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SIDE-CHAIN OXIDIZED OXYSTEROLS AS METABOLIC REGULATORS IN LIVER AND BRAIN

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To my beloved family

ABSTRACT

Oxysterols are oxygenated derivatives of cholesterol characterized by a very short half-life and their ability to pass lipophilic membranes easily, thus they are considered as important intermediates in cholesterol excretion pathways and its conversion to bile acids.

The two major oxysterols in the circulation of human and mouse are 24S-hydroxycholesterol (24S-OH) and 27-hydroxycholesterol (27-OH), which are formed by the cytochrome P450 enzymes cholesterol 24-hydroxylase (CYP46A1) and sterol 27-hydroxylase (CYP27A1), respectively. The two oxysterols 27-OH and 24S-OH are both strong inhibitors of cholesterol synthesis and activators of LXR *in vitro*. However, their role as physiological regulators under *in vivo* conditions is controversial.

The overall aim of this thesis was to investigate the regulatory role of side chain oxidized oxysterols as metabolic regulators *in vivo*. In particular we have studied the role of 24S-and 27-hydroxycholesterols (24S-and 27-OH) as regulators of cholesterol synthesis and activators of LXR. We used mouse models with increased levels of 27-OH (CYP27A1) transgenic mice and Cyp7b1 knock-out mice ($Cyp7b1^{-/-}$) as well as a mouse model with no detectable levels of 27-OH in their circulation, Cyp27a1 knock-out mice ($Cyp\ 27^{-/-}$). The latter mice were treated with cholic acid to compensate for the reduced formation of bile acids.

In **Paper I**, we studied a possible regulatory role of 27-OH and 24S-OH in the brain using human CYP27A1 transgenic mice(CYP27A1tg) and Cyp27a1 knock-out ($Cyp\ 27a1^{-/-}$) mice. The levels of 27-OH were increased about 12-fold in the brain of CYP27A1tg mice while the levels of 24S-OH was decreased by about 25%, most probably due to increased metabolism by the CYP27A1 enzyme. The mRNA levels of HMG-CoA reductase and HMG-CoA synthase in the brain were increased. In accordance with increased cholesterol synthesis, most of cholesterol precursors were also increased. The increased cholesterol synthesis is likely due to reduced inhibition by 24S-OH. 27-OH is an activator of LXR and in spite of this, there was no upregulation of the LXR-target genes in the brain of the transgenic mice. In contrast, some of the genes were downregulated. In $Cyp27a1^{-/-}$ mouse brain, cholesterol synthesis was slightly increased with increased levels of cholesterol precursors. The increased synthesis is probably the consequence of the absence of an inhibitory effect of the flux of 27-OH into the brain. The results of this study are consistent with the possibility that both 24S-OH and 27-OH have a suppressive effect on cholesterol synthesis in the brain. Since there was no activation of the LXR-target genes in the brain of the transgenic mice, we concluded that 27-OH is not a general activator of LXR in the brain.

In **Paper II**, this study has examined the role of 27-OH in the liver using the above three mouse models. In the liver of CYP27A1tg mice we found a modest increase of the mRNA levels corresponding to the LXR target genes Cyp7b1, and Abca1. There was no effect on a number of other LXR-regulated genes. There were no significant effects on cholesterol synthesis at the transcriptional level and cholesterol precursors were not affected as well. However, there was a modest decrease in T-MAS levels in the liver of CYP27A1tg mice. In the liver of the $Cyp7b1^{-/-}$mice, there were also no effects on cholesterol synthesis neither at the transcriptional level nor in the levels of cholesterol precursors, with the exception of increase in desmosterol. In connection to the LXR-target genes in these mice, there were no differences in the expression between the $Cyp7b1^{-/-}$ and the wild type mice. If the high levels of 27-OH are important, the same effects would be expected in the two mouse models. In the liver of the $Cyp27a1^{-/-}$ mice there was a slight activation of some LXR-regulated genes, Abcg5, Abcg8, Fas and Srebp1c. If 27-OH is of importance as a normal activator of the above genes a suppressing effect would be expected. The overall results do not support the contention that 27-OH is an important regulator of cholesterol homeostasis or an activator of LXR-regulated genes under basal conditions in the liver.

In conclusion our results suggest that both 24S-OH and 27-OH may be of some regulatory importance for cholesterol synthesis in the brain but not in the liver. Under normal basal conditions 27-OH does not seem to be a general activator of LXR neither in the brain nor in the liver. The different effects on cholesterol synthesis in the two organs may be related to the fact that almost all oxysterols in the brain are in the free form whereas most of them are esterified in the liver.

LIST OF PUBLICATIONS

I. **ALI, Z.***, HEVERIN, M.*, OLIN, M., ACIMOVIC, J., LOVGREN-SANDBLOM, A., SHAFAATI, M., BAVNER, A., MEINER, V., LEITERSDORF, E. & BJORKHEM, I. 2013. On the regulatory role of side-chain hydroxylated oxysterols in the brain. Lessons from CYP27A1 transgenic and Cyp27a$^{-/-}$ mice. *Journal of lipid research*, 54, 1033-43.

II. **ALI, Z.**, HEVERIN, M., OLIN, M., LEITERSDORF, E., WARNER, M., GUSTAFSSON, J. Å. & BJÖRKHEM, I. On the regulatory importance of 27-hydroxycholesterol in mouse liver. *Manuscript*.

* Authors contributed equally to the work

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette, subfamily A, member 1
ABCG1	ATP-binding cassette, subfamily G, member 1
ABCG5	ATP-binding cassette, subfamily G, member 5
ABCG8	ATP-binding cassette, subfamily G, member 8
ACAT	Acyl-CoA: cholesterol acyltransferase
AMK	Adenosine monophosphate protein kinase
APOE	Apolipoprotein E
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CSF	Cerebrospinal fluid
CTX	Cerebrotendinous xanthomatosis
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
FXR	Farnesoid X receptor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
INSIG	Insulin-induced gene
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LRH1	Liver receptor homolog-1
LRP	Low density lipoprotein receptor related protein
LXR	Liver X receptor
RCT	Reverse cholesterol transport
SCAP	SREBP cleavage-activating protein
SHP	Small heterodimer partner
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSD	Sterol sensing domain
VLDL	Very low density lipoprotein

1 BACKGROUND

1.1 CHOLESTEROL

Cholesterol is a hydrophobic molecule with the molecular formula $C_{27}H_{45}OH$ and four rings in its structure (Figure 1). It is a vital structural component of all mammalian cell membranes that is necessary for proper membrane permeability and fluidity. In addition to that cholesterol functions as an important precursor for the biosynthesis of steroid hormones and bile acids.

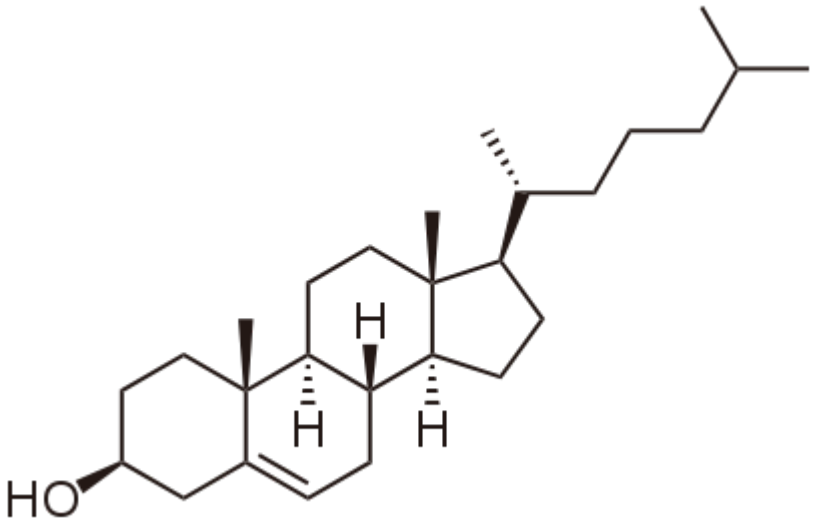

Figure 1. The Chemical structure of cholesterol molecule.

