Damjana Rozman
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Contents

Sterols from the Post-Lanosterol Part of Cholesterol Synthesis: Novel Signaling Players

Cene Skubic and Damjana Rozman

Introduction

Cholesterol is the major sterol in all mammalian cells and is crucial for viability of cells. It is the only lipid not dedicated to metabolism of energy and its storage. The majority of cholesterol resides in cellular membranes, where it influences the order of phospholipid chains and contributes to membrane fluidity, integrity and heterogeneity. Further roles of cholesterol also include cell cycle regulation and protein modification, as well as being the starting point for the synthesis of steroid hormones and bile acids (Fig. 1).

The synthesis of cholesterol is a housekeeping pathway and supposedly takes place in all mammalian cells. In mammals, there are more than 100 genes associated with cholesterol synthesis and synthesis regulation [1]. Cholesterol synthesis itself includes over 20 reactions, starting from acetyl coenzyme A [2]. This chapter focuses on the second part of cholesterol synthesis, from lanosterol on (Fig. 2). It is called the post-squalene or sometimes post-lanosterol portion of cholesterol synthesis since lanosterol is the first sterol intermediate in the pathway. Initially, the post-squalene pathway has been divided into the Bloch and Kandutsch–Russell branches [3]. In the Bloch branch, the final reaction is the conversion of desmosterol to cholesterol by sterol-Δ24-reductase (DHCR24); thus, all intermediates from lanosterol to desmosterol contain Δ24 double bonds. In contrast, in the Kandutsch–Russell branch, DHCR24 acts already on lanosterol; thus all intermediates from 24,25-dihydrolanosterol to 7-dehydrocholesterol contain a saturated side chain. Since DHCR24 can, in principle, metabolize any cholesterol synthesis intermediate from lanosterol on, the two branches cannot be treated separately. Study of DHCR24 substrate specificity in vitro showed 24-dehydrolathosterol as the most

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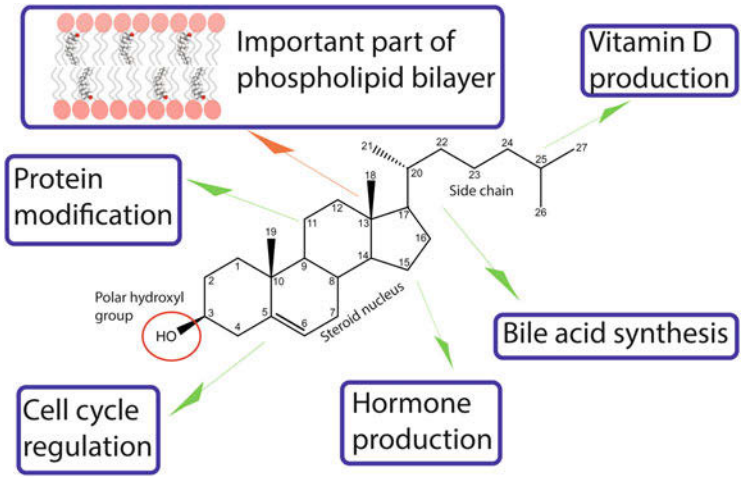


Fig. 1 The molecular structure of cholesterol with carbon atoms numbering based on IUPAC-IUB recommendations. Red ring represents the polar hydroxyl group of cholesterol molecule and the rest of molecule is nonpolar. The figure represents major roles of cholesterol in the human body. The major function of cholesterol is in the membrane as the basic part of phospholipid bilayer

reactive substrate, suggesting that cholesterol synthesis preferentially starts with the Bloch branch from lanosterol to 24-dehydrolathosterol and is then shifted to Kandutsch–Russell branch via lathosterol. In this case, 7-dehydrocholesterol presents the last intermediate before cholesterol. According to theoretical predictions, there could be many more intermediates in cholesterol synthesis [4]. The predicted number of sterol metabolites from lanosterol to cholesterol is 72, comprising a metabolic network. This has been calculated by an algorithm that applied a binary code, labelling each C atom of the sterol ring by either 1, if containing a substituents, or 0 for no substituents. However, two important facts were not considered during enzyme activity coding. First, the enzymes SC4MOL, NSDHL, and HSD17B7 remove the methyl groups at position 4 in a specific order, where methyl group at position a is removed first. After its removal, methyl group at position b rotates to position a and is removed in the following step. The coding system was designed to remove the 4b methyl group first to create molecules that have one methyl group at position 4a. Second, a single bond at position 7(8) seems to completely inhibit activity of SC5DL, as no molecules with single bond at 7 (8) and double bond at 5(6) were found in any reports. A single bond at 7(8) may influence positioning of hydrogen atoms at position 6 and thus may prevent interaction of substrate with SC5DL [4].

We will now describe the major intermediates of cholesterol synthesis, their roles as signaling molecules, and links to human diseases.

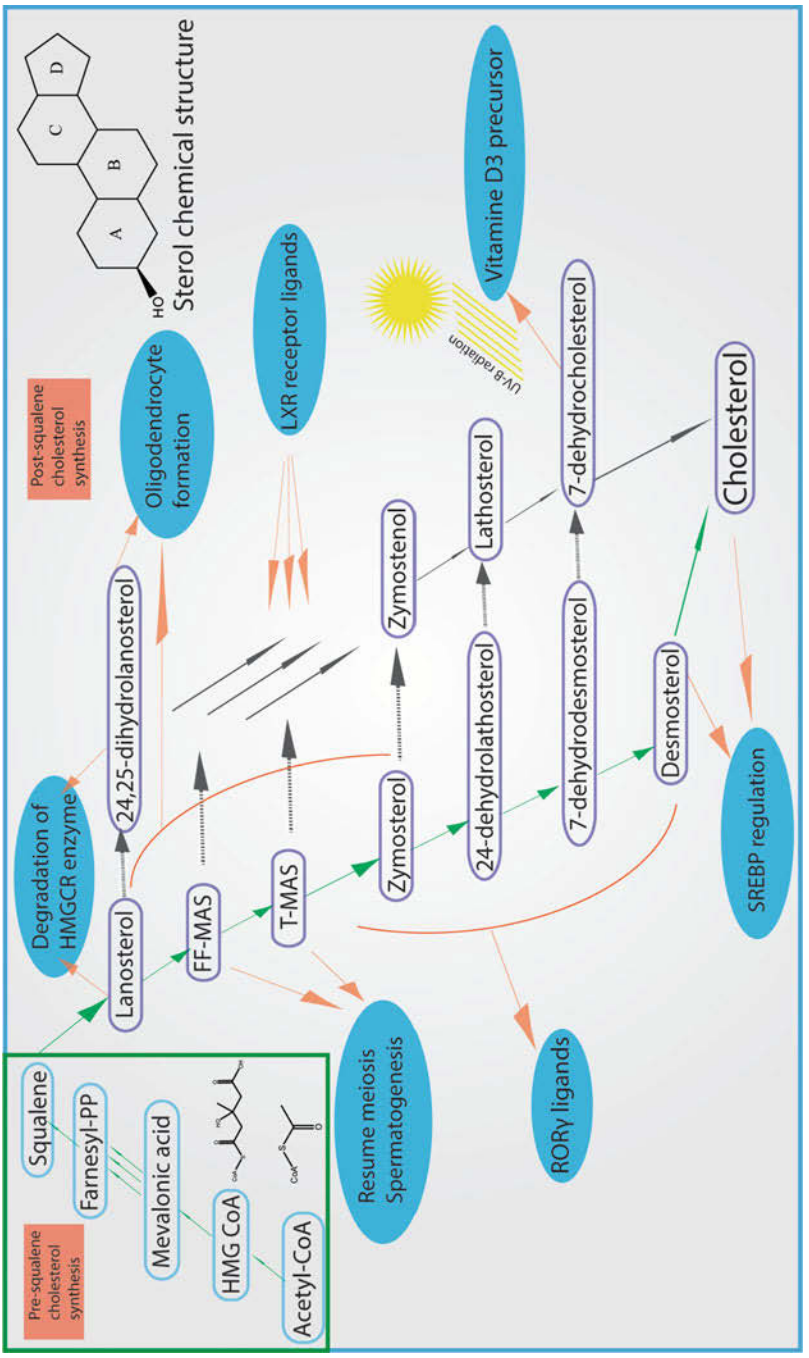


Fig. 2 Cholesterol synthesis with its intermediates. Figure represents the major cholesterol intermediates and their physiological functions in blue circles. The synthesis is from two parts, the pre-squalene in green square (not discussed in this work) and post-squalene in which lanosterol is the first molecule with sterol structure. With green arrows, the most probable synthesis pathway is shown

Sterol Intermediates from Lanosterol to Cholesterol

Lanosterol and 24,25-Dihydrolanosterol: The Branch Points of Sterol Synthesis

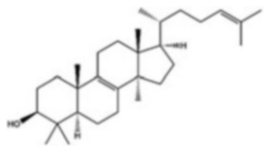
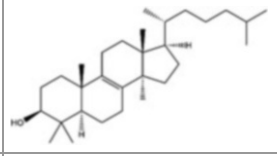
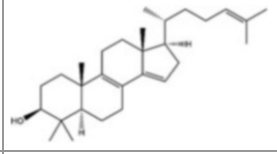
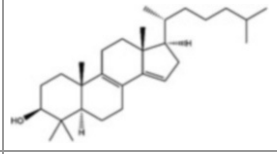
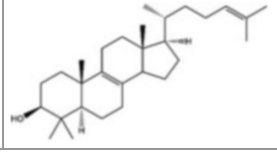
Lanosterol is the first cyclic intermediate of cholesterol synthesis pathway and the first sterol molecule in the post-squalene part of the synthesis (Sterol structures in Table 1). Lanosterol is formed from squalene by squalene epoxidase/monooxsyenase (SQLE) and lanosterol synthase (LSS) [5]. From lanosterol on the cholesterol synthesis is done by enzymes that are membrane bound in endoplasmic reticulum [6]. In the Bloch branch lanosterol is converted into FF-MAS (Follicular fluid meiosis-activating sterol) by CYP51 enzyme [7].

Addition of lanosterol efficiently stabilizes co-chaperon CHIP. CHIP acts as a molecular switch for both proteasomal and lysosomal mechanisms, which reduce aggregation of misfolded proteins, that often cause pathological conditions [8]. According to some studies, lanosterol surprisingly reverses protein aggregation in cataracts of animals. Lanosterol can dissolve precipitates and even amyloid-like fibril structures, which are the cause of cataracts in individuals. Lanosterol effectively treats cataracts in rabbit and dog lenses in vivo. Treating the cataract lenses with lanosterol was not yet successful in humans [9]. The "dissolving" ability of lanosterol seems to be partially controversial. Sterol intermediates of cholesterol synthesis, in particular desmosterol (discussed later), have been accused to cause cataracts and resulted in removal of a cholesterol lowering drug Triparanol from the market in early 1960s, as reviewed [10]. Triparanol was supposed to lower the level of cholesterol in the blood and reduce the risk of heart attacks. However, many people who took the drug went blind from an unusual form of cataract. It was suggested that accumulating desmosterol is causing the cataracts, and consequently, that all sterol intermediates toward cholesterol, from lanosterol and on, likely have harmful effects and should not accumulate in the cells. This also negatively influenced further drug development efforts to find novel hypolipidemic drugs with non-statin properties, that would inhibit enzymes in the late part of cholesterol synthesis [10].

