The role of vitamin A in bile acid synthesis and transport and the relevance for cholestatic liver disease
Hoeke, Martijn Oscar

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hoeke, M. O. (2013). The role of vitamin A in bile acid synthesis and transport and the relevance for cholestatic liver disease. Groningen: s.n.
Chapter 6

General Discussion

Martijn O. Hoeke and Klaas Nico Faber

Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, University of Groningen, The Netherlands
The research presented in this thesis sheds new light on the interconnection between bile salt homeostasis and vitamin A homeostasis. Well established are the fat-emulsifying properties of bile salts that are required for the effective absorption of fat-soluble vitamins, including vitamin A. Moreover, the liver is the central organ in controlling vitamin A homeostasis, where 80% of the total body content of vitamin A is stored in lipid droplets in hepatic stellate cells. Work described in this thesis, however, shows that vitamin A directly affects transcription of genes that control bile salt synthesis and transport. In addition, vitamin A affects the protein level and/or function of the corresponding gene products. Vitamin A deficiency in furthermore healthy rats leads to mild cholestasis. When exposed to obstructive cholestasis, these vitamin A deficient (VAD) rats develop severe liver damage that quickly progresses to liver failure. Taken together, these studies show that vitamin A is important to maintain normal bile homeostasis and prevents excessive liver damage during obstructive cholestasis. Thus, the vitamin A status deserves more clinical attention in the treatment of liver diseases.

Hypovitaminosis A
Vitamin A deficiency may develop from low dietary intake, insufficient intestinal uptake or increased release from the liver. Vitamin A deficiency is common in developing countries especially in children. In developed countries, vitamin A deficiency is a condition that may develop in patients with chronic disease of the digestive tracts, such as inflammatory bowel disease, biliary atresia or chronic hepatitis. One of the first clinical symptoms of vitamin A deficiency is night blindness, which is due to impaired regeneration of rhodopsin in the rod cells of the eye and may progress to permanent damage to the eye and ultimately blindness. At earlier, subclinical stages, vitamin A deficiency may already exert pathological effects as it is crucial for optimal functioning of the immune system. For instance, hypovitaminosis A is associated with a reduced number of lymphocytes and natural killer cells and a reduced antigen-specific immunoglobulin response. In early age, vitamin A deficiency is associated with increased risk of HIV infection from mother to child and an increased risk of mortality as a result of diarrhea (1). On the other hand, low vitamin A levels were shown to relief symptoms of Asthma (2).

In general, the symptoms of hypovitaminosis can be treated with vitamin A supplementation and night vision can be restored within 8 days (3). In developed countries, hypovitaminosis A is not a common problem in otherwise healthy people. Vitamin A reserves are high in humans and up to 1.5 years may be required before healthy volunteers develop the earliest manifestations of deficiency when they take a vitamin A-free diet (4). However, newborns have limited reserves of vitamin A. In combination with cholestasis these infants are in a precarious nutritional state, with limited ability to build up stores even though vitamin A is present in their diet (5). Therefore, infants suffering from cholestasis are at high risk of developing vitamin A deficiency.
Hypervitaminosis A

Hypervitaminosis A is typically the result of self-medication. Following excessive intake the liver becomes saturated with retinylesters, which eventually spill over into the general circulation. The plasma retinyl ester levels in hypervitaminotic humans can be as high as 67% of the total plasma vitamin A (6), while in healthy individuals only 5.5% of the total vitamin pool in blood consists of retinyl esters (7). Excessive intake of carrots and/or other vitamin A-rich vegetables may lead to a condition called carotenemia that is characterized by high serum levels of carotene (pro-vitamin A) and causes the skin to turn yellow. This is a benign condition, as the turnover from carotene to vitamin A is slow and prevents vitamin A poisoning (8).

Pathological symptoms of chronic vitamin A intoxication include hair loss, pruritus, and dryness of mucus membranes, bone fractures and hepatitis. In addition, there is also a growing concern for subtoxicity without clinical signs of toxicity, because nowadays the intake of preformed vitamin A often exceeds the recommended daily allowance (RDA) in the developed countries. Osteoporosis and hip fractures have been associated with an intake of preformed vitamin A of twice the RDA (9).