1.1.1 Cholesterol synthesis and its regulatory mechanisms

Cholesterol is synthesized by almost all types of mammalian cells, in particular in liver and intestine (Dietschy et al., 1993). Cholesterol synthesis occurs in five main steps: 1) Condensation of three acetyl-CoA molecules to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) (Bloch, 1987); 2) Conversion of HMG-CoA to mevalonate. This reaction is catalyzed by the enzyme HMG-CoA reductase (HMGCR), which is the rate limiting step in the cholesterol synthesis pathway (Goldstein and Brown, 1990); 3) Formation of the five carbon structure isopentenyl-PP from mevalonate. Isopentenyl-PP is the precursor not only for cholesterol but also for many non-steroidal isoprenoid molecules that are of importance for prenylation of different proteins within the cell; 4) Generation of the 30 carbon atom structure squalene from the isopentenyl-PP through multiple steps; 5) conversion of squalene into lanosterol, which is then converted to cholesterol through a series of reactions (Figure 2).

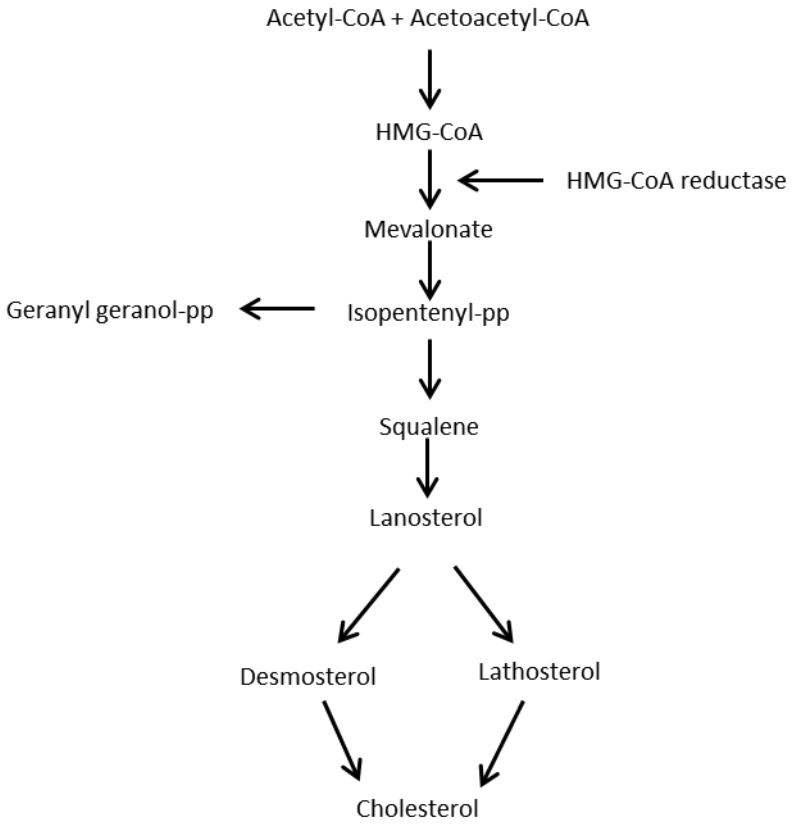

Figure 2. A simplified overview of cholesterol biosynthesis. *Cholesterol synthesis also results in production of non-steroidal isoprenoid molecules (geranyl geranol-PP). PP; pyrophosphate.*

Cholesterol biosynthesis, uptake and turn-over by the cells are among the most strongly regulated processes in the cells. The rate limiting enzyme in cholesterol biosynthesis, HMGCR, is competitively inhibited by the cholesterol lowering drug statin (Stancu and Sima, 2001). Sterol regulatory element binding proteins (SREBPs) are ER membrane-bound transcription factors able to regulate multiple genes involved in cholesterol and fatty acids biosynthesis and uptake (Brown and Goldstein, 1999). There are three isoform of SREBPs. SREBP-1a and SREBP-1c are produced by the same gene and mainly control genes involved in the fatty acid synthesis. SREBP-2 is encoded by a separate gene and controls the transcription of genes involved in cholesterol synthesis (e.g. HMGCR) and uptake (low density lipoprotein-receptor (LDL-R)) (Brown and Goldstein, 1997). In the ER membrane, SREPB-2 is bound to the SREBP-cleavage activating protein (SCAP) which forms a complex with the anchor protein insulin-induced gene (Insig) in the presence of high cholesterol concentrations. SCAP serves as a cholesterol level sensor due to the presence of a specific intra-membrane sequence called sterol sensing domain (SSD). When cholesterol levels drop in the cell, the SREBP-2/SCAP complex is released from the anchor protein Insig and transported to the Golgi, where it is subjected to enzymatic cleavage by two proteases with subsequent release of the active form of SREBP-2. The latter compound enters the nucleus and exerts its action by binding to the sterol regulatory elements (SREs) in target genes (Goldstein *et al.*, 2006) (Figure 3).

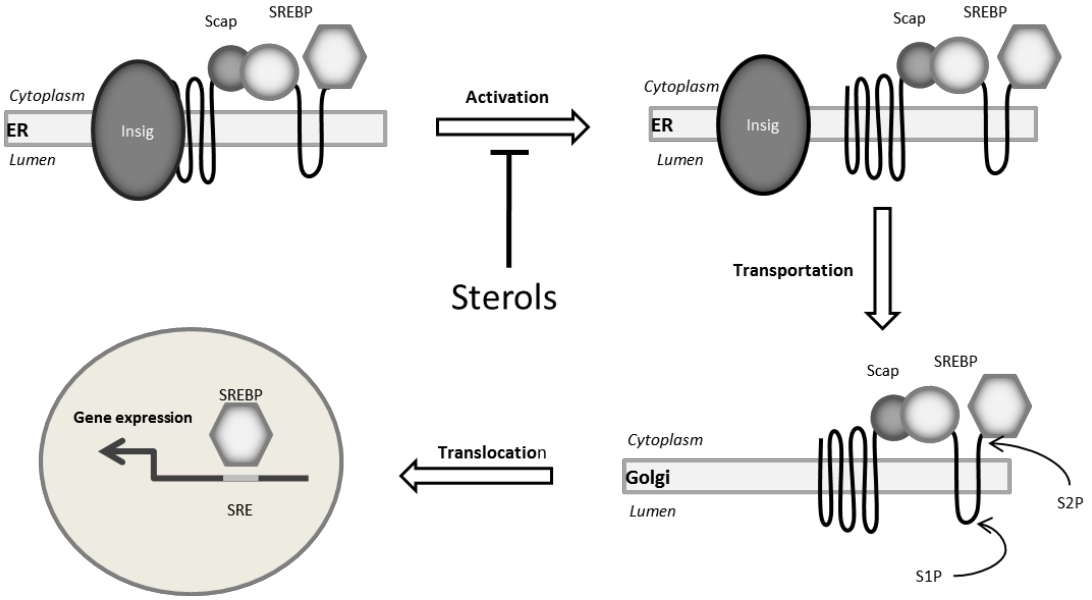

Figure 3. Schematic picture of the SREBP pathway. *In the ER membran, SREBP forms a complex with an escort protein called SCAP that facilitates the transportation of SREBP from the ER to the Golgi where two proteases, S1P and S2P, act on the SREBP/SCAP to release the active transcription factor domain of the SREBP from the membrane. This transcription factor domain is then translocated to the nucleus and enters the nucleus and activates genes encoding enzymes required for the synthesis of cholesterol and fatty acids through binding to the SRE. This process is blocked by cholesterol and oxysterols, so when the levels of cholesterol or oxysterols are sufficient in the cell, the anchor protein Insig binds to the SREBP/SCAP complex and prevents its movement to the Golgi.*

HMGCR has a sterol sensing domain (SSD) similar to that in SCAP. When the concentration of sterols increases in the ER, this will induce binding of the reductase enzyme to the ER proteins Insig-1 and Insig-2. This will lead to recruitment of a membrane-associated ubiquitin ligase that initiates ubiquitination and subsequent degradation of the HMGCR (Sever *et al.*, 2003). The cholesterol synthesis pathway can also be rapidly regulated by phosphorylation and dephosphorylation of the enzyme HMGCR. This enzyme is most active in its unmodified form and phosphorylation by adenosine monophosphate protein kinase (AMK) decreases its activity (Corton *et al.*, 1994; Hardie, 2003).