For lanosterol, it was proven to have toxic effects in CHO-7 cells. Addition of lanosterol to cholesterol auxotrophs failed to support growth and killed the cells. Surprisingly, lanosterol killed also the wild-type cells, which underlines its toxic effect. The explanation has been that lanosterol indirectly suppressed cholesterol synthesis [11] and promoted the degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA reductase), and in this way downregulated cholesterol synthesis [12]. Alternative explanations claimed that sterols that have two methyl groups at position 4, such as lanosterol, cannot replace cholesterol in membranes, which results in defective membranes and cell death.

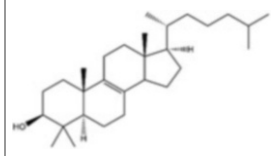
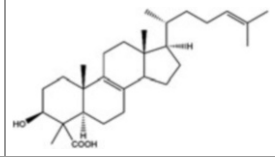
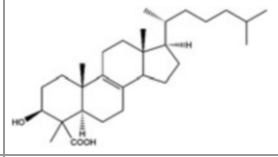
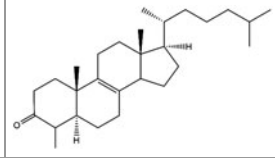
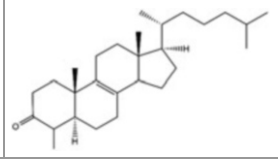
Recently Hubler et al. discovered new roles of sterols that have a double bond between C8 and C9 like lanosterol, FF-MAS, T-MAS, and zymostenol. Their results are showing that these sterols have an important role in oligodendrocytes formation.

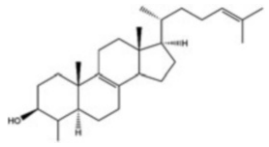
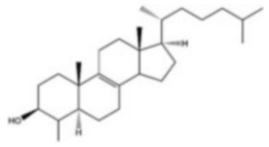
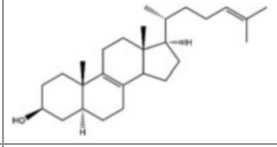
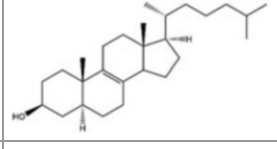
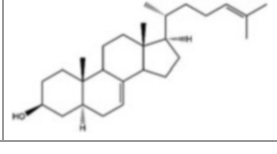
Table 1 The 19 known cholesterol intermediates, from lanosterol toward cholesterol

Trivial name	Chemical name	Formula	Structure	Molecular weight (g/mol)	Converted by
Lanosterol	4,4,14α-Trimethyl-5α-cholesta-8(9),24-dien-3β-ol	$C_{30}H_{50}O$		426.72	CYP51A1
24,25-Dihydrolanosterol	4,4,14α-Trimethyl-5α-cholest-8(9)en-3β-ol	$C_{30}H_{52}O$		428.73	CYP51A1
FF-MAS	4,4-Dimethyl-5α-cholesta-8(9),14,24-trien-3β-ol	$C_{29}H_{46}O$		410.67	TM7SF2, LBR
Dihydro-FF-MAS	4,4-Dimethyl-5α-cholesta-8(9),14-dien-3β-ol	$C_{29}H_{48}O$		412.69	TM7SF2, LBR
T-MAS	4,4-Dimethy-5α-cholesta-8(9),24-dien-3β-ol	$C_{29}H_{48}O$		412.69	SC4MOL

(continued)

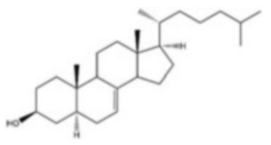
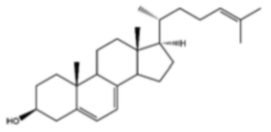
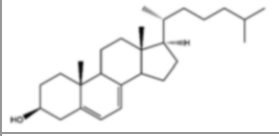
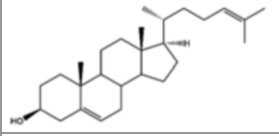
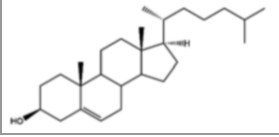
Table 1 (continued)

Trivial name	Chemical name	Formula	Structure	Molecular weight (g/mol)	Converted by
Dihydro-T-MAS	4,4-Dimethy-5α-cholest-8(9)-en-3β-ol	$C_{29}H_{50}O$		414.69	SC4MOL
4-Methyl-4-carboxyzymosterone		$C_{29}H_{46}O3$		442.34	NSDHL
4-Methyl-4-carboxy zymostenone		$C_{29}H_{48}O3$		444.36	NSDHL
4-Methyl zymosterone		$C_{28}H_{44}O$		396.34	HSD17B7
4-Methyl zymostenone		$C_{28}H_{46}O$		398.35	HSD17B7

4-Methyl zymosterol		$C_{28}H_{46}O$		398.35	SC4MOL NSDHL HSD17B7
4-Methyl zymostenol		$C_{28}H_{48}O$		400.37	SC4MOL NSDHL HSD17B7
Zymosterol	5α-Cholesta-8(9),24-dien-3β-ol	$C_{27}H_{44}O$		384.64	EBP
Zymostenol	5α-Cholest-8(9)-en-3β-ol	$C_{27}H_{46}O$		386.65	EBP
24-Dehydrolathosterol	5α-Cholesta-7,24-dien-3β-ol	$C_{27}H_{44}O$		384.64	SC5DL

(continued)

Table 1 (continued)

Trivial name	Chemical name	Formula	Structure	Molecular weight (g/mol)	Converted by
Lathosterol	5α-Cholest-7-en-3β-ol	$C_{27}H_{46}O$		386.66	SC5DL
7-Dehydrodesmosterol	5α-Cholesta-5,7,24-trien-3β-ol	$C_{27}H_{46}O$		386.66	DHCR7
7-Dehydrocholesterol	5α-Cholesta-5,7-dien-3β-ol	$C_{27}H_{44}O$		384.64	DHCR7
Desmosterol	5α-Cholesta-5,24-dien-3β-ol	$C_{27}H_{44}O$		384.64	DHCR24
Cholesterol	5α-Cholest-5-en-3β-ol	$C_{27}H_{46}O$		386.66	

Each sterol is represented with its molecule structure, chemical formula, molecular weight, and enzyme uses this sterol as substrate in the cholesterol synthesis pathway

By inhibition of enzymes in sterol synthesis, they found that 8,9-unsaturated sterols that accumulate in these cells are responsible for the promotion of oligodendrocyte formation and remyelination. The mechanism through which sterols promote oligodendrocyte formation is poorly understood. It is suspected that nuclear hormone receptors (NHR) have a role in this, but reporter assays with 20 different NHRs that are known to interact with sterols have not shown any activity in the case of oligodendrocytes. This means that the mechanism by which 8,9-unsaturated sterols promote oligodendrocytes is not yet fully understood [13].

There is no nuclear receptor identified that would be specific for lanosterol. It was shown that lanosterol can activate RORC but with a lower activity compared to some other sterols discussed below [14].

24,25-Dihydrolanosterol (DHL) can be converted from lanosterol by enzyme DHCR24 at the beginning of Kandutsch–Russell pathway of cholesterol synthesis. DHCR24 catalyzes the reduction of Δ^{24} bond in lanosterol [2]. But in most tissues lanosterol is predominantly converted to FF-MAS as proposed in the Bloch pathway. There is some evidence that 5–10% of lanosterol in the liver is converted to DHL, but not to downstream intermediates via CYP51 as proposed in Kandutsch–Russell pathway. There is possibility that some of hepatic DHL is converted to another sterol, the identity of which remains unknown [15].

DHL has a major role in posttranscriptional regulation of HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase), which catalyzes the reduction of HMG CoA (3-hydroxy-3-methylglutaryl-coenzyme A) to mevalonate. This is a rate-limiting step in the entire cholesterol synthesis. DHL inhibits HMGCR by triggering degradation and ubiquitination of the enzyme. DHL causes conformational changes in HMGCR to enable interactions with the INSIG proteins. INSIG proteins recruit the E3 ubiquitin ligase gp78 and proteasome-associated protein VCP, which results in ubiquitination and degradation of HMGCR. In this way, DHL affects cholesterol synthesis and its homeostasis [16]. Because DHL is supposedly converted from lanosterol mainly in the liver, the function of cholesterol regulation by DHL through HMGCR is likely valid only for the liver [15].

Meiosis Activating Sterols FF-MAS and T-MAS

Follicular fluid meiosis-activating sterol (FF-MAS) was first identified in human ovarian follicular fluid [17]. It is a cholesterol synthesis intermediate from the Bloch pathway. It is converted from lanosterol by the previously mentioned enzyme lanosterol 14α-demethylase (CYP51). CYP51 belongs to the cytochrome P450 superfamily and converts lanosterol (and DHL) into FF-MAS together with microsomal electron transferring protein NADPH-P450 reductase [18]. The highest concentrations of FF-MAS are found in the ovary, for instance in human preovulatory follicular fluid the concentration of FF-MAS is around 1.3 μM (T-MAS concentration is half of FF-MAS) [19]. The next step in cholesterol synthesis is reduction of Δ^{14} bond in FF-MAS to form testis meiosis-activating sterol (T-MAS) that will be discussed in detail in the next paragraph. This reduction can be done by

two different enzymes encoded by two different genes, the sterol-Δ14-reductase (DHCR14) and lamin B receptor (LBR) [2]. There is evidence suggesting that mice models with DHCR14 deletion were still able to normally synthesize cholesterol [20]. On contrary, HeLa cell lines with LBR knockout were unable to effectively sustain cholesterol synthesis, despite the presence of DHCR14, which means LBR is necessary for normal cholesterol synthesis [21]. FF-MAS can also be converted by enzyme DHCR24 to form FF-MAS with saturated side chain—dihydro-FF-MAS, which is a part of Kandutsch–Russell pathway [2].

Meiosis activating sterols (MAS) were first isolated from preovulatory follicular fluid from women undergoing treatment for infertility by in vitro fertilization (FF-MAS) and from bull testicular tissue (T-MAS). MAS function as stated in name, was first associated with function of activation of oocyte meiosis [17].

