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## Effects of bile salts on hepatic lipoprotein production

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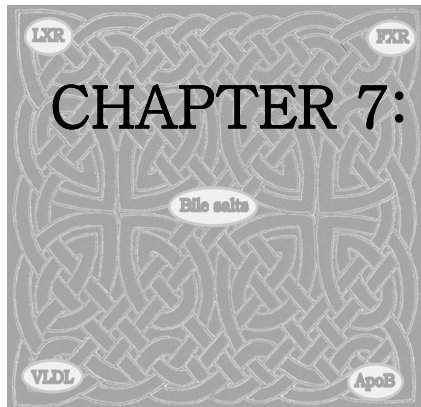
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## General Discussion

## GENERAL DISCUSSION

Most of the population of the Western societies live their lives under non-fasting conditions. As a consequence, they experience a continuous hepatic and intestinal production of TG-rich lipoproteins that are secreted into the blood. Overproduction and/or a delayed removal from the circulation of these TG-rich lipoproteins, i.e. VLDL and chylomicrons, result in elevated concentrations of plasma TG and cholesterol. An elevated plasma TG concentration (hypertriglyceridemia) has been recognized as a risk factor for cardiovascular disease, such as atherosclerosis, which is one of the major causes of death in the Western world. Hypertriglyceridemia is a common characteristic of several metabolic syndromes, like diabetes type II, obesity and familial combined hyperlipidemia [1].

Interruption of the enterohepatic circulation in humans with cholestyramine or after ileal exclusion, leading to *decreased* transhepatic bile salt fluxes, appeared associated with elevated plasma TG concentrations. A clinical study published in the 1970's demonstrated that plasma TG levels in patients with familial hypertriglyceridemia could be lowered by supplementing them with the bile salt chenodeoxycholate [2] that leads to *increased* transhepatic bile salt fluxes. This potentially beneficial effect of bile salts was further investigated *in vitro* in primary human and rat hepatocytes in our lab [3,4]. Bile salts were shown to dose-dependently inhibit secretion of VLDL-TG by hepatocytes. Experiments described in this dissertation were conducted to quantify the effects of bile salts *in vivo* and to unravel the underlying mechanism(s) by which bile salts inhibit VLDL production. In particular, the following questions were addressed: 1. whether bile salts influence VLDL production via regulation of genes involved in lipoprotein metabolism and 2. whether bile salts inhibit secretion of VLDL by interfering with intracellular lipoprotein assembly.

### **Quantification of the effects of bile salts on hepatic production of VLDL *in vivo* under physiological, non-pathological conditions**

Since inhibition of VLDL production by bile salts *in vitro* appeared to be direct and acute (VLDL secretion -50% within 4 hours [3,4]), we studied VLDL production *in vivo* in the bile-diverted rat model [5] in which we could determine acute effects of bile salts on VLDL production (**chapter 2**). Surprisingly, a one-week bile diversion or a direct infusion of bile salts into the duodenum did not affect VLDL-TG production rate, plasma

TG concentration and VLDL lipid composition in rats. These findings were in contrast to results from clinical studies that reported that interruption of the enterohepatic circulation by 18h-withdrawal of bile (instead of a week in our rat model) increased plasma TG concentrations in hypertriglyceridemia patients [6]. Interruption of the enterohepatic circulation in healthy humans with cholestyramine or after ileal exclusion was also reported to be associated with elevated plasma TG concentrations [6-8]. Davis and co-workers have postulated that hepatic production of VLDL is linked to the expression of cholesterol 7 $\alpha$ -hydroxylase (Cyp7A1), the rate-limiting enzyme in the conversion of cholesterol into bile salts [9,10]. Expression of *Cyp7A1* should induce expression of MTP and genes involved in *de novo* lipogenesis, through transcription factors SREBP-1a and 1c. In their model, VLDL-triglyceride production is directly linked to *de novo* lipogenesis. Our data from bile-diverted rats do not support this hypothesis. Despite the fact that bile diversion in rats has been reported to significantly upregulate expression of *Cyp7A1* [11] no differences in VLDL-TG production were observed between control intact rats and bile-diverted rats.

