activated FXR was repressing apo(a) expression in a more physiological condition, we fed transgenic apo(a) and FXR-deficient transgenic apo(a) mice with bile acids. Bile acid feeding lowered apo(a) plasma concentration as well as gene expression and protein levels in transgenic apo(a) mice, an effect abolished in FXR-deficient transgenic apo(a) mice. Furthermore, *in vitro* activation of FXR by bile acids or a non steroidal FXR agonist lowered apo(a) gene expression, in a time and dose dependent manner due to a transcriptional mechanisms.

In normal individuals, plasma Lp(a) levels have been shown to correlate significantly with the synthesis rate of apo(a) (Krempler *et al.*, 1980; Rader *et al.*, 1994) and appear to be minimally affected by its catabolism. Thus, pharmacological FXR activation could constitute a novel and promising approach to treat hyper-Lp(a) individuals and significantly reduce adverse coronary events in a high risk population. Interestingly, novel FXR agonists were shown already to display anti- atherosclerotic effects in mice (Miyazaki-

Anzai *et al.*, 2010) and to normalize dyslipidemia (Evans *et al.*, 2009) in rodent models lacking apo(a) expression. It will therefore be of interest to measure Lp(a) in ongoing human clinical trials using FXR agonist such as INT-747. Bile acid binding resins, probably by depleting FXR ligand and therefore lowering FXR activation, were shown to reduce the incidence of coronary disease (1984a) due to the lowering of cholesterol and glucosuria (1984b). Novel resins with higher bile acid affinity, specificity and binding capacity are currently available and constitute a safe therapeutic option either in combination with HMGCoA reductase inhibitors (statins) or in statin resistant patients. However, our results allude that resins and intestinal bile acid uptake inhibitors should be carefully monitored in a patient population with dyslipidemia and potentially elevated Lp(a). Conversely, introducing Lp(a) as a screening parameter could lead to improved resins without side effects and being even more cardiovascular protective.

Here we show that FXR directly repress apo(a) gene promoter activity by binding to a DR-1 site shared with HNF4α leading to suppression of transcription. Several molecular regulators were found to bind to and modulate the promoter region of the apo(a) gene. These include binding sites for HNF1, HNF4α, RXR, LINE amongst others (Hixson *et al.*, 1996; Puckey *et al.*, 2003). FXR is highly expressed in the liver and was found to bind to IR-1 response elements in promoters as a heterodimer with RXR, as well to various DR-1 to DR-5 (Laffitte *et al.*, 2000), thereby trans-activating cognate target genes. In addition, FXR can bind monomeric response elements and hence directly repress gene transcription (Barbier *et al.*, 2003; Claudel *et al.*, 2002; Laffitte *et al.*, 2000; Lu *et al.*, 2005). Recently, the location and sequence of FXRE was systematically studied *via* ChIP and sequencing (Chong *et al.*, 2010). In this work, Chong *et al.,* identified 1656 binding sites, including 10% located in the proximal 2kb of the promoter. Moreover, up to 25% of these FXREs were not classical IR-1. In our study, by combining, reporter assays, site directed mutagenesis, EMSA and ChIP, we identified unambiguously a DR-1 located at -826bp upstream of the transcription start site as a new negative FXR response element in the promoter of apo(a). This new site is therefore compatible with the architecture of a *bona fide* FXR response element. Since Chong *et al*., used a

mouse liver, not expressing apo(a) chromatin- enriched material, our new response element could not be found in their database. Thus we for the first time provide evidence that the DR-1 located at the -826 to -814bp region is found to be bound and activated by HNF4α as shown by transfection, EMSA and ChIP. In addition, HNF4α was competitively displaced by FXR as demonstrated in Figure 32. HNF4α is well known to be involved in lipid, glucose and bile acid homeostasis (Hayhurst *et al.*, 2001). A competition between FXR and HNF4α was previously found in the promoter of apoCIII (Anisfeld *et al.*, 2003; Claudel *et al.*, 2003; Laffitte *et al.*, 2000). It is therefore tempting to speculate that the balance between FXR and HNF4α binding on gene promoters could coordinate a network of genes involved in lipid homeostasis (Figure 33). The precise mechanism for this suppression including the events involved in a non-productive FXR binding to a response element requires additional studies.

FXR also trans-activates the mouse FGF15 a gene that is expressed almost exclusively in the terminal ileum, and its human ortholog, FGF19, expressed in the small intestine as well as in the liver. FGF-15/19 signals from intestine to the liver to repress the transcription of key enzymes of bile acid biosynthesis (Holt *et al.*, 2003; Inagaki *et al.*, 2005). FXR activation efficiently repressed apo(a) *in vitro* in primary mouse hepatocytes that do not express FGF15 indicating that FXR can regulate the apo(a) gene in FGF15/19 independent manner. Further studies will be required to clarify a possible additional role of FGF15/19 in apo(a) gene repression.

Another pathway described for FXR indicates that it can indirectly modulate gene expression *via* the induction of the small heterodimer partner (SHP; NR0B2) in the liver (Goodwin *et al.*, 2000). Although SHP is a transcriptional repressor, it has no DNA binding motif (Seol *et al.*, 1996), but interacts with several nuclear receptors, such as LRH-1 or HNF4α thereby interfering with gene transcription. Recently, the SHP/LRH-1/CYP7A1 signaling pathway was disproved and LRH-1 was identified as a master regulator of CYP8B1 (Mataki *et al.*, 2007),(Lee *et al.*, 2008). Since SHP is able to interact *in vitro* with multiple partners, the identification of the actual SHP targets is still an open quest. Our transgenic apo(a) mice fed with cholic acid or primary hepatocytes incubated

with FXR activators were found to have more SHP and less apo(a) gene expression. We therefore wondered whether SHP induction could repress apo(a). However, transfection experiments using a dose response of SHP showed that SHP did not repress and instead increased the apo(a) promoter activity in HepG2 as well as in COS-7 cells that do not express FXR nor HNF4α (Figure 25**)**. Conversely, FXR directly repressed apo(a) promoter activity by binding to a DR-1 also recognized by HNF4α. This was verified by ChIP assay which impressively confirmed that the DR-1 element at -826/-814bp region of apo(a) promoter is occupied by HNF4α, whereas CA-activation leads to a switch of occupancy of the site by FXR (Figure 32). Taken together, our data suggest that SHP does not regulate apo(a) promoter, in contrast to FXR.

In view of the present results, FXR agonists could constitute a new therapeutic avenue to treat hyper Lp(a) states and may be useful in the treatment of atherosclerotic disease and myocardial infarction. In addition, these results suggest that present and future FXR partial agonists, also called BARM (Bile Acid Receptor Modulator), have to be monitored for possible adverse effects on plasma Lp(a) levels in human clinical trials.

6 Number of Figures

7 Number of Tables

8 References

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