Meiosis is crucial for sexual reproduction of animals and forming haploid genetically balances gametes. In mammals, oogonia enter meiosis and are transformed into oocytes. Oocytes are arrested in late prophase of the first meiotic division at diploid stage. Meiosis does not resume until the follicular unit is not mature, fully grown, and can respond to gonadotropin hormones (FSH, LH), which resume meiosis and ovulation [19]. FF-MAS has the ability to resume meiosis of oocyte [17] in the gonadotropin-dependent mechanism. FF-MAS probably works through receptor-mediated mechanism and efforts have been invested to find specific MAS receptor. Liver X receptor (LXR) is a nuclear receptor that binds FF-MAS and several oxysterols. It was a candidate for receptor-mediated mechanism of oocyte meiosis resumption, but was excluded because FF-MAS has lower affinity for LXR than oxysterols [22]. Another reason is that none of oxysterols that can bind to LXR was able to resume oocyte meiosis [19]. Data indicate that MAS signaling may work through a G-protein-coupled mechanism and may be mediated by binding to an oocyte plasma membrane-associated protein with high affinity for FF-MAS. There is a lot of evidence suggesting the role of FF-MAS in resumption of oocyte meiosis, but another set of data suggested that FF-MAS might not be a universal mediator of hormone-induced meiotic maturation. In vivo role of MAS is not clear and stays enigma [23], some experimental evidence oppose the in vivo roles of MAS. The delay in germinal vesicle breakdown after addition of MAS or AY-9944, an inhibitor of DHCR7, a later enzyme of cholesterol synthesis, was at that time the strongest evidence against the suggested role of MAS as an essential mediator of luteinizing hormone in meiosis resumption [24]. It was, on the other side, proposed that the MAPK signaling pathway is required for the MAS-like resumption of meiosis activated through one of the hormonal upstream pathways [25].

T-MAS is converted from FF-MAS with enzymes DHCR14 and LBR [2]. It was first isolated from bull testicular tissue using high-performance liquid chromatography [17]. T-MAS is the part of cholesterol synthesis pathway and is generated in all cholesterol-synthesizing cells. In most cell types T-MAS immediately converted to zymosterol [26] through several intermediates catalyzed by sterol 4,4-dimethylase enzyme complex. The first step is carboxylation of one of the 4-methyl groups by enzyme sterol-C4-methyl oxidase (SC4MOL) to form 4-methyl-4-carboxyzymosterone. 3β-hydroxy-Δ5-steroid-dehydrogenase (NSDHL)

then converts 3β-hydroxy group to 3-keto by removing one of the CO_2 and 4-methylzymosterone is formed, which is then converted to 4-methylzymosterol by enzyme 3β-keto-reductase (HSD17B7) by restoring 3β-hydroxy group. Enzymatic process is then repeated one more time and zymosterol is formed [2]. FF-MAS and T-MAS both accumulate at high concentrations in ovaries and testes, due to low expression of respective downstream enzymes [26]. In mammalian testes T-MAS concentration can be above 30 μg/g, FF-MAS in testes is present only in trace amounts [19]. The large fraction of T-MAS that is synthesized in testes is not converted to zymosterol, but to sterol that remains unidentified and is not a part of the downstream cholesterol synthesis [15]. The elevated production and accumulation of T-MAS in the testis may result from transcriptional regulation of cholesterol synthesis pathway and inhibition of the enzymes converting T-MAS into downstream cholesterol synthesis intermediates. CYP51 can escape the SREBP regulation under certain physiological conditions in the testes and can be upregulated via cAMP-dependent stimuli and cAMP-responsive element modulator (CREMτ). CREMτ is expressed only in spermatids and regulates genes associated with maturation and development of spermatids [23].

It is still not firmly established whether T-MAS is as FF-MAS associated with the resumption of meiosis. In vitro T-MAS and FF-MAS have interchangeable roles and are able to induce meiosis resumption in similar concentrations. T-MAS is more efficient in germinal vesicle breakdown assay in naked oocyte [23]. T-MAS concentration in testes is high, but its function is not clear. It may play a role in fertilization, spermatogenesis, and resumption of second meiotic division [19]. Since CYP51 that converts lanosterol to FF-MAS, unequivocally localizes to acrosomal membranes of male germ cells, it was proposed that sperm cells can synthesize MAS sterols in situ [27]. To test the role of sterols MAS in vivo, we generated a conditional male germ cell-specific knockout of *Cyp51* in the mouse. As expected, metabolic profiling revealed elevated CYP51 substrates lanosterol and 24,25-dihydrolanosterol and diminished levels of MAS, the immediate products of CYP51. To our surprise the germ cell-specific ablation of *Cyp51* did not affect testicular morphology, sperm production, or reproductive performance of the males. These results failed to show that de novo synthesis of MAS and cholesterol in male germ cells is most likely not essential for spermatogenesis [28].

Zymosterol and Zymostenol

Zymosterol is synthesized from T-MAS with removal of two methyl groups on C4 in the process involving enzymes SC4MOL, NSDHL, and HSD17B7. In the Bloch pathway zymosterol is then converted to 24-dehydrolathosterol by enzyme sterol-Δ^{8-7}-isometase (EBP), which shifts the double bond from position Δ^8 to position Δ^7. Like all cholesterol intermediates in the Bloch pathway, zymosterol can also be theoretically converted by DHCR24 to zymostenol in K–R pathway [2, 29]. But in the case of zymosterol, there is evidence that this may actually be one of the points of crossover between Bloch and the Kandutsch–Russell pathway. This was confirmed

on embryotic kidney cells HEK-293. Some data show that zymosterol is a better substrate for DHCR24 than lanosterol, which is the first intermediate in the Bloch pathway, which means that crossover between both pathways is probably downstream of lanosterol. Anyway, in most cells zymosterol is not considered to be the main substrate for DHCR24; its major physiological substrate is desmosterol on the end of Bloch pathway [15].

Zymosterol is synthesized like other sterols in rough ER. Some of the newly synthesized zymosterol in cultured human fibroblasts is rapidly (even faster than cholesterol) transferred to the plasma membrane. Then some of zymosterol is transferred back to ER, where it is converted to next sterol intermediates and finally to cholesterol. The function of this movement within the cell is unknown [30]. In studying zymosterol effect on artificial lipid monolayer, it was concluded that zymosterol possesses condensing and ordering abilities. However, the effect of zymosterol on lipid membranes is much less efficient compared to cholesterol [31].

Zymosterol is considered one of the ligands for the nuclear receptor RORγ (discussed in last chapter). Altered zymosterol concentration can through binding with RORγ [32] change the expression of genes associated with immunity, circadian rhythm, and metabolism [33]. Like other 8,9-unsaturated sterols zymosterol can also enhance the oligodendrocyte formation [13].

Zymostenol can be synthesized from zymosterol with enzyme DHCR24 [2] and is also one of the crossover points between Bloch and Kandutsch–Russell pathway of cholesterol synthesis in some tissues [15]. Zymostenol can theoretically be synthesized also from dihydro-T-MAS, an upstream cholesterol intermediate in Kandutsch–Russell pathway, through series of reactions with enzymes SC4MOL, NSDHL, and HSD17B7, like in T-MAS to zymosterol conversion [2], but this pathway is not supported with sufficient evidence. Sterols proceed down the Bloch pathway at least until zymosterol, when the demethylation of sterol nucleus is complete, and can then be converted by DHCR24 and cross into Kandutsch–Russell pathway [15].

Lathosterol and 24-Dehydrolathosterol

Lathosterol is the next cholesterol intermediate from Kandutsch–Russell pathway downstream of zymostenol. It is converted from zymostenol by enzyme EBP or from 24-dehydrolathosterol with enzyme DHCR24 [2, 29]. There is more evidence that physiologically lathosterol is synthesized from zymostenol and not from 24-dehydrolathosterol [15]. In the next step of cholesterol synthesis, lathosterol is converted to 7-dehydrocholesterol by enzyme SC5DL, which catalyzes the formation of double bond between C5 and C6.

24-Dehydrolathosterol is converted from zymosterol with changing double bond position form Δ^8 position to Δ^7 with enzyme EBP. In Bloch pathway enzyme sterol-C5-desaturase/lathosterol oxidase (SC5DL) formats double bond on C5 position and converts 24-dehydrolathosterol to 7-dehydrodesmosterol. 24-Dehydrolathosterol can also be converted by DHCR24 to lathosterol which is intermediate from Kandutsch–Russell pathway [2].

7-Dehydrocholesterol

7-Dehydrocholesterol (7-DHC) is the last intermediate in Kandutsch–Russell cholesterol synthesis pathway and it is converted from lathosterol by enzyme SC5DL. The last reaction before cholesterol is synthesized is reduction of Δ^7 double on 7-deydrocholesterol by enzyme DHCR7 [2, 34].

Apart from being precursor for cholesterol, 7-DHC is also precursor for vitamin D synthesis. Vitamin D_3 is produced in a two-step nonenzymatic process. UVB light (280–320 nm wavelength) brakes the B-sterol ring and pre-vitamin D_3 is formed, which then isomerizes to form vitamin D_3. This process happens in the skin and is dependent on UVB intensity and melanin (skin pigmentation level), which can block UVB from reaching 7-dehydrocholesterol [35].

It was shown that 7DHC can destabilize the HMGCR enzyme. In this way 7DHC can downregulate cholesterol production. This function of 7DHC was shown only in the case of 7DHC accumulation like in case of SLOS (discussed in next chapter) patients [36].

Desmosterol and 7-Dehydrodesmosterol

Desmosterol is synthesized from 7-dehydrodesmosterol by enzyme DHCR7, which removes double bond from Δ^7 position. It is the last intermediate in Bloch cholesterol synthesis pathway and is converted to cholesterol by enzyme DHCR24, which catalyzes the reduction of Δ^{24} double bond on the side chain of sterol ring [34].

Desmosterol also binds to LXR, which controls cholesterol export genes and represses inflammatory genes. Desmosterol can accumulate in macrophage foam cells and by LXR activation regulates metabolism and inflammatory response [29]. In mature testis, desmosterol can be found in high concentration (up to 12% of total sterols) and even higher in sperm where desmosterol can reach concentration up to 58% of total sterols (both data are for Rhesus monkeys). The high desmosterol level may be the consequence of lower levels of DHCR24 or its inhibition. In sperm, concentration of desmosterol is not homogenous, but desmosterol is mainly located in sperm tail. This effects the membrane fluidity and is possible to be necessary for normal motility of flagella [37].

Experiments done on J774 cells (mouse monocyte/macrophage) showed that cells substitute cholesterol with desmosterol in case of cholesterol depletion. Cells were able to survive and proliferate without cholesterol (DHCR24 mutation and medium without cholesterol). Because desmosterol and cholesterol differ only in double bond on position C24, and it is not surprising that desmosterol can substitute cholesterol in some of the processes. J774 cells with cholesterol depletion have a functional SREBP pathway and desmosterol can suppress SREBP pathway and gene expression [38]. Similarly to zymosterol, desmosterol also binds and activates the RORγ and affects the expression of genes controlled by this nuclear receptor [32].