Changes in total body stores of vitamin A are difficult to establish. The liver is capable of maintaining physiological optimal and constant retinol levels in the body despite declining storage or over-storage of vitamin A. As a result, plasma retinol concentrations do not reflect the hepatic vitamin A content and only change significantly when hepatic vitamin A levels are dangerously high or low (10). Moreover, plasma retinol levels are strongly affected by inflammation or magnesium deficiency (11, 12). As such, it is often not until clinical symptoms of hypo- or hypervitaminosis A develop, before patients are accurately diagnosed.

In this respect, monitoring changes in bile salt homeostasis is easier, as changes in bile salt concentrations can be readily detected in blood and/or feces. Moreover, a general obstruction in bile flow leads to jaundice, which is caused by the accumulation of bilirubin in blood and is often, but not strictly, associated by a changes in bile salt homeostasis.

Vitamin A co-regulates FXR target gene expression

Both hypo- and hypervitaminosis A have the potential to interfere with bile salt homeostasis. Under- or overstimulation of the retinoid X receptor alpha (RXRα), which serves as the obligatory partner of the farnesoid X receptor (FXR), potentially affects expression of key genes involved in bile acid synthesis and transport.

The data presented in this thesis show that the RXRα ligand 9cRA indeed co-regulates expression of FXR target genes. In chapter 2 and 3, we show that bile acid-induced expression of various FXR/RXR-target genes is modulated by the RXRα ligand. Depending on the FXR-target gene of interest the bile acid-induced expression is super-induced (SHP and FGF19), inhibited (BSEP and OSTα in HepG2 cells) or not affected (OSTα in the colon carcinoma cell line DLD-1) by the RXR ligand. In line
with these *in vitro* data, hepatic BSEP expression in cholate (CA)-fed mice is increased when they are VAD (Chapter 2). Figure 6.1 shows the effects of vitamin A on the regulation of hepatic and ileal genes involved in bile salt homeostasis.

**Figure 6.1. Vitamin A co-regulates bile salts transport and synthesis**

The farnesoid X receptor (FXR) acts as a heterodimer with the retinoid X receptor (RXR). This heterodimer regulates expression of key genes involved in bile salt transport (Bile salt export pump (BSEP)) and Organic Solute Transporter α/β (OSTα/β)) and negative feedback regulation on bile acid synthesis (small heterodimer partner (SHP) and fibroblast growth factor (FGF19)). FXR is activated by bile salts, while RXR is activated by vitamin A. In the presence of the FXR ligand, additional activation of RXR by vitamin A leads to a reduced expression of BSEP, while expression of OSTβ, SHP and FGF19 is super-induced under this condition. Vitamin A signaling via RXR-SHP and RXR-FGF19 leads to a decrease in bile acid synthesis (via CYP7A1). But vitamin A may also limit the expression of CYP7A1 via FXR/RXR-independent mechanisms (Cai et al. 2010).

**The FXR/RXRα Heterodimer Is “Non-Permissive” by Default**

The effect of the RXRα-ligand on the transcription of downstream targets of NR/RXRα heterodimers has earlier been subdivided in “permissive” and “non-permissive” (13). As RXRα ligands were found to induce and/or super-induce bile salt-induced expression of the FXR target genes *IBABP* (14) and *PLTP* (15), RXRα was proposed to act as a permissive partner for FXR (16, 17). The observation that bile salt-induced expression of BSEP is inhibited by 9cRA and synthetic RXRα ligands, prompted Kassam *et al.* to designate the FXR/RXRα heterodimer as “conditionally permissive” (18). The inhibitory effect of RXRα ligands on transcription of FXR target genes is not unique for *BSEP* as we also observed this phenomenon for *OSTα* in HepG2 cells. The fact that 9cRA did not repress OSTα mRNA levels in DLD-1 cells
indicates that there are cell type-specific factors that modulate the FXR/RXRα transcriptional machinery.

As the 9cRA-mediated repression of BSEP expression was not dependent on the specific nucleotide sequence of the FXR/RXRα binding site (IR-1), we proposed in chapter 2 that by default “RXRα acts as a nonpermissive partner for FXR”. More recent data presented in chapter 3 show that the IR-1 from SHP used in chapter 2 is actually not the main FXR-responsive element in the SHP promoter. Even though this IR-1 can function as an FXRE, it is not required for FXR/CDCA dependent activation of the SHP promoter (Figure 3.1). This underscores the presumed “involvement of additional binding sites or cofactors in case of co-stimulation by liganded FXR and RXRα” as stated in the discussion of chapter 2.