1.1.2 Cholesterol absorption and transportation

Cholesterol is a hydrophobic molecule, which must be associated with lipoproteins to enable its transport in the blood stream. Dietary cholesterol is absorbed from the intestinal lumen and delivered to the liver by chylomicrons. In the liver cholesterol may be stored as cholesteryl esters, by the action of the enzyme acyl coenzyme A: cholesterol acyltransferase (ACAT), converted to bile acids, secreted directly in the bile or packed in the very low density lipoprotein (VLDL) particles. In the circulation, the triglyceride core of VLDL is subjected to lipolysis by the lipoprotein lipase and there is a transfer of apolipoproteins A and C to HDL. Eventually, hydrolysis of VLDL remnants by hepatic lipase will lead to formation of low density lipoprotein (LDL), which contains relatively high cholesterol content. LDL delivers cholesterol to target tissues through interaction with the LDL-R.

1.1.3 Cholesterol elimination

Free cholesterol is toxic for the cells and is converted to cholesterol esters by the action of the enzyme ACAT in the cells and by the enzyme lecithin: cholesterol acyltransferase (LCAT) in the circulation. The unesterified cholesterol is transported to the liver in the HDL particles. Lipidation of HDL is facilitated by the transmembrane ATP-binding cassette transporter (ABCA1). The HDL is then taken up by the liver, where the cholesterol is metabolized. The flux of excess cholesterol from the peripheral tissues to the liver by HDL is called reverse cholesterol transportation (RCT).

Another major route for elimination of excess cholesterol from the body is by being secreted into the bile (via ABCG5/ABCG8) as free cholesterol for subsequent elimination in the faeces (Graf *et al.*, 2003).

Extrahepatic tissues can also convert cholesterol into hydroxycholesterol by the action of the cytochrome P-450 enzyme CYP27A1 (*cf.* below). This 27-hydroxylation of cholesterol allows a rapid passage through the cell membrane followed by transportation in the lipoproteins back to the liver.

1.2 BILE ACIDS

Bile acids are necessary for emulsification and absorption of dietary lipids, cholesterol and lipid-soluble vitamins in the intestine. The synthesis of bile acids takes place in the liver, where approximately 500 mg of cholesterol is converted into bile acids daily. After secretion of the bile into the intestine about 95% of the bile acids are reabsorbed and get back to the liver in what is called enterohepatic circulation. About 300-500 mg of bile acids avoids this cycle and are excreted in the faeces daily. The liver compensate for this loss by replacing it with a newly synthesized bile acids (~500mg/day) and this is one of the two major mechanisms to eliminate excess cholesterol from the body.

The process of bile acid synthesis involves multiple enzymes and takes two major pathways, the classical (neutral) and the alternative (acidic) (Russell, 2003). The first step in the classical pathway is catalyzed by the rate limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) resulting in the production of cholic acid (CA) and chenodexoycholic acid (CDCA) with CA as the major product. In the alternative pathway CDCA is formed predominantly.

The alternative pathway is initiated by the sterol 27-hydroxylase (CYP27A1) and is believed to contribute to about 25% of the formation of bile acids in rodents. Knocking out the gene Cyp27a1 in mice results in a severe reduction of bile acid synthesis (by about 70%). As a consequence of the decreased levels of bile acids, these mice also show decreased intestinal cholesterol absorption and increased cholesterol synthesis. As a compensatory mechanism, Cyp7a1 is upregulated in the mutant mice (Rosen *et al.*, 1998; Repa *et al.*, 2000). Feeding with cholic acids normalizes the metabolic disturbance in these mice. Unlike mice a mutation in the human gene CYP27A1 results in accumulation of xanthomas in the brain and tendons. This rare disease is called cerebrotendinous xanthomatosis (CTX) and is also characterized by dementia, ataxia and cataracts (Bjorkhem and Leitersdorf, 2000).

The bile acid pool is auto-regulated by the binding of bile acids to the nuclear receptor farnesoid X receptors (FXR) in the liver (Makishima *et al.*, 1999; Chiang, 2004). FXR inhibits CYP7A1 transcription by a mechanism involving activation of small heterodimer (SHP) and liver receptor homologue 1 (LRH1). Bile acids fluxing into the intestine regulate the plasma levels of intestinal fibroblast growth factor (FGF) 19 (in mice FGF15) that in turn regulate the activity of the enzyme CYP7A1 (Inagaki *et al.*, 2005; Lundasen *et al.*, 2006).

1.3 CHOLESTEROL METABOLISM IN THE BRAIN

The brain is very rich in cholesterol and its total cholesterol content is estimated to be about 25% of the total cholesterol in the body. Almost all of it exists as unesterified cholesterol. Most of the brain cholesterol is localized to myelin (about 70%). The myelin sheath is essential for the proper functioning of the brain. Unlike other tissues in the body, the brain cholesterol pool is totally isolated from other pools in the body due to the presence of the blood-brain barrier which is impermeable to lipoprotein-bound cholesterol in the circulation. All cholesterol found in the brain is thus synthesized in situ. Oligodendrocytes, the cells responsible for myelination, and astrocytes are the major cells responsible for cholesterol synthesis in the brain (Bjorkhem and Meaney, 2004).

Cholesterol synthesis in the brain is relatively high during the development period but reaches a very low level in the adult state. As a consequence, the cholesterol in the adult human brain has a long half-life that approximated to be five years (Bjorkhem *et al.*, 1998; Dietschy and Turley, 2004). In spite of this long half-life, the brain still needs to eliminate some of its cholesterol to maintain constant levels. About 1 – 2 mg/day of apolipoprotein E-bound cholesterol (APOE) is excreted from the brain into the cerebrospinal fluid (CSF). However, the conversion of cholesterol into 24S-OH is considered to be the quantitatively major mechanism for elimination of the excess cholesterol from the brain (*cf.* below). The amount of cholesterol eliminated from the human brain through this pathway is estimated to be about 6-7mg/day (Bjorkhem *et al.*, 1998).

Neuronal cells are able to synthesize cholesterol but this synthesis is reduced during its maturation. Therefore neurons are dependent on cholesterol provided by astrocytes (Pfrieger, 2003). Astrocytes are also considered to be the main producer of APOE in the brain, which is the main transporter protein for cholesterol in the central nervous system. APOE-bound cholesterol is taken up by the neurons and other cells via LDL-R and LDL receptor-related protein (LRP). Astrocytes also express the membrane transporter ABCA1, which is also important for cholesterol flux in the brain (Bjorkhem and Meaney, 2004).

1.4 SIDE-CHAIN OXIDIZED OXYSTEROLS

Oxysterols were first identified by Lifschütz about one century after the characterization of cholesterol. They are oxygenated derivatives of cholesterol and can

be formed enzymatically and non-enzymatically. They are characterized by very short half-life and ability to pass lipophilic membranes more easily than cholesterol. The latter property makes them easily redistributed in the body (Lange *et al.*, 1995; Meaney *et al.*, 2002). In particular this is valid for side-chain oxidized oxysterols like 24S-hydroxycholesterol (24S-OH), 27-hydroxycholesterol (27-OH) and 25-hydroxycholesterol (25-OH). Due to these physical property oxysterols are considered as important intermediates in cholesterol excretion pathways and conversion to bile acids.