In mice, manipulation of the hepatic bile salt flux for 3 weeks did affect VLDL-TG production rate, plasma TG concentration and VLDL lipid composition. Supraphysiological bile salt fluxes (4-fold increased compared to control mice) resulted in decreased plasma VLDL-TG concentrations and a minor decrease in VLDL-TG production. In mice, an inverse relationship between bile salt flux rates and either plasma TG concentration or TG production rate was found. Results described in **chapter 2** demonstrate that VLDL-TG production in rodents *in vivo* is not acutely inhibited by bile salts, which is in contrast to effects of bile salts on VLDL-TG secretion by primary rat and murine hepatocytes *in vitro* [4,12]. Several factors could be responsible for the observed discrepancy between the *in vivo* results and the previously obtained *in vitro* data. The *in vivo* model involves polarized hepatocytes with an efficient hepatobiliary bile salt transport. Rat hepatocytes *in vitro* that are incubated with bile salt concentrations similar to postprandial concentrations in portal blood probably reach higher intracellular bile salt concentrations than those reached during their flux through the hepatocyte *in vivo*. Intracellular bile salt concentrations reached in hepatocytes could be a more important parameter for inhibition of VLDL secretion than the actual flux of bile salts through the hepatocyte. **Chapter 3** shows that significantly increased bile salt fluxes in the same mouse model used in chapter 2 have relatively minor effects on hepatic expression of the bile salt transporters Ntcp (basolateral) and Bsep (canalicular) *in vivo* in

mice. This suggests that these transporters are abundantly expressed under normal physiological conditions. Bsep and Ntcp are homogeneously distributed along the liver acinus, while bile salt transport is predominantly localized to the periportal zone. At higher bile salt fluxes, more hepatocytes along the acinus will be effectively involved in bile salt transport thus preventing intracellular bile salt concentrations to raise inside the periportally localized hepatocytes. This phenomenon could support the hypothesis that the height of intracellular bile salt concentrations is a more important parameter in affecting VLDL secretion rather than the actual bile salt flux.

Our data in **chapter 2** support the hypothesis that the transhepatic bile salt flux can affect VLDL secretion, but do not support an important regulatory role for bile salts in control of hepatic secretion of VLDL-TG *in vivo* in rodents under normal, physiological conditions.

### **Effect of activation of the Liver x Receptor on hepatic VLDL production**

Oxysterols, cholesterol metabolites and putative intermediates in bile salt biosynthesis, are natural ligands for the nuclear liver X receptor. The liver X receptor  $\alpha$  (LXR $\alpha$ ; NR1H3) and LXR $\beta$  (NR1H2) are involved in the control of important genes in cholesterol and fatty acid metabolism. Activated LXR was shown to induce *Cyp7a1* gene expression [13,14]. Pharmacological activation of LXR leading to accelerated reverse cholesterol transport has been advocated as a potential novel treatment or prevention of atherosclerosis [15], but results described in **chapter 4** demonstrate that the beneficial raise of HDL levels is accompanied by potentially adverse effects on TG metabolism. In mice, a 2-fold induction of VLDL-TG production upon LXR activation was observed which was exclusively due to the formation of large, TG-rich particles, since the increased VLDL-TG production exactly matched the increased VLDL particle volume and the apoB content of isolated VLDL fractions was not affected upon treatment.

LXR activation caused TG accumulation in perivenous hepatocytes and in periportal hepatocytes resulting in the development of a severe hepatic steatosis, which was also reported previously [16]. The development of hepatic steatosis is likely related to increased *de novo* lipogenesis in combination with an increased free fatty acid (FFA) flux towards the liver probably caused by an enhanced release of FFA in the circulation through increased lipolytic activity of LPL. It is tempting to speculate that LXR-

stimulated *de novo* lipogenesis and subsequent increased supply of FFA to the liver is the primary cause of the raised VLDL-TG secretion. However, in a limited series of experiments rat hepatoma McA-RH7777 cells were kept under fixed FFA concentrations and simultaneously incubated with the LXR agonist. In these *in vitro* studies, genes involved in *de novo* lipogenesis were upregulated and cellular TG secretion was increased, again without changes in apoB production. This demonstrates that the observed LXR-stimulated VLDL-TG secretion was independent of increased FFA flux. In addition, it should be noted that increased hepatic *de novo* lipogenesis is often *not* associated with increased VLDL-TG secretion, as was demonstrated by other studies from our lab in which strong induction of *de novo* lipogenesis upon inhibition of glucose-6-phosphatase activity in rats is not associated with increased VLDL production and in ob/ob mice in which a massive increase in *de novo* lipogenesis was not accompanied by a stimulated hepatic VLDL production [17].