**Human SHP Promoter Contains a 9cRA Response Element Outside the FXRE**

Super-induction of FXR-target gene transcription by the RXR ligand, as observed for SHP and FGF19, requires additional RXR/9cRA- responsive elements that overcome the non-permissive effect of the FXR/RXR at the FXRE. We delineated such an 9cRA responsive element in the SHP promoter and surprisingly found that this RXR/9cRA responsive element overlaps with the IR-1 sequence previously identified as an FXRE (19). Besides the IR-1, this DNA region also contains a DR-4 (binds LXR/RXRα; (20)) and a DR-2 (binds RXR/RXR and RXR/RAR) (21) (Figure 3.56). These three NR-response elements make use of the same half site TGACCT. Therefore, various NR/RXRα dimers could compete for binding to the same half site. In chapter 2, we placed this half-site in the context of the BSEP promoter and found that this SHP IR-1 sequence is activated by FXR and repressed by 9cRA (Figure 2.2). However, in the context of the SHP promoter this IR-1 sequence is dispensable for the FXR/CDCA-mediated transcription of SHP (Figure 3.1). Unfortunately, this half site is mutated in all the constructed IR-1 mutants of the SHP promoter and therefore it is currently impossible to relate the 9cRA-mediated induction of SHP to either the IR-1, DR-2 or DR-4. Site-directed mutagenesis is required to pinpoint the functional retinoic acid-responsive element and the NR/RXRα dimer that is involved.

**CDCA/FXR Response Element in hSHP Promoter Co-locates with LRE**

The newly identified CDCA/FXR response element in the SHP promoter co-locates with a previously identified LRH-1 response element (LRE; consensus sequence: TCAAGGTTG) and is conserved in human and rodents. The sequence of this LRE-region (GGAG_TCAAGGTTG) strongly resembles the reverse complement sequence of a previously identified atypical FXRE in the promoter region of apolipoprotein A-I (Apo-AI) (AGAGTTCAAGGATC; LRE in italic; non-matching nucleotides between SHP and Apo-AI are underscored) (22). FXR associates as a monomer with this atypical response element and represses transcription of Apo-AI (22). This is the opposite effect as we observed for FXR-mediated regulation of SHP. Maybe the orientation of this atypical FXRE determines its function as a positive or negative regulatory element. Interestingly, the FXR target genes BSEP and MRP3 require LRH-1
for maximal expression (23, 24). However, this does not seem to be the case for the FXRE/LRE site in the SHP promoter. Co-transfection experiments with FXR, RXR and LRH-1 expression plasmids showed that LRH-1 modulates the basal level of SHP promoter activity, but the maximal FXR/CDCA-dependent SHP promoter activity is not different in the absence or presence of LRH-1 (Figure 3.S3). Previously, it was shown that LRH-1 may recruit FXR to the promoter of genes involved in lipid metabolism (a.o. Sec14l2, Scarb1, Srebp2, Lcat, Fdft1, Prkag2 and Ldlrap1) (25). However, we show that FXR regulates SHP expression via this element in the presence and absence of LRH-1, eliminating LHR-1 as a bridging factor in the FXR-mediated regulation of SHP. Moreover, LRH-1 does not seem to compete for FXR binding at this site in the SHP promoter, as increasing amounts of LRH-1 did not significantly change the FXR/CDCA-induced SHP promoter activity (Chapter 3, Figure 3.S3).

**Physiological role of FXR target gene co-regulation by the RXR ligand vitamin A**

Vitamin A absorption depends on the emulsifying action of bile salts and vitamin A metabolites directly affect expression of genes involved in bile salt synthesis and transport. The putative role of vitamin A in bile salt physiology is discussed in the following section, where we hypothesize that local vitamin A concentrations are sensors to control optimal bile function.