The two major oxysterols in the circulation of man and rodents are 24S-OH and 27-OH (Bjorkhem, 2009). 24S-OH is formed by the enzyme cholesterol 24-hydroxylase (CYP46A1) that is located almost exclusively to the brain in humans (Bjorkhem *et al.*, 1998; Lund *et al.*, 1999). Unlike cholesterol, this mono-oxygenated molecule is able to cross the BBB to the circulation, where it is taken up by the liver and converted into bile acids and conjugates of un-metabolized or partially metabolised 24S-OH (Bjorkhem *et al.*, 2001). Conversion of cholesterol into 24S-OH is considered to be the main mechanism for the elimination of excess cholesterol from the brain in both man and rodents.

27-OH is formed by the action of the mitocondirial enzyme sterol 27-hydroxylase (CYP27A1) that is widely distributed all over the body. There is a constant flux of 27-OH and metabolites of this oxysterol from extra-hepatic tissues to the liver where it is metabolized into bile acids (Lund *et al.*, 1996). There is a significant uptake of 27-OH by the brain where it is rapidly metabolized into a steroid acid, 7α-hydroxy-3-oxo-4-cholestenoic acid (Heverin *et al.*, 2005). This acid is also able to cross the blood-brain barrier and is rapidly eliminated from the brain and metabolized in the liver (Meaney *et al.*, 2007).

1.4.1 Regulatory roles of the side-chain oxidized oxysterols in cholesterol homeostasis

Under *in vitro* conditions, side-chain oxidized oxysterols are able to regulate critical genes in cholesterol turnover pathways. As mentioned above, cholesterol synthesis is regulated by the controlled movement of SREBP from the ER to the Golgi, where it is subjected to a series of cleavage with subsequent release of the active form and its translocation into the nucleus. This movement is inhibited by either cholesterol itself or oxysterols, thus stopping cholesterol synthesis. Cholesterol induces conformational changes in SCAP and as a consequence SCAP binds to Insig. On the other hand, oxysterols inhibit cholesterol synthesis by binding to Insig followed by binding of Insig to SCAP. The latter binding prevents the transportation of the SREBP/SCAP complex to the Golgi (Radhakrishnan *et al.*, 2007). Side-chain oxidized oxysterols can also induce formation of the HMGCR/Insig complex that leads to ubiquitination and degradation of the enzyme resulting in a rapid inhibition of cholesterol (Sever *et al.*, 2003; Goldstein *et al.*, 2006). It has been reported that 27-OH enhance HMGCR ubiquitination and degradation under *in vitro* conditions (Radhakrishnan *et al.*, 2007).
It should be emphasized that the above mechanisms for regulation of cholesterol homeostasis have been demonstrated in vitro and the importance of oxysterols in

regulation of cholesterol homeostasis in vivo in the presence of great excess of cholesterol, is not known with certainty.

Some of the oxysterols effects on cholesterol homeostasis are believed to be mediated by the nuclear liver X receptor (LXR). There are two isoforms LXRα and LXRβ that form a heterodimer with the retinoid X receptors (Willy *et al.*, 1995). LXRs have an important physiological role in cholesterol and lipid homeostasis as shown by several studies in mice lacking LXR (Peet *et al.*, 1998; Schuster *et al.*, 2002). In rodents, pharmacological activation of LXR results in an upregulation of Srebp-1c, Fas, Abca1, Abcg5, Abcg8, Cyp7a1 and a decrease in Cyp7b1 (Uppal *et al.*, 2007). Activation of the LXR-target genes (ABCA1, ABCG5, ABCG8, and CYP7A1) can be regarded as a defence mechanism preventing accumulation of cholesterol in tissues.

Lehmann *et al.* demonstrated that oxysterols are natural ligands for LXR under *in vitro* conditions (Lehmann *et al.*, 1997). It has been found that 24(S), 25-epoxycholesterol, 24S-OH, and 25-OH are the most potent LXR-activating oxysterols. 27-OH is a less potent activator of LXR than the aforementioned oxysterols but this oxysterols is however, present at relatively high levels and has therefore been suggested as a physiological activator of LXR. Several *in vitro* studies have demonstrated the ability of 27-OH to activate the LXR-responsive genes, ABCA1, ABCG1and APOE (Kim *et al.*, 2009). Fu and his colleagues also reported that 27-OH is an activator of ABCA1 and ABCG1 genes in cholesterol-loaded fibroblast (Fu *et al.*, 2001).

Oxysterols are present in trace amounts only in the circulation and tissues and is always accompanied by 10^3- to 10^6- fold excess of cholesterol. In most tissues this excess of cholesterol in relation to oxysterol, might affects the binding ability of oxysterols to LXR (Bjorkhem, 2009). In the brain, however, the ratio between a side-chain oxidized oxysterol and cholesterol is higher than 1 to 1000. Therefore there is a higher potential for LXR-mediated regulation in the brain than in other tissues.

Neurons are believed to depend on astrocytes for their cholesterol supply that is delivered in an APOE-bound form. Based on *in vitro* studies, it has been postulated by Pfrieger that the flux of cholesterol from astrocytes to neurons is regulated by 24S-OH, which is considered as one of the most efficient activators of LXR *in vitro*. Flux of 24S-OH from the neurons to the astrocytes would be expected to results in transcription of the LXR-target genes ABCA1and APOE in glial cells with subsequent increase in cholesterol efflux (Pfrieger, 2003). This contention is supported by another *in vitro* experiment demonstrating that 24S-OH induces expression of APOE and APOE-mediated efflux of cholesterol via an LXR pathway (Abildayeva *et al.*, 2006).

1.4.2 *In vivo* studies in mice with different levels of side-chain oxidized oxysterols

More than 30 years ago, based on *in vitro* studies, it was hypothesized that oxysterols are important physiological regulators of cholesterol homeostasis (Kandutsch *et al.*, 1978). Since then, many *in vitro* and *in vivo* studies have been done to test the validity of the hypothesis. The important role of oxysterols in bile acid synthesis and in

cholesterol transportation is well documented. However their physiological role as regulators of cholesterol homeostasis *in vivo* is still unclear.

Experiments on mice lacking the enzyme Cyp46a1 revealed a reduction in cholesterol biosynthesis in the brain of the mutant mice by about 40%, but not in other organs (Lund *et al.*, 2003). This reduction in cholesterol synthesis is mainly due to reduced elimination of cholesterol from the brain by its conversion into 24S-OH. In spite of this, the total cholesterol levels in the brain of the mutant mice are normal. The effect of the knocking-out of the enzyme Cyp46a1 on LXR-target genes in the brain was not studied. Behavioral studies in these mice demonstrate severe memory defects (Kotti *et al.*, 2006).

A transgenic mouse model with high levels of 24S-OH has been recently generated by our group (Shafaati *et al.*, 2011). These mice are overexpressing the human enzyme CYP46A1 that consumes cholesterol and converts it to 24S-OH. The over expression induces cholesterol synthesis in these mice with unchanged cholesterol levels in the brain. It is known from *in vitro* studies that 24S-OH is one of the most efficient LXR activators (Janowski *et al.*, 1999). Contrary to expectations, there were no stimulatory effects on the LXR-target genes in the brain and liver of these transgenic mice (Shafaati *et al.*, 2011). These results are consistent with other results acquired by local over expression of CYP46A1 in the cortex and hippocampus of APP23 mice. This group also failed to detect any activation of LXR-target genes in the brain of these mice in spite of the elevated 24S-OH (Hudry *et al.*, 2010).

Knocking-out the enzyme (Cyp27a1) responsible for the formation of 27-OH in mice results in approximately a 75% decrease in bile acid pool, reduced intestinal cholesterol absorption and increased hepatic cholesterol synthesis. Cholic acid supplementation of the diet of these mice reverses all these symptoms (Repa *et al.*, 2000). In a separate study by our group, we found that these mice have a modest upregulation of cholesterol synthesis in the brain as predicted by high lathosterol levels (Bavner *et al.*, 2010). This increase in cholesterol synthesis is observed in the brain of mice treated with cholic acid as well as in non-treated mice. This result is consistent with a possible regulatory role of 27-OH for cholesterol synthesis in the brain. However, the effects of the knocking-out of the gene on LXR-target genes were not studied in this model.