In the Bloch pathway 7-dehydrodesmosterol is converted from 24-dehydrolathosterol by the enzyme SC5DL, which catalyzes the formation of

double bond on position C5. In the next step sterol-Δ^7-reductase (DHCR7) catalyzes the reduction of Δ^7 double bond in 7-dehydrodesmosterol to form desmosterol. In the Kandutsch–Russell pathway DHCR24 converts 7-dehydrodesmosterol to 7-dehydrocholesterol. Both desmosterol and 7-dehydrocholesterol are direct precursors of cholesterol [34].

Alternative Pathways

It is becoming clear that not all of the lanosterol and post-lanosterol intermediates are intended for cholesterol biosynthesis. One of the branches is DHL, which in liver regulates HMG-CoA reductase and is possibly converted to unknown sterol. It is known that not all of 7DHC is converted to cholesterol, but in skin also to vitamin D_3 [15]. Part of both FF-MAS and T-MAS (and dihydro-MAS forms) in testes are diverted from cholesterol synthesis and are converted to unknown sterols. A mathematical model suggested that unknown enzymes can drive the nonpolar cholesterol intermediates from the pathway. It was then experimentally confirmed that some of the enzymes from the cytochrome P450 superfamily (CYP) can catalyze non-classic reactions in cholesterol synthesis. CYP7A1, CYP11A1, CYP27A1, and CYP46A1 are enzymes for which it was shown that they can catalyze reaction with zymosterol, lathosterol, 7-DHC, desmosterol, and cholesterol. In vivo reactions of these CYP enzymes with cholesterol intermediates are not confirmed, but products could potentially have important biological activities [7].

CYP27A1 is gene encoding the mitochondrial sterol 27-hydroxylaso enzyme, which in normal physiological conditions transforms cholesterol to bile acids. By cloning CYP27A1 to *E. coli*, they found out that the enzyme is not only cholesterol specific, but can also metabolize cholesterol precursors (zymosterol, desmosterol, 7-dehydrocholesterol, and lanosterol) to form 27-hydroxy derivatives. Their function and levels in normal physiological conditions is unknown. In cells, the CYP27A1 enzyme is located on the matrix side of inner mitochondrial membrane and probably more than enzyme specificity, the mitochondrial membrane permeability for sterol molecules is crucial for the selection of the substrate [39]. CYP27A1 can theoretically convert also FF-MAS and T-MAS in the testis [7].

Accumulating Sterols Can Cause Malformations in Humans

Antley–Bixler Syndrome (ABS) Is Characterized by Accumulation of Lanosterol and 24,25-Dihydrolanosterol

Antley–Bixler syndrome (ABS) represents a group of heterogeneous disorders characterized by skeletal, cardiac, and urogenital abnormalities that have frequently been associated with mutations in fibroblast growth factor receptor 2 or cytochrome P450 oxidoreductase (*POR*) genes [40]. ABS is a rare congenital multiple malformation syndrome. Patients often have craniosynostosis and other craniofacial

anomalies, as well as other skeletal defects. Many patients also demonstrate defects in steroidogenesis and sexual development. One of the causes is mutation in a gene encoding POR. In cytochrome P450 enzymatic reactions taking place in endoplasmic reticulum, POR acts as an electron donor. This is the case also for the conversion of lanosterol to FF-MAS or 24,25-dihydrolanosterol to dihydro-FF-MAS by CYP51, which in the case of mutated POR results in accumulation of lanosterol and DHL [41].

CYP51A1 is an enzyme from the first part of post-lanosterol cholesterol synthesis and converts lanosterol in FF-MAS. To study the involvement of CYP51A1 in the ABS, the mouse model was prepared with deletion of *Cyp51* gene. Lanosterol and 24,25-dihydrolanosterol are accumulated in this knockout mice. These mice showed several ABS-like features and *Cyp51* deletion led to embryonic lethality on day 15. Lethality was caused by heart failure resulting from hypoplasia, ventricle septum, epicardial and vasculogenesis defects [42]. Some ABS patients show lower CYP51A1 activity and accumulation of lanosterol and 24,25-dihydrolanosterol substrates, suggesting that CYP51A1 is at least indirectly involved in ABS disease pathology [43].

Rare Diseases with Accumulation of 4,4′-Dimethylsterols, 4-Carboxysterols, and 14-Methylsterols

The first enzyme of T-MAS conversion to zymosterol is SC4MOL and its mutations cause a disorder called SC4MOL deficiency. It is a very rare disorder that can cause severe ichthyosiform erythroderma affecting entire body, cataracts, microcephaly, and delayed skeletal and sexual development. Disorder can be diagnosed by measuring the elevated 4,4′-dimethylsterols in the skin. There is no treatment available [44].

NSDHL is the enzyme responsible for conversion of T-MAS to zymosterol and is also associated with severe pathologies. Mutation in NSDHL causes rare disorder called CHILD syndrome, which is an X-linked and generally lethal disorder in males. CHILD syndrome causes severe skeletal defects such as aplasia of entire limb, phocomelia, limp hypoplasia, and hypodactyly. Skin is also affected and patients have inflammatory nevus with skin lesions that often follow lines of X inactivation (lines of Blaschko). Mild cognitive and heart problems are also possible [45]. The best marker for diagnosis is biochemical detection of 4-carboxysterols in affected skin flakes. Due to random X inactivation, the blood sterol profile is not always the best option [44].

Pelger–Huët anomaly and Greenberg skeletal dysplasia are two disorders associated with a defective LBR enzyme. Pelger–Huët anomaly is characterized by hypolobulation of nuclei in granulocytes and is classified as laminopathia. Greenberg skeletal dysplasia (also hydrops-ectopic calcification-moth-eaten dysplasia) is a perinatal lethal condition, with abnormal bone development and excessive fluid accumulation. Patients with Greenberg skeletal dysplasia have defect sterol metabolism. LBR is a bifunctional membrane protein located in inner nuclear

membrane. N-terminus of LBR is located in the nucleus and interacts with chromatin and intermediate filaments. Unconnected to the first function, the other function of LBR on the C-terminal end is the sterol C14 reductase activity [46]. The causes of pathological conditions associated with LBR are mutations in LBR that substantially reduce NADPH binding affinity due to changes in NADPH binding pocket site, what causes abnormal sterol metabolism [21].

Rare Diseases with Accumulation of Zymosterol and Zymostenol

Conradi–Hünermann–Happle syndrome, or X-linked dominant chondrodysplasia punctate (CDPX2, OMIM no. 302960), is a rare genetic disorder characterized by skeletal dysplasia, stippled epiphyses, cataracts, transient ichthyosis, and atrophic residua in a mosaic pattern. It is a rare disorder and in most cases lethal for males. Mutations in the gene encoding the emopamil-binding protein (EBP) have been identified as an underlying cause. Because EBP is unfunctional, the zymosterol accumulates in the tissue [40, 41]. There are over 50 different variations known in the EBP enzyme that cause missense, nonsense, frameshift, and splicing mutation in the gene. The phenotype is very variable, because of random X-inactivation, in females, linear ichthyosis and severe erythroderma at birth are common [47]. CDPX2 diagnosis can be made by detection of elevated levels of zymostenol (cholesta-8(9)-en-3b-ol) [44].

Lathosterolosis

The syndrome caused by mutation in SC5DL gene (located on chromosome 11q23.3) is called lathosterolosis (OMIM no. 607330) [41]. Because of gene mutation, enzyme is unable to convert both lathosterol and 24-dehydrolathosterol to 7-dehydrocholesterol or 7-dehydrodesmosterol. Patients with lathosterolosis have elevated levels of lathosterol in tissues; diagnosis can be made on blood, skin fibroblast, or lymphocyte samples [44]. Lathosterolosis is an extremely rare autosomal recessive disorder with few documented cases. Patients have, similar to other cholesterol synthesis disorders, axial and appendicular skeletal, craniofacial and neurological abnormalities. Also common are cataract formation, intellectual disability, neonatal microcephaly, dystrophic calcification and others [40, 41]. Treatment of one patient was successful, but with only four known patients, it is hard to conclude on treatment efficiency [48].

Smith–Lemli–Opitz Syndrome

Smith–Lemli–Opitz syndrome (SLOS, OMIM no. 270400) is autosomal recessive disorder caused by different mutations in gene encoding DHCR7 enzyme, which converts 7-dehydrodesmosterol and 7-dehydrocholesterol to desmosterol or

cholesterol [44]. SLOS is the most frequent disorder from group of cholesterol synthesis defect disorders, with frequency 1:20,000–50,000. It is a multisystemic disorder, where treatment is possible only for mild cases [48]. There is high prevalence of SLOS in Northern Europe, supposedly because advantage of heterozygotes with DHCR7 mutation. This causes 7-dehydrocholesterol accumulation and more vitamin D3 production what brings advantage in areas with reduced exposure to sunlight [29]. Disorder phenotypes are extremely broad. There are patients with very mild form; on the other hand, the severely affected cases die in utero or very soon after birth. The mild cases have minor physical findings and learning problems. Most of the SLOS patients have distinctive facial appearance [41], with microcephaly, ptosis, and midface hypoplasia. Central nervous system, limb defects, urogenital and gastrointestinal anomalies are common in patients. There are many other abnormalities and defects caused by SLOS disorder. Diagnosis can be made by measuring cholesterol and its intermediate levels in plasma, amniotic fluid, or cultured skin fibroblasts, with gas chromatography combined with mass spectrometry. Cholesterol levels can be reduced or normal, 7-dehydorcholesterol and 8-dehydrocholesterol levels are elevated [44] up to 50 times. Some patients respond positively to treatment with cholesterol supplementation and they show symptoms of improvement. In addition, simvastatin therapy has been investigated, but there is no final conclusion about simvastatin efficiency [41]. Interestingly, accumulated 7-dehydorcholesterol and 8-dehydrocholesterol in SLOS patients can both be partially converted to bile acids by CYP27A1 enzyme. This unnatural bile acid synthesis pathway is happening due to the high concentration of both sterols [49].

Diseases with Accumulation of Desmosterol

Desmosterolosis (OMIM no. 602398) is disorder caused by missense mutation of DHCR24 gene (chromosome 1p32.3) that encodes the last enzyme in Bloch pathway of cholesterol synthesis, which converts desmosterol to cholesterol. DHCR24 can theoretically convert any of intermediates from lanosterol on, but its mutation causes elevation of desmosterol levels in tissue [44]. Concentration of desmosterol in patient's serum is elevated up to 120 times. Phenotype of desmosterolosis is very similar to SLOS and is associated with central nervous system and skeletal abnormalities [50].