Although activated LXR was demonstrated to induce *Cyp7A1* gene expression [13,14], Plösch *et al* [18] recently showed in a series of similar experiments that hepatic *Cyp7A1* gene expression in LXR-agonist treated mice was not statistically different from control values. Biliary bile salt secretion rates were also similar in controls and treated mice. These findings together with our data in **chapter 4** do not support the concept that hepatic VLDL production is directly linked to expression of *Cyp7a1*, as postulated by Davis and co-workers [9,10]. These data are in agreement with results in **chapter 2**, concerning bile-diverted rats and cholestyramine-treated mice. LXR-induced *Cyp7A1* expression coincided with increased expression levels of *Srebp-1c* and an increased VLDL-TG production similar to mice overexpressing the human *Cyp7A1* gene reported by Davis and co-workers [10].

### **Effects of Farnesoid x receptor–deficiency on production of TG-rich lipoproteins *in vivo***

Recently, bile salts were shown to be natural ligands for the Farnesoid X Receptor (Fxr, Nr1H4) and were demonstrated to repress their own synthesis by inhibition of *Cyp7a1* gene transcription via activation of FXR [19-21]. Bile salt-activated FXR may also provide a link between bile salt and TG metabolism, since Sinal *et al* reported that disruption of FXR leads to hypertriglyceridemia in mice [22]. To provide a more

mechanistic basis for the latter finding we investigated the role of FXR in hepatic and intestinal formation of TG-rich lipoproteins.

The data presented in **chapter 5** demonstrate that FXR deficiency in mice results in the formation of differently structured TG-rich lipoproteins by liver and intestine. The hyperlipidemic plasma profile of *Fxr*<sup>(-/-)</sup> mice was not accompanied by hepatic steatosis, as was reported for *Fxr*-null mice generated by Cre-lox technology used by Sinal *et al* [22]. The different observations on hepatic fat content may be related to strain specificity or differences in diet composition.

Despite the significant hyperlipidemia in *Fxr*<sup>(-/-)</sup> mice, similar hepatic VLDL-TG production rates were measured in control mice and in *Fxr*<sup>(-/-)</sup> mice. This is in contrast to observations recently reported by Lambert *et al* [23] showing a more than 2-fold increase in plasma TG accumulation, reflecting TG production rate, in their *Fxr*-null mice compared to wild type controls. However, plasma TG accumulation in *Fxr*<sup>(-/-)</sup> mice in our study was only slightly lower than reported in *Fxr*-null mice studied by Lambert *et al* (985 mg/dl vs. ~1300 mg/dl). It should be noted that TG accumulation data from Lambert *et al* were determined in non-fasted mice (in contrast to mice in our study), indicating that TG originating from intestinal chylomicrons at least partly contributed to the total plasma TG accumulation. In addition, plasma TG accumulation in their control mice was 1.5-2 fold lower than reported by us and others for wild type C57Bl6 mice (600 mg/dl vs. 903-1327 mg/dl) [12,17,24-27].

Although TG production rates were similar in all groups, *Fxr*<sup>(-/-)</sup> mice were shown to produce larger particles enriched in TG. The increase in particle volume was balanced by a decreased production of the amount of VLDL particles, represented by a ~60% decrease in VLDL-apoB protein content. Since each VLDL particle contains a single apoB molecule, secretion of apoB molecules corresponds with the number of VLDL particles produced [28].

From studies in diabetic models, large TG-rich VLDL particles (VLDL1) were demonstrated to have a longer retention time in plasma than smaller VLDL particles (VLDL2) [29-31]. In addition, processing of VLDL1 particles was shown to result in formation of small dense LDL particles (pattern B LDL), which is associated with increased risk of development of cardiovascular disease [32]. In **chapter 4**, pharmacological activation of LXR also resulted in formation of larger, TG-enriched VLDL particles. In contrast to *Fxr*<sup>(-/-)</sup> mice, LXR agonist-treated wild type mice did not

show a hypertriglyceridemic profile. Although larger VLDL particles (VLDL1) are considered to have a longer plasma retention time, they were very efficiently processed and cleared from the circulation in LXR agonist-treated mice. However, hepatic mRNA levels of ApoC-II, a stimulatory co-factor for lipoprotein lipase, were significantly reduced in *Fxr*<sup>(-/-)</sup> mice compared to controls. In contrast, LXR activation has been shown to induce expression of *apoC2*. In *Fxr*<sup>(-/-)</sup> mice a reduced ApoC-II presence on VLDL and chylomicron particles could result in a decreased susceptibility to LPL-mediated lipolysis.