Overexpression of the human CYP27A1 in mice results in 3-5 fold increase in 27-OH in the circulation and tissues. There is no marked effect on cholesterol homeostasis in these mice (Meir *et al.*, 2002). The most striking effect in these mice is the reduced levels of 24S-OH in the circulation of the overexpressor mice, possibly due to metabolism by the overexpressed enzyme (Bjorkhem *et al.*, 2001). Disruption of oxysterol 27-hydroxylase (Cyp7b1) in mice also results in markedly high levels of 27-OH in the circulation and tissues (Li-Hawkins *et al.*, 2000). These mice have normal cholesterol levels in the circulation and normal cholesterol synthesis in the liver. The possible consequences of the high levels of 27-OH on the expression of LXR-target genes were never studied in any of these two models.

A triplet knock-out mouse model that lacks 24S-OH, 25-OH and 27-OH in the circulation and tissues was generated previously (Chen *et al.*, 2007). Due to the

consequences of the knock-out of Cyp27a1, the mice were treated with cholic acid in diet. Challenging these mice with high cholesterol diet failed to induce expression of some established LXR-target genes in the liver. This result led to the conclusion that some of the effects of cholesterol feeding on LXR-target genes are likely to be mediated by side- chain oxidized oxysterols.

The above *in vivo* studies do not support the contention that side-chain oxidized oxysterols are important for cholesterol synthesis and turnover under normal conditions, with the possible exception of the situation in the brain. Except for the situation with high levels of 24S-OH, the effects of high levels of side-chain oxidized oxysterols on LXR-targeted genes have not been studied previously.

2 AIMS

The intention of the present study was to use transgenic mouse models with markedly changed levels of side-chain oxidized oxysterols to evaluate if such oxysterols are of importance in the regulation of cholesterol homeostasis and LXR-target genes in the brain (**Paper I**) and in the liver (**Paper II**).

3 MATERIALS AND METHODS

The following is a brief account of materials and methods used in this thesis. For more details, please refer to the respective papers.

3.1 MATERIALS

3.1.1 Animals

All the mice were between 2-4 months old when experiments were conducted. The mice were specifically generated for these experiments from our on-going breeding colonies in Huddinge University Hospital Animal Facility. The colonies were maintained by breeding heterozygous pairs. The animals were housed under standard environmental conditions with free access to food and water. All the mice were euthanized by CO_2 inhalation at the same time in the morning except Cyp27a1 knock-out mice, which were euthanized by cervical dislocation. Blood and tissues were collected and stored at -80°C.

All experiments in these studies were performed in accordance with the guide lines from the Swedish national board for Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and approved by the Southern Stockholm Ethical Committee.

3.1.1.1 CYP27A1 transgenic mice (CYP27A1 tg)

These mice were generated previously using the b-actin promoter (Meir *et al.*, 2002). The founders were originally obtained from our collaborator Eran Leitersdorf and they were on C57BL/6 background. In this experiment C57BL/6 mice were used as a control group and they were purchased from Charles River Laboratories, Germany.

3.1.1.2 Cyp27a1 knock-out mice (Cyp27a1$^{-/-}$)

This strain was also generated previously on C57BL/6 background (Rosen *et al.*, 1998). Homozygous knock-out mice and their corresponding wild type controls were generated from breeding of heterozygous mice. The diet of the mice was supplemented with 0.05% cholic acid at the age of 6 weeks. This supplementation compensates for the reduced bile acid synthesis in these mice (Repa *et al.*, 2000).

3.1.1.3 Cyp7b1 knock-out mice (Cyp7b1$^{-/-}$)

The Cyp7b1$^{-/-}$ mice used in this project were generated in another laboratory as has been described previously (Rose *et al.*, 2001). They were on C57BL/6 background. In the same way as for Cyp27a1$^{-/-}$ mice, homozygous knock-out mice and their corresponding wild type controls were generated from breeding of heterozygous mice.

3.2 METHODS

3.2.1 Lipid extraction and analysis

Livers and brains were extracted according to Folch method. Folch solution (chloroform/methanol 2:1,v:v), 3 and 10 ml, were added to about 100 mg liver tissue and half of a brain or a hippocampus or a cortex from half of a brain, respectively. After 24 hours at room temperature extracts were transferred to new vials and evaporated under argon. After evaporation the extracts were re-dissolved in 1 ml Folch (liver) or 10 ml Folch (brain) and stored at -20°C until required. Sterols and oxysterols were measured in the Folch extract by gas chromatography–mass spectrometry after alkaline hydrolysis using deuterium-labelled internal standards as previously described (Dzeletovic *et al.*, 1995; Acimovic *et al.*, 2009). In some experiments the hydrolysis step was excluded in order to measure the fraction of unesterified oxysterol.

3.2.2 RNA preparation and real-time PCR

Total RNA was extracted from liver and brain tissues by TRIzol reagent. 1 µg of total RNA was used for the cDNA synthesis. The relative expression levels of target genes were determined by Fast Real-time PCR System (Applied Biosystems). All values were normalized to Hypoxanthine phosphoribosyltransferase (HPRT) mRNA concentrations.

3.2.3 Statistics

Measurements of mRNA are shown as mean ± range as described by Livak and Schmittgen (Livak and Schmittgen, 2001). Determinations of sterols are shown as mean ± SEM. Statistical analysis was done using the unpaired Student t-test.

4 RESULTS AND DISCUSSION

4.1 ON THE REGULATORY ROLE OF SIDE-CHAIN HYDROXYLATED OXYSTEROLS IN THE BRAIN. LESSONS FROM CYP27A1 TRANSGENIC AND CYP27A1-/- MICE (PAPER I)

4.1.1 Effects of overexpression and knocking-out of the gene CYP27A1 on cholesterol synthesis

The plasma and brain levels of 27-OH were about 6- and 11- fold higher in the transgenic mice than in the controls, respectively. 27-OH is a suppressor of cholesterol synthesis under *in vitro* conditions. Therefore we would expect a suppression of cholesterol synthesis in the brain of the transgenic mice. There was, however, an increase in cholesterol synthesis in the brain of the CYP27A1tg mice as shown by the elevated mRNA levels of Hmgcr, Hmgcs and Srebp2 in the brain of the CYP27A1 transgenic male mice (Figure 4d). In accordance with this, most of the cholesterol precursors in the brain of these mice were also increased (Figure 4a).

There was an approximate 25% reduction in the major cerebral oxysterol 24S-OH. It has been demonstrated previously that 24S-OH is metabolized by CYP27A1 (Bjorkhem *et al.*, 2001), which could be the cause of the low levels of 24S-OH. It is most likely that this reduction in the levels of 24S-OH, which is known to be a suppressor of cholesterol synthesis in neuronal cells (Wang *et al.*, 2008), is the reason for the increased cholesterol synthesis. In spite of the high levels of 27-OH in the brain of transgenic mice compared to the controls, the levels are still much lower than the concentration of 24S-OH in the brain.

There is another possibility that the increased cholesterol synthesis in the brain of the transgenic mice is a compensatory mechanism due to the increased consumption of the brain cholesterol by the overexpressed enzyme. It seems less likely that such a metabolic pathway could be of major importance, since the normal expression of CYP27A1 in the brain is low and it is believed that most of the cerebral 27-OH originates from the circulation (Heverin *et al.*, 2005). There was no tendency to reduced levels of cholesterol in the CYP27A1 transgenic mice (Figure 4a).