Sterols Signaling Through Nuclear Receptors

RORγ

Nuclear hormone receptors (NHR) are transcription factors that are regulating cell processes like immune response, metabolism, reproduction, and differentiation. On the N terminus, NHR has DNA-binding domain and on the C terminus, there is

ligand-binding domain. Among the members of NHR are also retinoic acid-related receptors (RORs) that have three isoforms (α, β, γ). RORγ activation was for a longer time an enigma. Cholesterol biosynthesis intermediates and metabolites were recently proposed as ligands [14]. In a study focusing on differentiation of TH17 cells that have important role in immunity, it was shown that RORγ has a role in cell differentiation. It was found that desmosterol is an agonist and can activate the RORγ. Desmosterol is in this way very important in the control of TH17 cells differentiation [32]. RORs are also associated with circadian expression of some genes, like the core clock genes *Bmal1* and *Clock* [51] RORγ can influence the expression of some cytochromes P450 genes (*Cyp2b10*, *Cyp2b13*, *Cyp2f2*, and *Cyp4a14*) [52, 53]. In this way, altered concentration of cholesterol intermediates can change the expression of many genes and importantly affect the cell homeostasis.

Liver X Receptor LXR: A Nuclear Receptor-Binding Multiple Nonpolar Sterols

LXR receptor is probably not responsible for meiosis resumption, but FF-MAS is still its ligand though physiological relevance of this interaction is still unknown [54]. A number of post-lanosterol cholesterol intermediates can serve as activating ligands on LXR. These are DHL, T-MAS, FF-MAS, and desmosterol [29]. LXR is one of the ligand-activated transcription factors and is highly expressed in the liver, kidney gut, spleen, and adipose tissue. It forms heterodimers together with retinoic X receptor (RXR), which binds to specific DNA sequence LXR response element [54]. LXR function is to protect cells from cholesterol overload. Together with RXR they stimulate the expression of enzymes and protein transporters that are responsible for reverse cholesterol transport, cholesterol to bile acid conversion, and bile acid transport [26]. ATP-binding cassette transporter A1 is one of the cholesterol transporters whose expression is regulated through LXR/RXR heterodimers [54]. It has been reported that LXR regulates cholesterol synthesis, by downregulating the gene expression of CYP51A1 and FDFT1 (dimerization of farnesyl diphosphate) and in this way lowering the levels of cholesterol [29]. De novo fatty acid synthesis is also regulated through LXR activation, which then affects SREBP-1, acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase gene transcription [54]. LXR is also associated with repression of inflammatory genes and dopaminergic differentiation of embryonic stem cells [29].

SREBP

Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors regulating the cholesterol synthesis pathway and are activated by low sterol levels. Almost all genes that encode enzymes in cholesterol synthesis (exception is LBR located on nucleus membrane) are target of SREBPs [29]. They are regulated

by negative cholesterol feedback loop. When cholesterol levels drop below 5% of endoplasmic reticulum lipids, activation of SREBPs happens. After cholesterol levels rise above 5%, SREBPs dissociate from promotors of cholesterogenic genes. Proteins involved in this cyclic feedback regulation include the insulin-induced genes (INSIG1 and 2), the SREBP cleavage-activating protein (SCAP), site 1 and 2 proteinases (S1P and S2P) and sterol regulatory element-binding proteins SREBPs, where SREBP2 is the cholesterol-dependent transcription activator of the majority of cholesterol synthesis genes. Other signaling pathways are involved in the regulation of cholesterogenic genes in various physiological, tissue-specific, developmental, or pathophysiological conditions [10].

Conclusions

It is now evident that sterols are very active molecules and that majority have multiple roles. From lanosterol and on sterols in the cholesterol biosynthesis pathway are evidently intermediates toward cholesterol. However, we still do not understand what are concentrations and half-lives of these sterols in different tissues. Without this knowledge, it will be difficult to reveal the entire spectrum of signaling potential that these nonpolar molecules have. Meiosis activating sterols, for example, got their name due to their ability to resume meiosis in oocytes even if it was later never possible to prove the exact mechanism behind that. Even if they bind to nuclear receptor LXR, albeit with lower affinity, we do not understand how their signaling activity is performed. Discovery that the retinoic acid orphan receptor gamma binds different nonpolar sterols was a surprising fact that gave the sterol research a novel boost. While the in vitro binding capacity of RORC to zymosterol derivatives and desmosterol has been revealed in 2015 by two research groups, we still await conformation of roles in vivo. It is possible to expect that different sterols would prove to be efficient agonists of RORC depending on the tissue. It also is possible to speculate that some might be more active in regulating the circadian clock while others, for example, in promoting the immunity. It is now clear that research efforts in the area of nonpolar sterols is rejuvenated and we are awaiting novel exiting news in the near future.

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Genetic Variability in Cholesterol Metabolism

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Introduction

Cholesterol is a vital hydrophobic compound with biologic activities ranging from a structural component of cell membranes to hormone signaling for many cellular processes. Cholesterol biosynthesis is, therefore, a highly conserved cellular process by which cells regulate the synthesis and localization of cholesterol. Despite its highly conserved nature, some genetic variability does exist within the enzyme genes in the cholesterol biosynthesis pathway. In rare cases in which genetic mutations cause considerable change in enzyme function, profound disruptions result. Population variation in enzyme sequences is associated with many complex and chronic conditions. This chapter discusses genetic variability in ten enzymes in the post-squalene cholesterol biosynthesis pathway—*CYP51A1, DHCR14, LBR, NSDHL, MSMO1, HSD17B7, EBP, SC5D, DHCR7,* and *DHCR24* (Fig. 1).

CYP51A1

Cytochrome P450 family 51 subfamily A member 1, encoded by *CYP51A1*, is the most evolutionarily conserved member of the cytochrome P450 superfamily of enzymes, as it is present in all biological kingdoms [1]. CYP51A1 localizes to the endoplasmic reticulum in hepatocytes where it functions as lanosterol 14-α-demethylase and participates in cholesterol synthesis. Specifically, CYP51A1 catalyzes the conversion of lanosterol to Δ8,14,24-dimethylsterol. Despite its highly conserved nature, sequence variability does occur within the gene. Genetic variability in *CYP51A1* has been reported in association with a multitude of common complex conditions which are described below.

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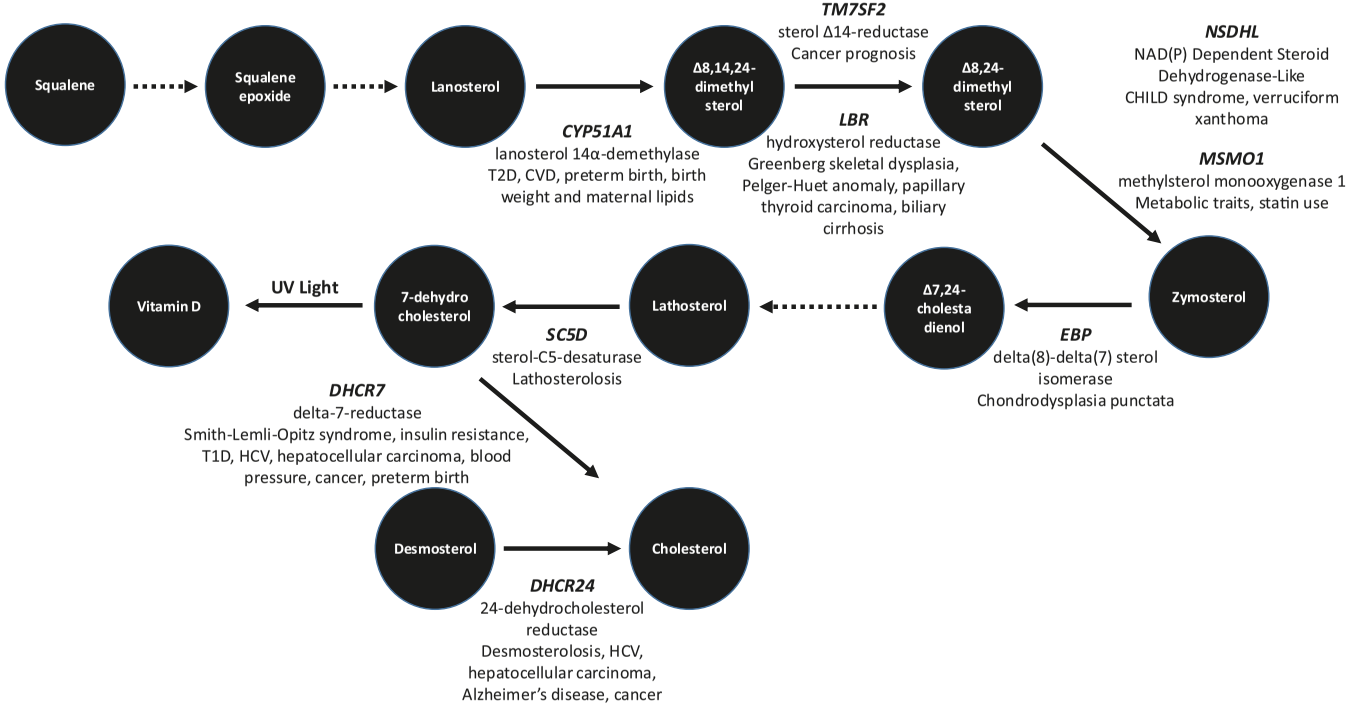

Fig. 1 Cholesterol biosynthesis pathway

Statins, Type 2 Diabetes, and Cardiovascular Disease

CYP51A1 has been identified as a potential target for cholesterol-lowering drugs. This research has largely been driven by the presence of side effects associated with statins, a common class of cholesterol-lowering drugs. Researchers have developed novel compounds targeting cholesterol biosynthesis, specifically at the enzymatic steps of CYP51A1 and DHCR14 [2]. In a study of human hepatocytes, the experimental compound LK-935 was investigated for its effects on cholesterol biosynthesis at the CYP51A1 step. LK-935, a pyridylethanol(phenylethyl)amine derivative, was found to inhibit post-squalene cholesterol synthesis and can mediate statin-induced side effects when taken in conjunction with atorvastatin [3].

A transcription and methylation study of a subset of 1254 American participants in the Multi-Ethnic Study of Atherosclerosis cohort investigated gene expression and epigenetic methylation in association with obesity-related traits and diseases. A network of cholesterol genes, including *CYP51A1* and *MSMO1*, were associated with BMI, type 2 diabetes, and coronary artery calcium. Increased expression of *CYP51A1* and *MSMO1* was associated with increasing BMI. These findings were replicated in a population of 1285 German participants in the Gutenberg Heart Study [4]. In combination with other genes in the cholesterol metabolism gene network, CYP51A1 acts as a mediator of the association between obesity and its associated inflammation and cardiometabolic disease.