In contrast to hepatic VLDL production, intestinal chylomicron production was impaired by ~60% in *Fxr*<sup>(-/-)</sup> mice and chylomicron particle size was reduced. The chylomicron fraction of *Fxr*<sup>(-/-)</sup> mice contained more apoB48, indicating that more particles were produced. FXR deficiency appeared to result in formation of small postprandial VLDL-like chylomicrons, which are also considered as pro-atherogenic lipoprotein particles and associated with increased plasma TG levels. Small chylomicrons are suggested to compete with VLDL particles for lipolysis, thereby increasing the retention of VLDL in the circulation [31]. The 60% decrease in chylomicron production rate may be caused by impaired assembly or secretion of chylomicrons by the enterocytes, but microscopical examination of the intestines of *Fxr*<sup>(-/-)</sup> mice under non-fasted conditions did not show accumulation of intracellular lipids. It is also not very likely that a disturbed activity of the microsomal triglyceride transfer protein (MTP) plays an important role in decreasing the chylomicron size, since impaired MTP lipid transfer activity was shown to decrease secretion of chylomicron particles similar to the inhibitory effect on VLDL secretion [33] instead of decreasing TG content.

Instead, FXR may play a role in intestinal phospholipid metabolism. Recently, Kirby and co-workers demonstrated that mice deficient in pancreatic bile salt-stimulated carboxyl ester lipase (CEL) showed similar alterations in chylomicron composition and secretion as described for *Fxr*<sup>(-/-)</sup> mice in this study [34]. CEL deficiency is associated with intracellular accumulation of ceramide in the intestinal cells due to a decreased capacity to break down ceramide. Ceramide, in turn, has been demonstrated to reduce secretion of intestinal lipoproteins [35]. The underlying mechanism is still unknown.

Absence of the farnesoid x receptor in mice *in vivo* results in development of an atherogenic lipid profile; formation of pro-atherogenic large VLDL particles and small, almost VLDL-sized chylomicrons that most probably contribute to the hyperlipidemia in



Fxr-deficient mice. Under normal circumstances bile salt-activated FXR may have the opposite effect and may promote formation of smaller VLDL particles and large chylomicrons that can be optimally processed in the circulation.

### **Effect of bile salts on VLDL secretion *in vitro* in cultured Fxr-deficient hepatocytes**

Under fixed fatty acid concentrations in control medium secretion of VLDL-TG *in vitro* by Fxr-deficient ( $Fxr^{(-/-)}$ ) hepatocytes was decreased by ~30% compared to control hepatocytes ( $Fxr^{(+/+)}$ ), despite a ~2-fold increased gene expression of the microsomal triglyceride transfer protein (MTP), a well-known rate-controlling enzyme in VLDL formation. Decreased TG secretion by  $Fxr^{(-/-)}$  hepatocytes indicates that either less particles were secreted or that smaller particles were produced. Incubation with the bile salt taurochate (TC) inhibited VLDL-TG secretion both in  $Fxr^{(-/-)}$  hepatocytes and wild type hepatocytes to the same extent as was observed earlier in TC-incubated primary human, rat and wild type mouse hepatocytes [3,4,12]. These results indicate that bile salts inhibit secretion of VLDL-TG (and probably of apoB) independent of a pathway controlled by FXR *in vitro*.

From the *in vivo* and *in vitro* data on involvement of the nuclear receptors LXR and FXR in VLDL production we may conclude that *in vivo* both *activation* of LXR and *FXR-deficiency* contribute to a hyperlipidemic profile (**chapters 4 and 5**). On the other hand, activation of FXR by bile salts under normal physiological conditions seemed not to be associated with a profound effect on production of VLDL-TG *in vivo* (taurocholate-fed mice, **chapter 2**). The small inhibitory effect of bile salts on TG production in TC-fed mice (**chapter 2**) could well be FXR-independent, as was also shown in TC-incubated  $Fxr^{(-/-)}$  hepatocytes.

### **Bile salts inhibit secretion of apolipoprotein B independent of its assembly into a TG-rich lipoprotein *in vitro***

Theoretically, bile salts could impair VLDL secretion via interfering with lipidation of apoB (assembly of TG-rich VLDL) or, alternatively, by a mechanism independent from the lipidation. In **chapter 6** we investigated whether assembly of apoB into a lipoprotein particle is required for this inhibitory action of bile salts on VLDL secretion.

As the *in vitro* model we used McArdle rat hepatoma cell lines stably transfected with either carboxyl-truncated human apoB18 (h-apoB18; N-terminal 18% of apoB100), or full-length human apoB100 (h-apoB100) and the rat bile salt transporter Ntcp. h-ApoB18 is secreted from these cells at a density above 1.23 g/ml [36], i.e., at the density of non-lipoprotein secretory proteins and is thus secreted in a virtually lipid-free form. In contrast, h-apoB100 requires assembly with neutral lipids and is secreted as a buoyant lipoprotein particle with the density of VLDL (d~1.006 g/ml). These cells were incubated with taurocholate and apoB secretion and intracellular degradation were determined.