Knocking-out the Cyp27a1 gene also resulted in increased cholesterol synthesis in the cortex of the brain of the Cyp27a1$^{-/-}$ mice as judged by increased cholesterol precursors (Figure 4c). This result is consistent with the result of a previous work on the brain of this model (Bavner *et al.*, 2010). The most likely explanation for the increased cholesterol synthesis in these mice is the lack of normal flux of 27-OH into the brain that has a slight inhibitory effect on cholesterol synthesis. It should be pointed out that these mice were treated with cholic acid to compensate for their reduced bile acid synthesis. In theory bile acid on itself could have some effects on cholesterol homeostasis in the brain. In the previous work, however, the same results were obtained both with treated and un-treated mice with cholic acid.

4.1.2 Theoretical model for regulation of cholesterol homeostasis in the brain

The main players in the regulation of cholesterol homeostasis in the brain seem to be HMGCR and CYP46A1. Similar to the situation in the liver, HMGCR is subjected to a tight regulation in the brain in order to keep the cerebral cholesterol pool at a constant level. On the other hand, CYP46A1 is subjected to a very little regulation at the transcriptional level (Ohyama *et al.*, 2006). Combining the present results with the previous results (Lund *et al.*, 2003; Wang *et al.*, 2008; Bavner *et al.*, 2010; Shafaati *et al.*, 2011), we arrived to the regulatory model shown in figure 5. This model is consistent with all present results, *in vivo* and *in vitro*, but further studies may be needed for confirmation.

Given the fact that 24S-OH is a substrate for the enzyme CYP27A1, we believe that the increased activity of this enzyme is the explanation for the reduced levels of 24S-OH seen in the brain and the circulation of our mouse model. It should be emphasized that the reduced levels of 24S-OH in the brain may be the consequence not only of an increased metabolism in the brain but also outside the brain (Figure 5). Reduced levels of 24S-OH in the circulation lead to an increased concentration gradient of the oxysterol between the brain and the circulation, which may lead to increased flux from the brain.

After publication of the above results, additional support has been obtained for the contention that the level of 24S-OH in the brain is of importance for cholesterol synthesis in this organ. In a recent unpublished study, our group characterized cholesterol homeostasis in the brain of a mouse model with pericyte-deficiency resulting in a leaking blood-brain barrier (Saeed *et al*, *unpublished study*). The defective blood-brain barrier resulted in a flux of cholesterol from the circulation into the brain and an increased flux of 24S-OH from the brain into the circulation. The latter flux leads to a reduction in the level of 24S-OH in the brain that was similar to the reduced levels of 24S-OH in the brain of our CYP27A1tg mice. Also in this case the reduced levels of 24S-OH were associated with increased cholesterol synthesis in the brain. If the increased levels of 27-OH in the brain of CYP27A1tg mice had been the reason for the increased cholesterol synthesis, the same effect would have been expected in the brain of $Cyp7b1^{-/-}$ mice. However, neither cholesterol synthesis genes nor cholesterol precursors (Figure 4b), were affected by the high levels of 27-OH in this mouse model and the levels of 24S-OH were similar to those of the controls (*unpublished results*).

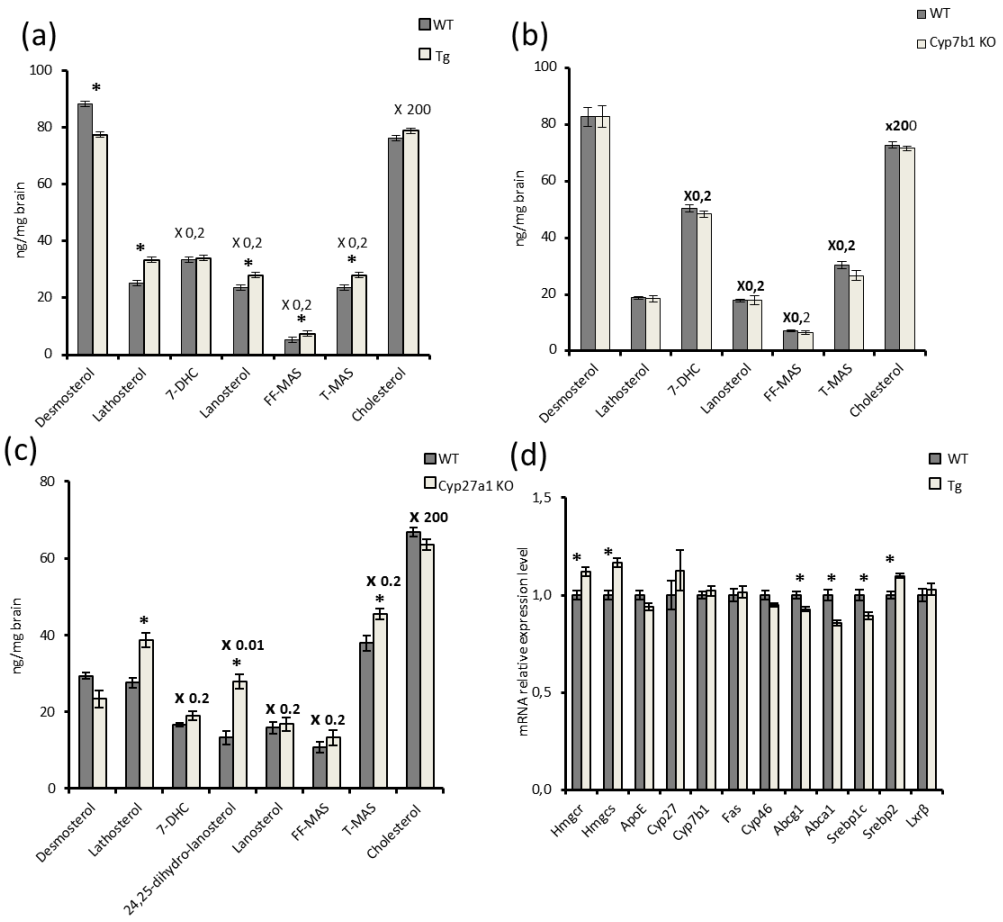

Figure 4. The effects of 27-OH on the cholesterol synthesis and LXR-target genes in the adult brain of male mice from different mouse models. *(a), the effect of overexpression of human CYP27A1 on the levels of cholesterol and cholesterol precursors in the brain of CYP27A1 transgenic mice; (b) levels of cholesterol and cholesterol precursors in the brain of Cyp7b1 knock-out mice; (c) levels of cholesterol and cholesterol precursors in the brain of Cyp27a1 knock-out mice; (d) mRNA levels of SREBP-regulated and LXR-regulated genes in the brain of CYP27A1 transgenic mice. n = 7-10 in each experiment. Data are presented as mean ± SEM. * P < 0,05.*

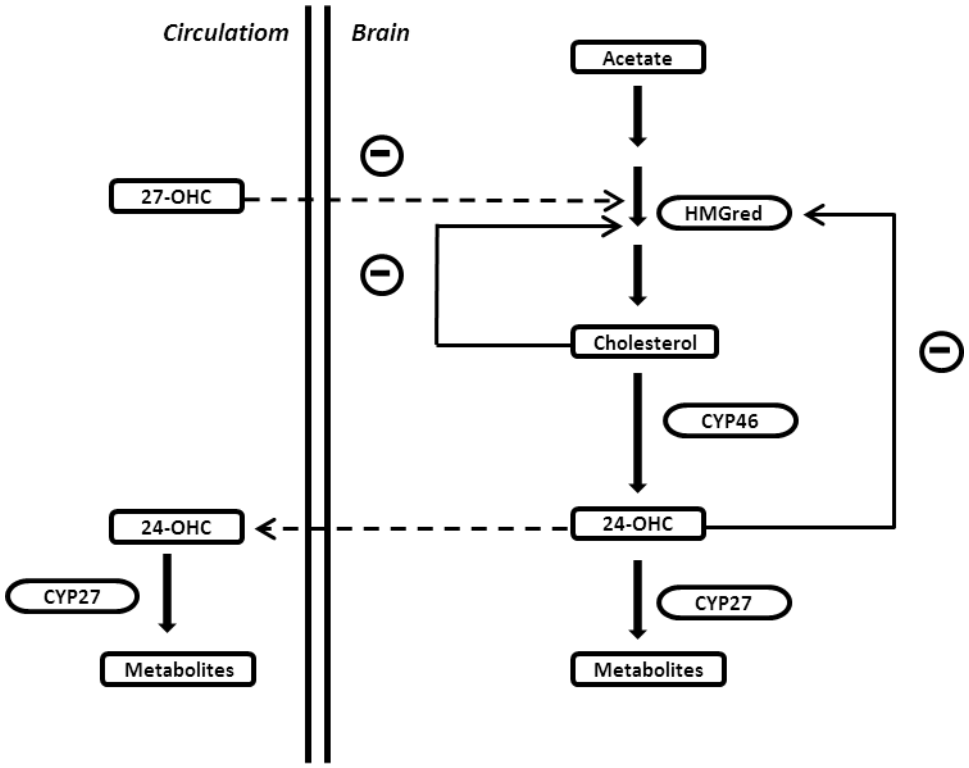

Figure 5. Theoretical model for the regulation of cholesterol homeostasis in the brain.