Preterm Birth and Maternal Lipids

A targeted sequencing study of 188 women with spontaneous preterm birth and 188 unrelated preterm infants identified 22 variants in *CYP51A1*, of which 10 were novel. None of the 12 previously identified variants were associated with preterm birth. The novel variant, Tyr145Asp, found in one neonate, was predicted to be damaging, although it was not associated with preterm birth. A common polymorphism, rs6465348, was associated with lower total cholesterol and lower LDL among women in the second trimester of pregnancy [5]. These findings demonstrate the role of CYP51A1 pregnancy lipids and highlight the need for future studies to investigate the role of novel variants in preterm birth.

TM7SF2

Transmembrane 7 superfamily member 2, encoded by *TM7SF2*, functions as a sterol Δ14-reductase. It is localized to the endoplasmic reticulum where it catalyzes the conversion of 7-dehydrodesmosterol to desmosterol. Recently, *TM7SF2* was suggested to be enzymatically redundant with *LBR* [6]. TM7SF2 is thought to be the primary enzyme for conversion of 7-dehydrodesmosterol to desmosterol, whereas the role of LBR in the cholesterol biosynthesis pathway is less clear [7].

Cancer

TM7SF2 expression has been implicated in cancer prognosis. Tissue samples from seven subjects with histologically confirmed metastatic follicular carcinoma and eight matched subjects with invasive nonmetastatic follicular carcinoma were analyzed for gene expression differences. *TM7SF2* expression was 2.59 times higher among nonaggressive tumors compared to metastatic tumors ($P = 0.0056$) [8]. In a study of 54 adrenocortical tumors from 49 patients, *TM7SF2* expression was 4.28 times lower among adrenocortical carcinoma compared to benign adrenocortical tumors ($P < 0.01$) [9]. Both of these studies report that *TM7SF2* expression is downregulated in more aggressive tumors, and this combined evidence suggests that *TM7SF2* may function as a tumor suppressor gene.

LBR

Greenberg skeletal dysplasia (OMIM: 215140), also known as hydrops-ectopic calcification-moth-eaten (HEM) skeletal dysplasia, is caused by mutations in the lamin B receptor gene, *LBR*. The lamin B receptor is localized to the inner nuclear envelope where it acts as a hydroxysterol reductase. The disorder is inherited in an autosomal recessive pattern as a homozygous or compound heterozygous mutation.

The first cases of Greenberg skeletal dysplasia were reported in 1988 in a pair of affected siblings born to consanguineous parents [10]. The disease is typically fetal-lethal. Symptoms include short-limb dwarfism, hydrops, "moth-eaten" appearance of long bones, and unusual ossification centers in bone. Subsequent cases have been suspected at ultrasound and are either preemptively terminated or result in fetal death. Recently, *TM7SF2* was suggested to be enzymatically redundant with *LBR* [6] and some researchers suggest that Greenberg skeletal dysplasia should be considered a laminopathy rather than an inborn error of cholesterol biosynthesis [6].

Pelger–Huet anomaly (OMIM: 169400) is an autosomal dominant trait caused by mutations in the lamin B receptor gene, *LBR*. The disorder is the result of heterozygous *LBR* mutation. It is characterized by abnormal nuclear shape and chromatin distribution in neutrophils. Normal neutrophils have multilobed nuclei; neutrophil nuclei with Pelger–Huet anomaly have bilobed nuclei [11]. The worldwide prevalence of the anomaly ranges from 0.01 to 0.1%, although it is as high as 1% in regions of Sweden and Germany [11, 12].

The lamin B receptor is involved in histone activity, chromatin structure, and nuclear envelope formation. The lamin B receptor binds heterochromatin and tethers it to histones located near the inner nuclear envelope [13]. Specifically, the *LBR* tudor domain (residues 1–62) recognizes histone H4 lysine 20 dimethylation, which triggers chromatin compaction [14]. The entire nucleoplasmic region (residues 1–211) is required for transcriptional repression [14]. The lamin B receptor is also necessary for nuclear envelope assembly at the end of mitosis [15]. Lamin B receptor function has been implicated in papillary thyroid carcinoma and biliary cirrhosis.

Papillary Thyroid Carcinoma and Biliary Cirrhosis

Papillary thyroid carcinoma (PTC) is the most common thyroid and endocrine carcinoma. Diagnosis is microscopically confirmed by irregular nuclear shape and abnormal chromatin distribution, suggesting that lamin B receptor function is compromised in the etiology of PTC. Primary biliary cirrhosis (PBC) is an autoimmune disease in which intrahepatic bile duct cells are destroyed [16]. In studies of 150 French patients with PBC and 159 American patients with PBC, four subjects had autoantibodies that recognized LBR [17, 18]. A subsequent study of these four subjects with autoantibodies against LBR found that the autoantibodies recognize the nucleoplasmic amino-terminal domain within the first 60 amino acids of the LBR protein [16]. This suggests that lamin B receptor function is compromised in PBC as the result of a targeted autoimmune response.

NSDHL

CHILD syndrome (congenital hemidysplasia with ichthyosiform nevus and limb defects, OMIM:308050) is an X-linked dominant, male lethal disorder caused by mutations in the NAD(P)H steroid dehydrogenase-like gene, *NSDHL*. CHILD syndrome was first reported in 1968 when clinicians reported two female siblings with a novel syndrome, both of whom died within a few days of delivery [19]. Dysmorphic features occur in a unilateral pattern, in contrast with chondrodysplasia punctata, which presents in a bilateral pattern. A study of unrelated female patients with CHILD syndrome identified 14 unique point mutations in *NSDHL*, including 4 stop mutations, 8 missense mutations, 1 splice site mutation, and 1 gene deletion [20].

Verruciform xanthoma is a rare mucocutaneous lesion consistent with the lesions observed in CHILD syndrome. A targeted sequencing study of nine cases of sporadic verruciform xanthoma, in absence of CHILD syndrome, investigated mutations in exons 4 and 6 of *NSDHL* based on the association between mutations in these exons and CHILD syndrome. The study identified a novel missense mutation in exon 6 of *NSDHL* in several subjects [21]. These findings suggest that unique mutations in exon 6 of *NSDHL* contribute to verruciform xanthoma and are distinct mutations from those causing CHILD syndrome.

To our knowledge, variability in *NSDHL* has not been reported in association with other diseases or traits.

MSMO1

MSMO1 encodes methylsterol monooxygenase 1, also known as sterol-C4-methyl oxidase-like protein (*SC4MOL*). The enzyme localizes to the endoplasmic reticulum and is one of three enzymes catalyzing the conversion of Δ8,24-dimethylsterol to zymosterol. Genetic variability in *MSMO1* is associated with metabolic traits.

Metabolic Traits

In a targeted genotyping study of 3575 adults in rural Netherlands, the G allele of rs17585739 was associated with lower plasma HDL-C levels [22]. A genome-wide association study (GWAS) of 927 nondiabetic African Americans and 570 nondiabetic West Africans revealed significant associations between rs17046216 in *MSMO1* and fasting insulin and insulin resistance [23]. As described previously, *CYP51A1* and *MSMO1* expression and methylation were associated with BMI, type 2 diabetes, and coronary artery calcium, independent of serum lipid levels in two independent populations. Increased expression of *CYP51A1* and *MSMO1* was associated with increasing BMI [4]. These findings suggest that *MSMO1*, in combination with other genes in the cholesterol metabolism network, is a mediator of the relationship between obesity and its associated inflammation and cardiometabolic disease.

HSD17B7

Hydroxysteroid 17-beta dehydrogenase 7, encoded by *HSD17B7*, was identified in 1999 as the enzyme responsible for estradiol production in the corpus luteum during pregnancy. The enzyme is also present in placenta and other steroid target tissues such as testis, prostate, and breast tissue where it converts estrone to estradiol [24]. Researchers later reported a dual functionality of *HSD17B7*, in which the enzyme is involved in both steroidogenesis and cholesterol biosynthesis. For both functions, the enzyme is localized to the endoplasmic reticulum [25]. Given the enzyme's role in estradiol production, genetic variability in *HSD17B7* has been associated with hormonal breast cancer and preterm birth.

Breast Cancer

Estradiol levels are significantly higher in breast cancer tissue compared to plasma. In a Norwegian study of premenopausal ($N = 11$) and postmenopausal ($N = 23$) women undergoing mastectomy, tumor specimens were analyzed for gene expression. *HSD17B7* expression was significantly positively correlated with intratumoral estradiol levels ($r = 0.59$, $P < 0.001$) [26]. However, targeted sequencing of 50 French-Canadian families at high risk for breast cancer without BRCA1/2 mutations and 70 healthy French-Canadian families failed to identify germline mutations in *HSD17B7* associated with breast cancer risk [27]. These findings suggest that although *HSD17B7* expression is associated with estradiol levels due to its enzymatic function, sequence mutations in the gene may not be responsible for these hormone level changes in breast cancer tissue.

Preterm Birth

In a targeted genotyping case-control study of 279 African American women, maternal rs1780019 was associated with preterm birth in genotypic analysis ($P = 0.03$) but not allelic analysis ($P = 0.92$) [28]. In a targeted genotyping study of an American population and a Norwegian population fetal rs4656381 was associated with preterm birth in genotypic analysis (0.0029) and allelic analysis ($P = 0.03$) in the Norwegian population but not the American population [29]. These studies demonstrate that both maternal and fetal genotypes in HSD17B7 can contribute to risk for preterm birth.

EBP

Chondrodysplasia punctata (CDPX2, OMIM: 302960), also known as Conradi–Hunermann–Happle syndrome, is caused by mutations in the emopamil binding protein gene, *EBP*. Emopamil-binding protein (EBP) functions as the delta(8)-delta (7) sterol isomerase. It is localized to the endoplasmic reticulum where it catalyzes the conversion of zymosterol to Δ7,24-cholestadienol. The disorder is inherited in an X-linked dominant pattern.

Symptoms include skin defects, abnormal bone calcification, and skeletal abnormalities. A case review of nine families of individuals with CDPX2 found that skin defects, typically in the form of ichthyosis following the lines of Blaschko, were present in more than 95% of cases. Chondrodysplasia punctate (punctiform calcification of the bones), cicatricial alopecia, and asymmetric shortening of the limbs were present in about 80% of cases, and cataracts were present in about 60% of cases. Short stature and other bone defects have been also found frequently [30].

To our knowledge, genetic variability in *EBP* has not been reported in association with other diseases or traits.

SC5D

Lathosterolosis (OMIM: 607330) is caused by mutations in the sterol-C5-desaturase enzyme, encoded by *SC5D*. This enzyme catalyzes the conversion of lathosterol to 7-dehydrocholesterol. The disorder is inherited in an autosomal recessive pattern as a homozygous or compound heterozygous mutation.

Lathosterolosis was first described in 2002 [31] and again in 2007 [32]. To date, only two cases of lathosterolosis have been reported in a pair of sisters born to non-consanguineous parents. Symptoms include microcephaly, polydactyly, syndactyly and liver disease, manifesting as jaundice, mental retardation and psychomotor delay. Additional dysmorphic features included anteverted nares, micrognathia, and a high arched palate. The effects of lathosterolosis on cholesterol include elevated levels of lathosterol, absent 7-dehydrocholesterol, and low plasma cholesterol

[31, 33]. Treatment for the only surviving patient with lathosterolosis included a liver transplant at the age of eight [34].