**Ileal Absorption of Vitamin A as an Additional Signal to Fine Tune Bile Acid Synthesis**

Vitamin A levels in the terminal ileum may control bile salt synthesis in the liver. Increased circulating bile acid levels suppress hepatic Cyp7a1 expression, however, this feedback inhibition on bile acid synthesis is impaired in obstructive cholestasis. This observation led to the identification of FGF15 in mice (FGF19 in humans) as a second, besides SHP, feedback inhibition mechanism for bile acid synthesis. FGF15 is expressed in the ileum and controlled by FXR. FGF15 is secreted to the circulation and signals to the liver to suppress bile acid synthesis. Obstructive cholestasis decreases the ileal bile acid concentrations and thereby impairs the FGF15/19 controlled inhibition of Cyp7a1. Transcription of SHP and FGF19 is induced by 9cRA alone and it super-induces the CDCA-dependent transcription of both genes (Chapter 3). Schmidt et al. also reported the vitamin A-dependent expression of mouse Fgf15, which required FXR/RXR, but was independent of bile acids. Vitamin A-induced expression of Fgf15 led to a decrease in hepatic Cyp7a1 (26). Efficient absorption of vitamin A in the ileum thus contributes to repression of bile acid synthesis in the liver. In the liver, vitamin A also contributes to the repression of bile acid synthesis. Cai et al. recently reported that CYP7A1 transcription is repressed by all-trans retinoic acid (atRA) in HepG2 cells. In these cells, both FGF19 and SHP transcription were increased by atRA. atRA may be transformed into its isomers 13-cis-RA and 9-cis-RA, either by spontaneous isomerization or by isomerases and subsequently act as a ligand for RXR (27). However, the inhibitory effect on CYP7A1 transcription persis-
General discussion

This indicates that in the liver both FXR/RXR-dependent and -independent mechanisms contribute to suppression of CYP7A1 expression in response to vitamin A. Conversely, we showed in chapter 4 that vitamin A deficiency in rats increased bile acid levels in plasma 2.25-fold and that the increased expression of ileal Fgf15 is unable to efficiently repress Cyp7a1. Apparently, FXR/RXRα-mediated suppression of Cyp7a1 is overruled when ileal vitamin A levels are low. This may serve a direct physiological role in hypovitaminosis A as bile salt synthesis is maintained to support efficient intestinal absorption of vitamin A. The mechanisms that are interfering with FGF15-mediated signaling to suppress Cyp7A1 are presently unknown, but add an additional level of complexity to the regulation of bile salt synthesis. Together, these results show that efficient absorption of vitamin A in the intestine contributes to repression of bile acid synthesis via FXR-dependent (FGF19 and SHP) and FXR-independent mechanisms. Low levels of vitamin A entering the enterocyte maybe a signal for a limited action of bile salts in the intestinal lumen, reducing the negative feedback loop on bile acid synthesis. Figure 6.2 depicts the impaired negative feedback on bile acid synthesis as a consequence of vitamin A deficiency.

Absorption of Vitamin A as an Additional Signal to Fine Tune Bile Acid Transport

With low levels of ileal and hepatic vitamin A the negative feedback mechanism fails to repress bile acid synthesis (Chapter 4) while high levels of vitamin A reduce bile acid synthesis (26). Genes encoding bile acid importers NTCP/Ntcp and ASBT/Asbt are both established RAR/RXRα target genes in human and rodents (29-31). Bile salts inhibit expression of rat NTCP by increasing expression of SHP, which interacts with RAR/RXRα and prevents its binding to the Ntcp promoter (29). SHP expression itself is also positively regulated by vitamin A, thereby creating a negative feedback loop on NTCP expression. Thus, vitamin A has the potential to increase ileal and hepatic import of bile acids by stimulating expression of NCTP and ASBT, but at the same time provides a “break” on their expression by also stimulating SHP transcription that represses transcription of NTCP and ASBT. Interestingly, rat Ntcp and human and mouse ASBT/Asbt, but not rat Asbt expression is repressed by bile acids (31). This specific difference between species may contribute to the observation that vitamin A deficient rats develop mild cholestasis (Chapter 4), while this was not observed for VAD mice (Chapter 2).