**Chapter 6** shows that, upon TC incubation, the secretion of both truncated h-apoB18 and full-length h-apoB100 was inhibited to a similar extent as was observed in primary human, rat and mouse hepatocytes [3,4,12]. Since the expression of the apolipoprotein B gene and the synthesis rate of apoB protein are rather constant under widely varying conditions in liver and intestinal cells [37], the rate of apoB secretion is mainly controlled at a post-translational level, e.g., by control of intracellular degradation [38-43]. Therefore, we examined whether TC-impaired secretion of apoB was associated with increased intracellular degradation of apoB. The data demonstrate that intracellular degradation of apoB was enhanced by TC even when proteasomal degradation was inhibited by the proteasomal (calpain) inhibitor ALLN. This indicates that TC does not affect apoB at the cytosolic side of the ER, the site of proteasomal degradation, but merely affects secretion of apoB at a site after translocation of apoB into the ER lumen, i.e., at the level of ER lumen or Golgi. These data were in agreement with results of Tran *et al* that the major part of intracellular apoB degradation occurs in endoplasmic reticulum and Golgi in McA-RH7777 cells [44].

The truncated h-apoB18 that does not require lipidation before secretion was inhibited by TC to a similar extent as h-apoB100, indicating that a disturbed activity of microsomal triglyceride transfer protein (MTP), the rate-limiting enzyme involved in lipidation of apoB with neutral lipids, is unlikely. Moreover, in **chapter 2** we showed that mRNA expression or protein activity of MTP was not affected by bile salts. In an *in vitro* study (unpublished) VLDL-TG secretion in mouse apoE-deficient hepatocytes was also inhibited by bile salts to a similar extent as demonstrated for wild type mouse hepatocytes. This indicated that apoE was not involved in TC-mediated inhibition of VLDL secretion *in vitro*.

ApoB has been described to interact with the ER membrane during translocation and directly after for stability of the protein [45]. Rusinol *et al* demonstrated that changes

in ER membrane phospholipid composition led to decreased secretion of h-apoB18 and other apoB's of various lengths and a concomitant accelerated intracellular degradation of apoB [46]. This increased intracellular degradation specific for apoB could not be abolished with proteasomal inhibitor ALLN, which was similar as we observed in TC-incubated cells. Perhaps bile salts could affect phospholipid composition in the ER/ Golgi membrane that influence stability of apoB still resident in the ER and Golgi. In addition, bile salts have been shown to interact with several signal transduction pathways via activation of protein kinases C, JNK, ERK and PI3 kinase [47-49]. PI3 kinase has also been implicated in the insulin-mediated inhibition of apoB secretion [50-53]. Inhibition of PI3 kinase was shown to abolish inhibitory effect of insulin on apoB secretion in rat hepatocytes and McA-RH7777 cells [50,54]. Bile salts may inhibit secretion of apoB via a similar signal transduction route as has been proposed for insulin. Activation of PI3 kinase via bile salts can release intracellular calcium stores from ER and Golgi resulting in degradation of specific proteins inside the ER and Golgi [50,53-57].

In conclusion, the studies described in this dissertation demonstrate that bile salts and oxysterols differentially affect production of TG-rich lipoproteins via several pathways, involving both transcriptional and post-transcriptional regulatory routes. *In vitro*, the inhibitory action of bile salts on VLDL secretion appeared to occur at a post-translational level, via inhibition of apoB protein secretion. The underlying mechanism(s) are still unclear. On the other hand, activation of LXR in rat hepatoma cells promoted VLDL-TG secretion, but did not affect apoB secretion. Oxysterols might induce VLDL-TG secretion by affecting lipidation steps in the assembly of TG-rich particles. *In vivo* under normal physiological conditions, widely varying transhepatic bile salt fluxes had small effects on VLDL secretion in mice. However, extra stimulation of the transcription factor LXR or absence of the transcription factor and bile salt receptor FXR, considered as key regulators of genes in cholesterol, bile salt and lipid homeostasis, clearly affected formation of TG-rich lipoproteins by liver and intestine. Disturbances in LXR and FXR-regulated signaling pathways may contribute to development of an atherogenic lipid profile and, in the long run, to cardiovascular diseases. Since many groups have recently focused their research on the regulatory role(s) of LXR and FXR in lipid metabolism, more information about their roles will become available in the near future and may contribute to a better knowledge of the complex interactions between bile salt and lipid metabolism.

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