To our knowledge, genetic variability in *SC5D* has not been reported in association with other diseases or traits.

DHCR7

The earliest reported syndrome associated with errors in the cholesterol biosynthesis pathway is Smith–Lemli–Opitz syndrome (SLOS, OMIM: 270400). Although SLOS was first described in 1964 [35], the discovery of its genetic cause was not reported until 1998 [36]. SLOS is caused by mutations in the delta-7-reductase gene, *DHCR7*, which catalyzes the conversion of 7-dehydrocholesterol to cholesterol. The enzyme is localized to both the endoplasmic reticulum and the outer nuclear membrane. SLOS is inherited in an autosomal recessive pattern as a homozygous or compound heterozygous mutation.

SLOS is manifested as a syndrome of congenital anomalies. Craniofacial abnormalities are nearly universal, and symptoms include microcephaly, short nose, anteverted nares, ptosis, blepharoptosis, and mental retardation. The distinctive effects on cholesterol biosynthesis include low plasma cholesterol and elevated 7-dehydrocholesterol, which are detectable postnatally in plasma [37] and prenatally in amniotic fluid [38].

DHCR7 catalyzes the conversion of 7-dehydrocholesterol to cholesterol. 7-dehydrocholesterol is also the precursor for vitamin D, so increased activity of *DHCR7* reduces the amount of substrate available for vitamin D synthesis [39]. Thus, polymorphisms in DHCR7 have shown extensive association with vitamin D concentration. DHCR7 has also been reported in association with many complex conditions including insulin resistance, type 1 diabetes, hepatitis C (HCV) and hepatocellular carcinoma, blood pressure, cancer, and preterm birth.

Vitamin D

7-dehydrocholesterol present in skin cells is converted to cholecalciferol upon exposure to ultraviolet light. Cholecalciferol is then metabolized by the liver and kidney to the biologically active form of vitamin D, 1,25-dihydroxycholecalciferol [40]. Vitamin D synthesis is related to cholesterol synthesis in that it diverts a substrate, 7-dehydrocholesterol, from the cholesterol synthesis pathway to the vitamin D synthesis pathway. Thus, in addition to genes specifically within the vitamin D synthesis pathway, genetic variability in the cholesterol synthesis pathway can contribute to circulating vitamin D levels.

Two genome-wide association studies (GWAS) have investigated the role of genetic variability in vitamin D levels. In a multinational GWAS of 4501 subjects of European ancestry, rs1790349 was significantly associated with vitamin D ($P = 3.4 \times 10^{-9}$) [41]. A multinational GWAS of European/Caucasian individuals,

including 16,124 people in the discovery population and 17,744 in the replication population, identified six SNPs in *DHCR7* (rs12785878, rs7944926, rs12800438, rs3794060, rs4945008, rs4944957) that were associated with vitamin D concentration ($P \leq 1.43 \times 10^{-12}$) [42]. A genome-wide methylation study of 11 African American men with vitamin D deficiency and 11 controls found reduced methylation at a CpG site in *DHCR7* in cases relative to controls, suggesting that increased *DHCR7* expression is associated with vitamin D deficiency [43].

A targeted genotyping study of 652 African American men and 405 European American men analyzed eight SNPs for association with vitamin D. rs12800438 was associated with vitamin D deficiency in African American men but not European American men [39]. In a targeted genotyping study of 1873 Chinese participants of different ethnicities (945 Uygur and 928 Kazak), rs12785878 was associated with vitamin D deficiency (OR = 2.442, 95% CI: 1.224–4.873; $P = 0.011$) among the Kazak ethnic population but not among the Uygur ethnic population [44]. In a targeted genotyping study of 3210 Han Chinese, rs3829251 and rs1790349 were associated with lower plasma vitamin D ($P \leq 5.7 \times 10^{-5}$) [45]. A candidate gene study among 506 Han Chinese children found that the A allele of rs3829251 and the G allele of rs12785878 were associated with lower vitamin D levels [46].

In an Austrian targeted genotyping study of 545 women with PCOS and 145 women without PCOS, the GG genotype of rs12785878 was associated with vitamin D deficiency (OR: 3.27, 95% CI: 1.45–7.39, $P = 0.004$) compared to the TT genotype [47]. In a British targeted genotyping study of 720 cases of Type 1 diabetes and 2610 controls, rs12785878 was associated with vitamin D concentration ($P = 9.9 \times 10^{-4}$) [48]. In a study of 3418 individuals, of whom 929 had type 2 diabetes, the G allele of rs12785878 was associated with lower vitamin D ($P = 3.37 \times 10^{-4}$) and rs3829251 (A allele with lower vitamin D, $P = 8.48 \times 10^{-4}$) [49]. In a targeted genotyping study of 1708 American children followed over time, the minor allele of rs12785878 was associated with lower vitamin D ($P = 0.03$) [50].

These studies demonstrate the role of common variability in *DHCR7* on circulating vitamin D levels across diverse populations, including racial/ethnic groups, males and females, and adults and children.

Insulin Resistance and Type 1 Diabetes

In a targeted genotyping study of the effects of diet on insulin resistance among 732 overweight/obese subjects, the T allele of rs12785878 was associated with greater short-term improvements in insulin and HOMA-IR in response to a high protein diet, but no association among the low protein diet group ($P < 0.002$) [51]. A Mendelian randomization study evaluated SNPs associated with vitamin D metabolism among multiple populations of European descent and their relation to type 2 diabetes (T2D). SNP associations with vitamin D concentration were analyzed among 5449 subjects from two study populations. SNP associations with T2D and glycemic traits were analyzed among 28,144 T2D cases and 76,344 controls from five studies and 6368 Europeans without diabetes in the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC). The T allele of rs12785878 was

significantly associated with 2.40 nmol/L (95% CI: 1.42, 3.38) lower vitamin D concentration but was not associated with T2D or glycemic traits [52].

In a British targeted genotyping study of 8517 cases of type 1 diabetes (T1D) and 10,438 controls, rs12785878 was associated with T1D (OR for minor allele: 1.07, 95% CI: 1.02, 1.13, $P = 6.8 \times 10^{-3}$) [48]. In a study of 1708 American children followed over time, rs12785878 was associated with development of islet autoimmunity (HR for minor allele: 1.36, 95% CI: 1.08, 1.73) but not progression to T1D among children with islet autoimmunity. However, a targeted genotyping study of 1467 Danish families found no association between rs12785878 and risk of T1D ($P = 0.211$) [53].

These studies present discordant conclusions regarding the association between common genetic variability in *DHCR7* and T1D and T2D. Inconsistent findings suggest that future research is needed to identify potential effect modifiers to this relationship.

Hepatitis C and Hepatocellular Carcinoma

A nested case-control from two European and two Japanese cohorts analyzed genetic associations with hepatocellular carcinoma among 1279 chronic HCV cases and 4325 uninfected controls. rs7944926 and rs12785878 (which are in perfect linkage disequilibrium) were associated with hepatocellular carcinoma in the combined analysis of the four populations (OR = 1.42, 95% CI: 1.13, 1.78, $P = 0.003$) [54]. In a targeted genotyping study of 260 patients with biopsy-confirmed genotype-1 chronic HCV, the GG genotype of rs12785878 was associated with increased risk for severe liver fibrosis ($P = 0.03$) and vitamin D ($P = 0.003$) [55]. In a targeted genotyping study of 177 Japanese subjects with chronic genotype 1b HCV infection who were treated with pegylated interferon/ribavirin/protease inhibitor combination therapy, rs794492 was not associated with response to treatment or vitamin D [56].

Taken together, the results of these studies suggest that genetic variability in *DHCR7* is associated with liver disease progression, including carcinoma and fibrosis, among those infected with hepatitis C. However, genetic variability in *DHCR7* does not affect response to standard treatment.

Blood Pressure

Researchers have identified gender-specific associations from GWAS of blood pressure. In the Women's Genome Health Study (23,294 women of European ancestry) and International Consortium of Blood Pressure (69,395 men and women of European ancestry), rs12785878 in *DHCR7* was not associated with blood pressure traits in either population. The A allele of rs1790370 in *DHCR7* was associated with lower diastolic in the female population but not in the male population [57]. These studies suggest that the association between genetic variability in *DHCR7* and blood pressure exhibits sexual dimorphism and this interaction by sex may explain lack of significance in the large GWAS.

Cancer

In a study of 3583 pancreatic cancer cases and 7053 controls from the heterogeneous PanScan collaboration of GWAS, SNPs in *DHCR7* were not associated with pancreatic cancer risk or vitamin D [58].

A Swedish targeted genotyping study of 734 women with invasive breast cancer and 1435 controls found no association between rs1790349 and breast cancer, but the T allele was associated with higher vitamin D concentration ($P = 0.0002$) [59]. In another targeted genotyping study of 9456 breast cancer cases and 10,816 controls of European ancestry, rs12785878 was not associated with breast cancer risk individually or in a four-SNP risk score [60].

In a GWAS meta-analysis of 10,061 colorectal cancer cases and 12,768 controls drawn from 13 international studies, rs12785878 was not associated with colorectal cancer either independently or as part of a genetic risk score [61]. In a targeted genotyping study of 838 African Americans, eight SNPs in *DHCR7* were analyzed for association with colorectal cancer risk and found no association [62]. In a targeted genotyping study of 264 Austrian patients with histologically confirmed stage II and III colon cancer who had undergone surgical resection, *DHCR7* rs12785878 G>T not associated with time-to-recurrence [63].

A Chinese GWAS of 1942 esophageal squamous cell carcinoma (ESCC) cases and 2111 controls evaluated four SNPs in *DHCR7* (rs3794060, rs12800438, rs7944926, rs3829251) for association with risk for ESCC. None were associated with risk for ESCC [64].

These studies demonstrate that variability in *DHCR7* is not a likely contributor to cancer risk, including prostate, breast, and colorectal cancers and esophageal squamous cell carcinoma.

Preterm Birth

Many epidemiologic studies have identified an association between vitamin D deficiency and preterm birth. In general, lower vitamin D close to delivery most predictive of preterm birth [65]. However, combined data from two randomized controlled trials of vitamin D supplementation during pregnancy found no association between vitamin D intake and individual adverse pregnancy outcomes, including preterm birth [66]. A candidate gene study of 257 American and Danish families, including 492 preterm infants, found evidence of linkage between rs1790318 and preterm birth ($P = 0.009$) [67]. In a targeted genotyping study of 305 American singleton preterm infants, neonatal rs1630498 and rs2002064 were associated with birth weight ($P = 0.002$ and $P = 0.003$, respectively) [68]. Taken together, these studies suggest that genetic contribution to vitamin D levels, mediated by genetic variability in *DHCR7*, may contribute to preterm birth, while vitamin D supplementation is not associated with preterm birth.