Various FXR target genes are differentially regulated by the combination of FXR and RXRα ligands. A remarkable example are OSTα and OSTβ that together form a membrane-embedded bile salt transporter. In the colon carcinoma cell line DLD-1, 9cRA does not affect OSTα transcription while the CDCA-dependent transcription of OSTβ is enhanced by 9cRA. The contribution of the individual subunits of the obligatory complex OSTα/β to the transport activity is unknown as is the stoichiometric ratio in which the α- and β-subunits are present at the plasma membrane. However, one can envision that by fine tuning the ratio of α- and β-subunits vitamin A
could modulate transport efficiency and/or specificity of this bile acid transporter at the basolateral membrane of enterocytes and thereby control the intestinal absorption of bile acids to the circulation.

Vitamin A affects bile acid homeostasis not only at the transcriptional level, but also at the post-transcriptional level. Without an apparent reduction in Ntcp mRNA levels in cholate (CA)-fed VAD mice (Chapter 2), NTCP protein expression is markedly reduced in these animals compared to the vitamin A sufficient (VAS) controls (Figure 2.7). It is unclear whether this decrease is caused by the increased bile acid levels in plasma, or that the decrease in NTCP expression itself contributes to the observed increase in plasma bile acid levels.

Vitamin A may thus affect ileal and hepatic bile acid import and export directly by acting as a ligand to transcription factors that induce transcription of bile acid transporters and indirectly by activation of a negative feedback mechanism on transcription or by post-translational lowering of protein levels of these transporter proteins.

**Vitamin A (deficiency) and liver damage**

Both hyper- and hypovitaminosis A are known to affect liver function. There are multiple reports that excessive vitamin A intake leads to cholestasis in human (32-37). Notably, all described cases of vitamin A-induced cholestasis were reversible. Vitamin A-induced fibrosis correlates with stellate cell activation, although it remains to be elucidated whether vitamin A *per se* is the major cause of this phenomenon (38).

At the other end of the spectrum, vitamin A deficiency (3 months VAD diet in rats) led to spontaneous morphological changes in the liver. Apart from the expected loss of lipid droplets in stellate cells, the hepatic architecture was disturbed and the parenchyma disorganized with fat droplets in the hepatocytes. Furthermore, expression of markers of stellate cell activation e.g. alpha-smooth muscle actin (α-Sma), collagen IV, laminin 1 and fibronectin were increased by VAD (39). Although rats were fed a VAD diet for 4 months in our experiments, we did not observe stellate cell activation and/or morphological changes of the liver. In the study of Aguilar et al. rats were put on the VAD diet at 3 weeks of age, while at the beginning of our study animals were 6 weeks of age. Younger animals have a smaller liver and thus less storage of vitamin A and thus these rats may have become vitamin A deficient earlier. In our experiments, we monitored the serum retinol level carefully and sacrificed the animals at the moment when the rats were not able anymore to maintain normal serum retinol levels. It could very well be that the hepatic architecture is rapidly disturbed after prolonged exposure to a vitamin A deficient diet. Another important difference between these 2 studies is that Aguilar et al. used female rats where we used male rats and clear differences in vitamin A storage, serum levels and minimal requirements of this vitamin between male and females have been described (40, 41).

**Vitamin A Deficiency and Liver Injury Caused by Obstruction of Bile Flow**

Based on the research presented in chapter 4 we expected that the lack of vitamin A may contribute to liver injury in acute and chronic liver diseases as VAD alone led
General discussion

to mild cholestasis. Furthermore, the observed maximal expression of \textit{Bsep} mRNA under low retinol conditions (\textit{Figure 2.1} and (18, 42)) may also be harmful, in particular in obstructive cholestasis, as it maintains canalicular bile salt secretion while bile flow from the liver is compromised.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.2.png}
\caption{Vitamin A deficiency impairs negative feedback loop on bile acid synthesis}
Vitamin A deficiency leads to an increased bile salt concentration in plasma in rats. Despite increased expression of FGF15 (FGF19 is the human homolog) CYP7A1 levels are not down regulated. Expression of bile salt transporters was not majorly altered by vitamin A deficiency. The lack of vitamin A therefore overrules FGF15-mediated repression of Cyp7A1 expression in the liver. Whether FGF15 requires vitamin A to fulfill its signaling function to the liver, or whether FGF15 and vitamin A signal via completely independent mechanisms remains to be determined. This way not only the presence of bile acids in the intestinal lumen, but also its ability to successfully fulfill its function in absorption of fat (-soluble) compounds is a signal that adequate amounts of bile acids are in circulation.