4.1.3 Effects of overexpression and knocking-out of the gene Cyp27a1 on LXR-targeted genes

In contrast to our expectation the overexpression of CYP27A1 led to moderate changes only in the expression of LXR targeted genes. Some of the LXR target genes (Abcg1, Abca1, and Srebpc1) were slightly decreased by the overexpression rather than increased. On the other hand, there were no changes on the LXR-target genes in the brain of the Cyp27a1$^{-/-}$ mice.

It has been reported that sulphatation of 25-OH is an antagonist mechanism for the oxysterol-dependent activation of the LXR (Xu *et al.*, 2010). Therefore we considered the possibility that the opposite effect on LXR target genes that we observed could be due to a sulphated form of 27-OH. In a very recent study from our laboratory we found that the fraction of the total level of the sulphated 27OH in the CYP27A1tg mice is similar to that in the wild-type mice (Acimovic *et al.*, 2013). Thus it is seems less likely that 27OH sulphate is of importance for the effects observed.

An alternative explanation is that reduction in the pool of 24S-OH is the cause of the above opposite effect, since 24S-OH is an efficient activator of LXR *in vitro*. In a previous study on mice with high levels of 24S-OH in the brain, there was a normal expression of the LXR-target genes in the brain (Shafaati *et al.*, 2011). The same result was obtained from another recent study on the pericyte deficient mouse model that have reduced levels of 24S-OH in their brain (Saeed *et al, unpublished study*). The results from these two studies do not support the hypothesis that the reduced level of 24S-OH is the cause of the downregulation of LXR-target genes observed in the brain of the transgenic mice.

In conclusion our data in this study does not support the contention that 27-OH and 24S-OH are important activators of LXR in the brain, at least not under normal conditions. It seems likely, however, that 24S- and 27-OH have a suppressive effect on cholesterol synthesis in the brain.

4.2 ON THE REGULATORY IMPORTANCE OF 27-HYDROXYCHOLESTEROL IN MOUSE LIVER (PAPER II)

4.2.1 Effects of 27-OH on cholesterol synthesis in the liver

It has been shown that 27-OH is a strong inhibitor of cholesterol synthesis under *in vitro* conditions (Axelson and Larsson, 1995; Schroepfer, 2000). In this study we were interested to investigate the effect of 27-OH on cholesterol synthesis and LXR-target genes *in vivo*, particularly in the liver. We used two mouse models (Cyp7b1$^{-/-}$ and CYP27A1tg mice) with high levels of 27-OH in their circulation and liver, plus a model with undetectable levels of 27-OH in the circulation (Cyp27a1$^{-/-}$ mice). The advantage of using two different models with high levels of 27-OH is that, any effect due to 27-OH itself should be seen in both models.

There was no downregulation of cholesterol synthesis in the liver of Cyp7b1$^{-/-}$ and CYP27A1tg mice as judged by normal mRNA levels of cholesterol synthesis genes

(Hmgcr, Hmgcs and Srebp2) as well as normal levels of most cholesterol precursors in the liver (Figures 6 & 7).

Our results are in agreement with the previous results (Li-Hawkins *et al.*, 2000), where they failed to detect effects on cholesterol synthesis in the liver of Cyp7b1$^{-/-}$ mice. The results are also consistent with the previous work in our laboratory, in which a trial to demonstrate a critical role of the 24S-, 25 or 27-hydroxycholesterol for cholesterol-induced downregulation of Hmgcr in mouse liver failed (Lund *et al.*, 1992).

4.2.2 Effects of 27-OH on LXR-targeted genes in the liver

27-OH is less potent than 24S-OH in activation of the LXR mechanism. However, it is present at a higher concentration than 24S-OH in the circulation and all tissues apart for the brain and this increases it's potential to stimulate LXR. Since there were high levels of this oxysterol in the circulation and the liver of the Cyp7b1$^{-/-}$ and CYP27A1tg mice, we expected a possible upregulation of the LXR-targeted genes in these mice. There were no effects in the liver of the Cyp7b1$^{-/-}$ mice, while there was a slight upregulation of the genes Abca1 and Cyp7b1 in the CYP27A1tg mice. If such an effect is due to the high levels of 27-OH, it should be seen in both models. Activation of LXR would result in inhibition of the gene Cyp7b1 rather than activation (Uppal *et al.*, 2007). A previous study by our group (Shafaati *et al.*, 2011) also failed to demonstrate any activation of LXR-target genes in the liver of CYP46tg mice in spite of the fact that 24S-OH is a potent activator of LXR (Janowski *et al.*, 1996).

Unexpectedly, there was an activation of some LXR-targeted genes Fas, Abcg5, Abcg8 and Srebp1c in the liver of the Cyp27a1$^{-/-}$ mice (Fig. 7). Since these mice were treated with cholic acid, we cannot exclude the possibility that this effect could be due to the cholic acid rather than to 27-OH. The suppression of the gene Cyp7a1 is most likely a secondary effect to the cholic acid feeding. The other explanation for the upregulation of the above genes is the existence of an unknown activator of LXR that is normally metabolized by Cyp27a1. A lack of this enzyme can then be expected to be associated with increased activation of LXR. Further work is needed to discriminate between these two possibilities.

Most of the oxysterols in the liver and circulation (90%) are present in the esterified form (Dzeletovic *et al.*, 1995; Li-Hawkins *et al.*, 2000). The opposite is observed in the brain, where 90% or more of the cholesterol and oxysterols are in the unesterified form. We have found that 98% of 24S-OH and 90% of 27-OH in mouse brain are present in free form (*unpublished observation*). The presence of most 27-OH in the esterified form, which is believed to be the inactive form of the oxysterols, could be the explanation for the failure of 27-OH to exert any effect on cholesterol synthesis and LXR in the liver of Cyp7b1$^{-/-}$ and CYP27A1tg mice.

The results from different models suggest that 27-OH is unlikely to be an important regulator of cholesterol synthesis and LXR in the liver under basal conditions. However the situation might be different under some specific pathological conditions.