Indeed, vitamin A deficiency led to severe liver damage in bile duct-ligated rats, a model of obstructive cholestasis. However, VAD-mediated dysregulation of FXR/RXR target genes could not explain the observed liver damage. Compared to bile salt feeding, bile duct ligation is a more complex model of cholestasis, which also leads to inflammation and fibrosis and may involve the response of other liver cell types to VAD. Although vitamin A deficiency represses the negative feedback on bile acid synthesis, resulting in increased bile salt serum concentration in rats (\textit{Chapter 4}) and cholate-fed mice (\textit{Chapter 2}), it does not lead to liver disease when bile flow is not obstructed.
A remarkable observation was that VAD rats accumulated a significant volume of bile in front of the ligation resulting in swelling of the common bile duct, which was only minor in bile duct-ligated VAS animals. This implies that bile secretion is increased in VAD rats and that obstruction of the bile flow may lead to physical pressure in the biliary tree leading to excessive liver damage. However, treating these animals with vitamin A strongly repressed liver damage to a level observed in VAS-BDL rats, while these “bile sacs” were not reduced in size. This indicates that the putative increase in pressure in the biliary tree is not causing the excessive liver damage. Moreover, hepatic bile salt concentrations were not significantly increased in VAD-BDL rats compared to VAS-BDL. Still the VAD-BDL rats showed strongly increased serum γGT levels together with pronounced bile duct proliferation.

Thus, lack of vitamin A may affect the composition of bile, shifting the balance of individual bile salts or lowering the phospholipids and cholesterol content in bile. Toxic bile may be an important feature in primary sclerosing cholangitis (PSC) and contribute to the progression of this disease (43). A rapid normalization of bile salt composition in response to vitamin A therapy, without affecting total bile salt concentration/flow, could explain the beneficial effects of vitamin A therapy despite the occurrence of bile sacs in VAD-BDL animals.

From these data we concluded that vitamin A therapy and deficiency do influence bile salt homeostasis, but that the acute therapeutic effect of vitamin A on bile duct ligation-induced liver injury under vitamin A-deficient conditions is most likely not caused by aberrant FXR/RXRα signaling. However, the one week window of BDL we used in these experiments may be too long to detect the involvement of FXR signaling in the onset of liver damage, as the cholestatic condition and occurrence of liver damage was greatly accelerated by VAD and the majority of the hepatocytes might already be damaged or necrotic when the animals were sacrificed.

**Vitamin A Deficiency and Hepatic Stellate Cell Activation**

Hepatic stellate cells (HSCs) control vitamin A metabolism and contain up to 80% of the body stores of this nutrient. These cells appear to play a pivotal role in liver injury caused by both hypo- and hypervitaminosis A. Chronic liver damage leads to liver fibrosis, which is characterized by activation of HSCs that rapidly lose their vitamin A-containing lipid droplets. This prompted researchers to investigate the role of vitamin A in the activation of HSCs in relation to liver damage. Lecithin retinol acyltransferase deficient (Lrat−/−) mice cannot synthesize retinyl esters and therefore lack vitamin A-containing lipid droplets in the HSC. These mice do not spontaneously develop liver fibrosis (44). Thus, the absence of vitamin A per se does not lead to activation of HSCs.

Serum retinol concentrations are lowered during infection and retinol binding protein 4 (RBP4) synthesis and excretion is lowered by inflammation, leading to retinol accumulation in the liver (45, 46). BDL is accompanied by inflammation and consequently, we observed decreased plasma retinol levels (-51%) and increased hepatic
retinol levels (+61%) in VAS BDL rats (Table 5.2). This could be explained by recruitment of retinol to the liver or by conversion of retinyl palmitate to free retinol by activated HSC. Remarkably, \textit{Lrat}\textsuperscript{+/−} mice do not suffer more liver damage as a consequence of BDL (3 weeks) compared to WT mice. The absence of retinyl ester-containing lipid droplets in the HSC does not influence BDL-induced HSC activation/fibrosis (44). Although the \textit{Lrat}\textsuperscript{+/−} mice are unable to store vitamin A in the liver they are not vitamin A deficient. Dietary vitamin A in these animals is primarily absorbed as free retinol in chylomicrons. The storage function of vitamin A as retiny lesters is partially taken over by adipose tissue and results in normal serum retinol levels (47). Thus, the suppressive effect of vitamin A on the development of liver fibrosis seems not to be due to retinyl palmitate contained in the vitamin A droplets, but is likely mediated through free retinol.