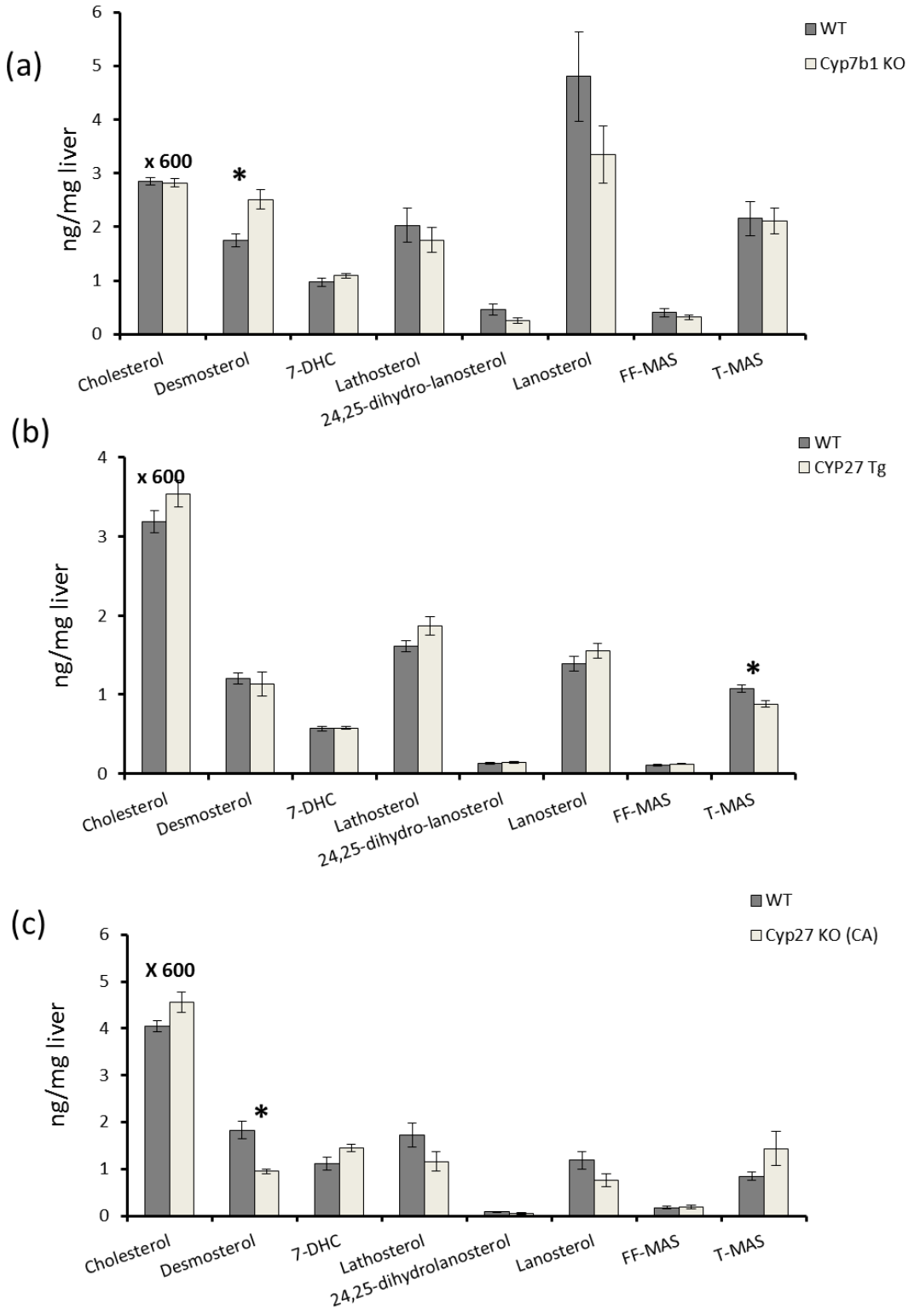

Figure 6. Levels of cholesterol and cholesterol precursors in the liver of Cyp7b1 knock-out mice (a), CYP27A1 transgenic mice (b) and Cyp27a1 knock-out mice (c). *n = 7-10 in each experiment. Data are presented as mean ± SEM. * P < 0,05.*

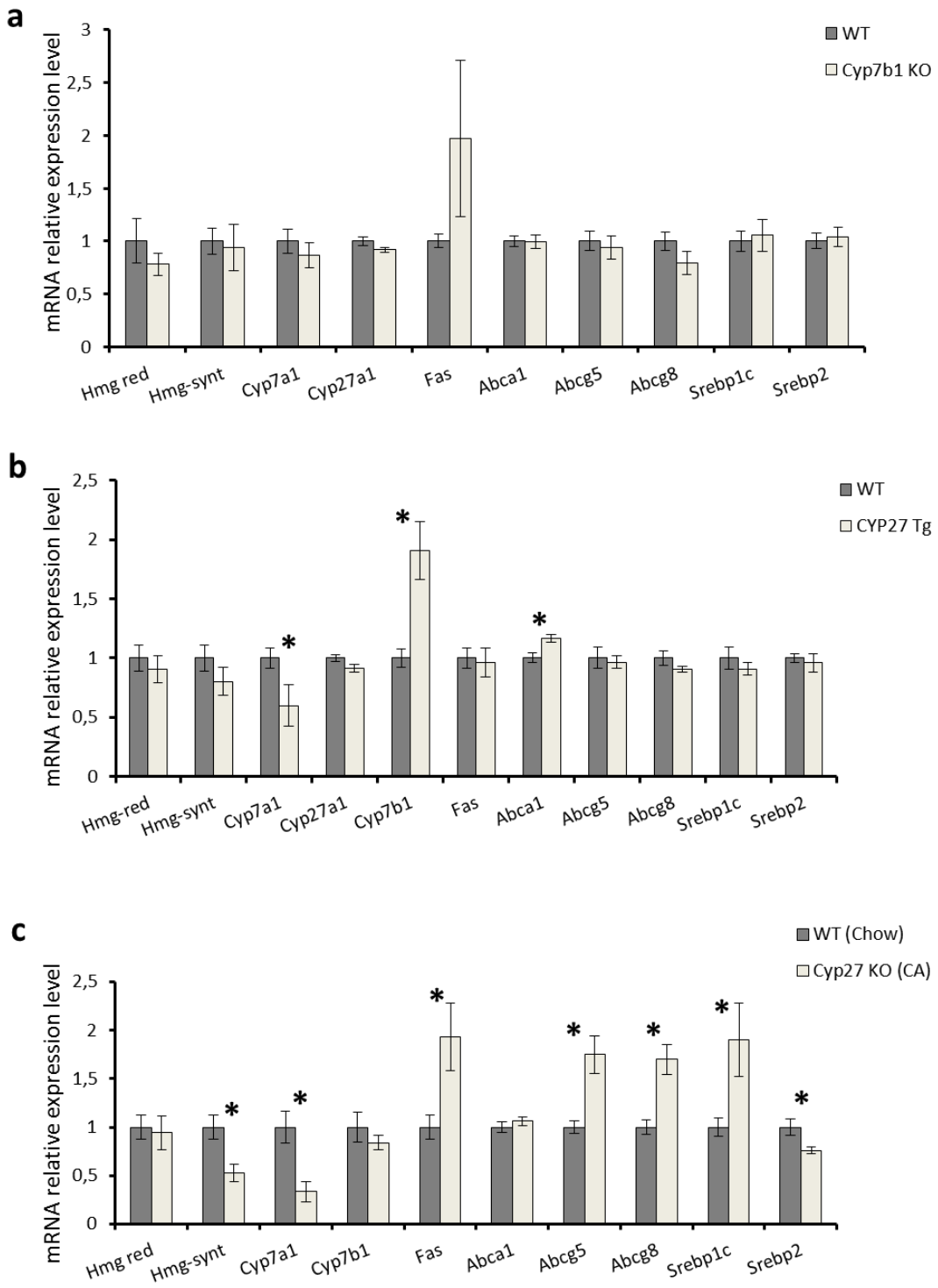

Figure 7. mRNA levels of SREBP-regulated and LXR-regulated genes in the liver of Cyp7b1 knock-out mice (a), CYP27A1 transgenic mice (b) and Cyp27a1 knock-out mice (c). *n = 7-10 in each experiment. Data are presented as mean ± SEM. * P < 0,05.*

5 CONCLUSIONS

Paper I – The results are consistent with the possibility that both 24S-OH and 27-OH are modest physiological suppressors of cholesterol synthesis in the brain but not important activators of the LXR-target genes in this organ.

Paper II – Using two mouse models with high levels of 27-OH, we failed to demonstrate any regulatory role for 27-OH on cholesterol synthesis or on LXR-target genes in the liver under normal conditions.

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