Like \textit{Lrat}\textsuperscript{+/−} mice, VAD rats have no retinoids stored in the HSC, but unlike \textit{Lrat}\textsuperscript{+/−} mice VAD rats cannot recruit free retinol to the liver since these animals lack vitamin A altogether. Consequently, BDL in VAD rats is accompanied by excessive liver damage and a strongly enhanced fibrotic response as shown by increased \textit{α-Sma} and collagen type I (\textit{Coll1a1}) expression (Chapter 5).

\textit{In vitro} studies have shown that HSC activation can be suppressed by vitamin A (48). Furthermore, various \textit{in vivo} studies reported the beneficial effect of (pro)vitamin A-derived compounds on the disease course of experimentally induced liver damage/cholestasis in vitamin A sufficient animals. Retinoic acid and β-carotene were shown to reduce CCl\textsubscript{4} induced hepatic fibrosis in rats (49-54). Release of vitamin A from activating stellate cells could therefore serve to prevent over activation of neighboring stellate cells.

In our study, vitamin A therapy did not increase hepatic retinol levels in control (VAS) animals. In contrast, serum and hepatic retinol levels were significantly increased after 1 week vitamin A therapy in VAD rats to 63% and 35% of the levels observed in VAS animals, respectively (Chapter 5). Bile duct ligation led to a decrease in serum retinol levels (-51%) and an increase in hepatic retinol levels (+61%) in control rats, indicating that bile obstruction leads to a redistribution of retinols to the liver even when vitamin A is sufficiently present. This process was even more pronounced in VAS-BDL rats receiving vitamin A therapy, which showed 60% lower serum levels and 140% higher hepatic levels of retinol. BDL also enhanced hepatic retinol concentrations in vitamin-A treated VAD animals, but these levels were still 70% lower than in vitamin A treated VAS-BDL rats. Vitamin A deficiency alone did not lead to a fibrotic response in rats. In contrast, BDL induced fibrosis in control (VAS) rats, which was not repressed by vitamin A therapy. The fibrotic response was strongly enhanced in VAD-BDL treated animals and was effectively suppressed by vitamin A therapy, while hepatic retinol levels were far below VAS-BDL levels. This indicates that absolute retinol levels in the liver are not associated with the fibrotic response and/or that only minor amounts or retinol are sufficient to prevent hepatic stellate cell activation.
Chapter 6

**Vitamin A Deficiency and Inflammation**

Bile duct ligation leads to hepatic inflammation and is strongly enhanced in the absence of retinol. Vitamin A is required for several aspects of the immune responses. Vitamin A deficiency impairs both innate and adaptive immune response to infection resulting in an impaired ability to counteract extracellular pathogens (55). Retinoic acid affects the balance between $T_{h1}$- and $T_{h2}$-cells in favor of the latter and promotes the proliferation of regulatory T-cells. Furthermore, vitamin A modulates nitric oxide (NO) production and inhibits the production of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2), thereby favoring a $T_{h2}$-$T_{reg}$ non-inflammatory environment (reviewed by Mora et al. (56) and Pino-Lagos et al. (57)). This is in line with the observation that vitamin A deficiency aggravates the clinical manifestations of inflammatory reactions (58).

Although we did not detect major effects of VAD on cytokine transcription (data not shown), $iNOS$ transcription is 21-fold increased in VAD-BDL rats compared to VAD-BDL animals. BDL gives rise to a sterile inflammatory response in which innate immune cells are activated (59). Kupffer cells (the hepatic macrophages) and attracted neutrophils produce ROS through increased expression of iNOS. $T_{h1}$ cells are more potent activators of macrophages than $T_{h2}$ cells and in vitamin A deficient condition the balance between $T_{h1}$- and $T_{h2}$- cells shifts towards the $T_{h1}$-cells favouring an environment in which macrophages and attracted neutrophils are stimulated to express more iNOS. However, VAD alone did not increase $iNOS$ mRNA levels (Figure 5.8 and Table 5.3), indicating that shortage of vitamin A itself is not the trigger of the sterile inflammatory response.

Pro-vitamin A β-carotene is a potent antioxidant (60, 61), but also retinol, retinyl esters and retinoic acid have been shown to prevent lipid peroxidation in vitro (62, 63). Various studies in rodents show that vitamin A deficiency affects hepatic antioxidant enzyme expression and/or activity, but contradictory results have been reported. Expression or activity of catalase (CAT) was found to be either up- (64-66) or down-regulated (67), while superoxide dismutase (SOD) was not affected (64, 66, 67) in liver, but reduced in lung (67). Glutathione peroxidase 1 (GPX1) was either up-regulated (66), unaffected (67) or down-regulated (64) by vitamin A deficiency. In our study, hepatic mRNA expression of both $Cat$ and $Gpx1$ was reduced in vitamin A-deficient rats (data not shown). These results show that vitamin A deficiency alters the antioxidant defense of the liver. On the other hand, vitamin A (atRA) supplementation restored SOD activity and hepatic glutathione (GSH) levels of bile duct ligated (4 weeks) rats to that of sham treated rats (53). However, in our one week BDL model we did not detect an effect on antioxidant enzymes ($Cat$, $Gpx1$ and $Sod$) in liver of VAS rats as a result of vitamin A therapy. Moreover, retinyl palmitate supplementation did not significantly increase expression of antioxidant enzymes in VAD-BDL treated rats.

In conclusion, vitamin A deficiency gives rise to production of pro-inflammatory cytokines and makes the liver more vulnerable to oxidative stress by altering expression levels of antioxidant enzymes, but did not induce an inflammatory response.
by itself in our study. However, it hereby increases the risk of acquiring irreversible tissue damage, as illustrated by our in vivo model of obstructive cholestasis.

**Suggestions for future research**

Although inadequate FXR/RXRα signaling could not be established as the major cause of liver damage in VAD-BDL rats, it may still play a role in the initial phase/onset of this phenotype. It is therefore essential to analyze the progression of liver damage and the hepatic process involved in VAD-BDL treated rats at earlier time points. Establishing FXR target gene expression at earlier time points may reveal whether FXR/RXRα signaling is involved in initiating excessive the liver damage. Since the excessive liver damage is characterized by bile duct proliferation and bile duct damage, it seems particularly interesting to analyze the composition of the bile produced by VAD animals and how this is changed upon bile duct ligation.

**In conclusion:**

In conclusion, the research described in this thesis shows that vitamin A co-regulates expression of various FXR target genes (such as BSEP, SHP, OSTα/β and FGF19) and is therefore an independent factor in controlling bile acid synthesis and transport. The RXRα ligand, 9cRA, inhibits binding of the FXR/RXRα-heterodimer to its responsive element (FXRE) as a default mechanism. We show that SHP is synergistically activated by RXRα/9cRA and FXR/CDCA through 2 spacely separated DNA elements, including a novel FXR binding site that shares high sequence homology to a LRH-1 binding element. These in vitro data show that the molecular mechanisms involving FXR and RXRα in regulating bile acid homeostasis is far more complex than simply binding of the FXR/RXRα heterodimers to the IR-1 element. The novel mechanisms that are required for synergistic regulation by RXRα/9cRA and FXR/CDCA are particularly relevant for the negative feedback on bile acid synthesis via SHP and FGF19. Vitamin A is not only important for gene regulation, but also affects bile salt physiology at post-transcriptional levels. This is evident from the mild cholestasis that develops in vitamin A deficient rats, while there are no significant changes in transcript levels of genes involved in bile salt synthesis and transport. Finally, vitamin A is essential to prevent excessive liver damage in a rat model of obstructive cholestasis. Without vitamin A (therapy), these animals die from progressive liver injury. The exact molecular events that lead to liver failure under these conditions remain to be established, but appear highly relevant for patients with chronic liver disease that are prone to develop hypovitaminosis A, including biliary atresia, autoimmune hepatitis and fibrosis.
Chapter 6

References