DHCR24

Desmosterolosis (OMIM: 602398) is caused by mutations in the 24-dehydrocholesterol reductase gene, *DHCR24*. The enzyme is localized to the endoplasmic reticulum where it catalyzes the reduction of desmosterol to cholesterol. It is inherited in an autosomal recessive pattern as a homozygous or compound heterozygous mutation.

The first case of desmosterolosis was described in 1998 in a female neonate who died shortly after birth [69]. To date, only eight individuals with desmosterolosis have been reported, six of whom were members of the same family. Craniofacial features include macrocephaly, microstomia, frontal bossing, and hypoplastic nasal bone. Other features include rhizomelia and ambiguous external genitalia. The distinctive effects on cholesterol biosynthesis include elevated levels of desmosterol and low plasma cholesterol [33].

In addition to rare mutations causing desmosterolosis, common genetic variability in *DHCR24* has been associated with hepatitis C infection and hepatocellular carcinoma, Alzheimer's disease, prostate cancer, and other cancers.

Hepatitis C and Hepatocellular Carcinoma

A Japanese study of 395 HCV-positive individuals and 24 health controls investigated the relationship between antibodies against *DHCR24* and hepatocellular carcinoma. The concentration of DHCR24 antibody was significantly higher in chronic HCV patients compared to controls ($P < 0.0001$), but significantly lower compared to HCV-positive hepatocellular carcinoma patients ($P < 0.0001$). DHCR24 antibody was highly sensitive for HCV-positive hepatocellular carcinoma (70.6%) compared to other biomarkers [70].

A study of a human hepatoblastoma cell line containing the entire HCV genome displayed increased *DHCR24* expression, whereas cell lines only expressing specific viral proteins (nonreplicating) did not have increased *DHCR24* expression. This suggests that viral replication is necessary to illicit increased *DCHR24* expression [71]. A study of HCV infection in human hepatocytes found that *DHCR24* expression is induced by HCV infection. Silencing of *DHCR24* reduced HCV replication, suggesting the DHCR24 is a critical enzyme in HCV infection [72]. In another study of human hepatocytes, *DHCR24* expression was increased in HCV-infected cells. *DHCR24* expression was also indicative of tumorigenicity, in which *DHCR24* overexpression suppressed the activity of the proapoptotic tumor suppressor protein, p53 [73].

The results of the clinical study suggest that antibodies against *DHCR24* are indicators of HCV infection and progression to hepatocellular carcinoma. This association is corroborated by studies in human cell lines, in which *DHCR24* expression was associated with HCV infection and tumor development.

Alzheimer's Disease

Two studies of the genetic contribution to Alzheimer's disease (AD) have identified gender-specific effects of polymorphisms on the risk for AD. A targeted genotyping study of 295 Hungarian adults with late-onset AD and 204 matched controls did not find an overall difference in genotype distribution for rs600491. However, when stratified by gender, the TT genotype of rs600491 was associated with risk for AD among men ($P = 0.022$) but not among women ($P = 0.804$) [74]. Targeted genotyping of four SNPs in 414 Finnish AD cases and 459 controls found an association between one SNP in *DHCR24* and AD in men. The T allele of rs600491 was associated with increased risk for AD among men (OR: 1.7, 95% CI: 1.2, 2.4, $P < 0.004$) [75]. None of the four SNPs were associated with AD among women [75].

Targeted exome sequencing of *DHCR24* in 100 Italian cases with familial AD did not identify any mutations associated with AD [76]. In a study of postmortem brain tissue from subjects with and without AD, *DHCR24* expression was downregulated by 33% in the temporal cortex of subjects with neuropathically confirmed AD [77]. Conversely, a study of postmortem frontal lobe tissue from subjects with dementia (9 with AD and 10 without AD) found that desmosterol was lower in AD tissue compared to the non-AD group ($P = 0.008$) suggesting greater expression or function of *DHCR24* in AD patients compared to non-AD patients with dementia [78].

The results of multiple studies are inconsistent in which some studies found associations between SNPs in *DHCR24* and expression of *DHCR24* and Alzheimer's disease while others found no association. Notably, multiple studies reported sexual dichotomy, in which SNPs in *DHCR24* and *DHCR24* expression were associated with AD among men but not among women.

Cancer

DHCR24 is expressed in epithelial prostate tissue. Its expression is elevated in low-grade prostate cancer and reduced in high-grade prostate cancer compared to normal tissue [79]. In a targeted gene expression study of prostate cancer tissues, *DHCR24* expression was significantly lower in metastatic castration-recurrent cancers compared to androgen-dependent primary prostate cancer [80]. In a study of the androgen receptor pathway and prostate cancer progression, lower expression of *DHCR24* in primary prostate cancers was associated with a higher incidence of metastases following radical prostatectomy [81].

A candidate gene study of adrenocortical adenoma and carcinoma tissues found lower *DHCR24* expression in adrenocortical carcinoma compared to adenoma and normal tissue. The study also found lower *DHCR24* expression in stage III/IV tumors compared to stage I/II tumors [82]. In another candidate gene study of adrenal carcinoma and normal adrenal gland tissues, *DHCR24* expression was significantly lower in adrenal carcinomas compared to normal tissue ($P = 0.01$)

and *DHCR24* methylation was significantly increased in adrenal carcinomas compared to normal tissue ($P = 0.02$) [83]. The increased methylation is consistent with lower gene expression, as methylation turns off gene expression.

Conversely, a candidate gene study of tissue specimens from 162 cases of non-muscle-invasive urothelial carcinoma found that *DHCR24* expression was significantly higher among high-grade tumors compared to low-grade tumors ($P = 0.003$) [84]. *DHCR24* expression was an independent predictor of tumor progression (HR: 5.464; 95% CI: 1.746, 17.099; $P = 0.004$) [84].

These studies demonstrate consistent inverse associations between *DHCR24* expression and prostate cancer progression and adrenocortical carcinoma, whereas a study of urothelial carcinoma found that higher *DHCR24* expression was associated with more severe disease. These findings identify the need for further investigation into the utility of *DHCR24* expression as a prognostic biomarker for certain cancers. The discordant directions of the associations between *DHCR24* expression different cancer types suggest that DCHR24 enzyme activity has a different effect on cancer progression depending on tissue type.

Summary

Cholesterol is vital to cellular function and its biosynthesis is a highly conserved metabolic process. However, rare and common genetic variability exist in the genes of the cholesterol biosynthesis pathway. In particular, genetic variability in *CYP51A1, DHCR14, LBR, NSDHL, MSMO1, HSD17B7, EBP, SC5D, DHCR7,* and *DHCR24* is associated with rare syndromes and common chronic diseases. The evidence presented in this chapter supports the need for further research into the role of cholesterol biosynthesis in chronic disease etiology and pathology.

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Side-Chain Oxidized Oxysterols in Health and Disease

Ingemar Björkhem and Ulf Diczfalusy

Introduction

Oxysterols are oxygenated derivatives of cholesterol that are important as intermediates or end products in the elimination of cholesterol from the body (For a previous review, see [1]). Introduction of an oxygen function in the steroid molecule can be regarded as a "death kiss" and will markedly reduce the half-life of the molecule in the body. In view of this, it is understandable that the metabolic control of cholesterol excretion pathways is assigned to specific monooxygenases that catalyze the rate-limiting oxidative step in the elimination. Among the many oxidized derivatives of cholesterol present in biological systems, those carrying the oxygen function in the steroid side-chain are of particular importance. The physical properties of such steroids allow them to pass biomembranes at a much higher rate than cholesterol itself [2, 3]. As an example, side-chain oxidized oxysterols are able to pass across the blood–brain barrier which is not the case with cholesterol itself. Oxysterols are always present together with cholesterol in biological systems and the excess of cholesterol normally varies between about 500 and 100,000 or more. From this point of view, addition of pure oxysterols to different in vitro systems is clearly highly unphysiologic and very difficult to compare with the in vivo situation.

Based on their potent biological effects mainly studied in vitro, side-chain oxysterols have been suggested to be important in connection with cholesterol homeostasis, atherosclerosis, necrosis, inflammation, immunosuppression, and development of [1]. More recently, they have also been suggested to have roles in development of breast cancer, osteoporosis, hereditary spastic paresis, and neurodegenerative diseases like Parkinson's disease and Alzheimer's disease. They have also been suggested to be involved in cell differentiation during embryonic development. Evidence has been presented that the flux of oxysterols in the brain is

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important for cognition and memory. During the last few years, one of the side-chain oxidized oxysterols, 25-hydroxycholesterol, has been shown to be of importance in connection with immunologic defense reactions.

In the present review we discuss the biological roles of 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24(S),25-epoxycholesterol as well as the enzymatic systems responsible for synthesis of these steroids. Emphasis is put on in vivo studies, in particular clinical consequences of increased or decreased production of these steroids.

There is a previous excellent short review on oxysterols, not only side-chain oxidized, covering the most important literature up to 2009 [4].

27-Hydroxycholesterol

Structure of 27-Hydroxycholesterol

The structure of 27-hydroxycholesterol is shown in Fig. 1. The stereochemistry of the hydroxylated methyl group may be either 25R or 25S. It has been suggested that the preferred nomenclature for 27-hydroxylation and 27-hydroxycholesterol should be (25R)26-hydroxylation and (25R)26-hydroxycholesterol, respectively [5]. In the early literature, the compound was called 26-hydroxycholesterol. In accord with most recent literature, we will however use the nomenclature 27-hydroxycholesterol here.

Sterol 27-Hydroxylase (CYP27) and Fluxes of 27-Hydroxycholesterol: Antiatherogenic Effects of CYP27

27-Hydroxycholesterol is formed by the mitochondrial cytochrome P-450 enzyme CYP27. This enzyme is present in most organs and tissues but hepatocytes, macrophages, and in particular, lung alveolar macrophages have the highest expression (for a review, see [6]). The enzyme has the capacity to oxidize the C27-methyl group not only into a CH_2OH group but also into a carboxylic acid (cholestenoic acid) with the corresponding aldehyde as an unstable intermediate [7]. The enzyme is located at the inner mitochondrial membrane and the transport of cholesterol into

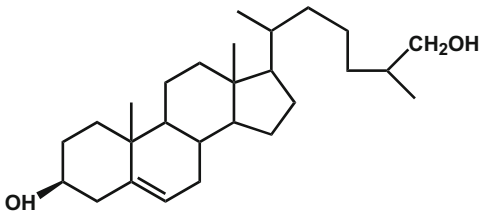

Fig. 1 Structure of 27-hydroxycholesterol

the mitochondria may be a limiting step for the overall hydroxylation [8]. This transport is facilitated by a STAR protein (STAR5D) [9].

Sterol 27-hydroxylase has a broad substrate specificity with a preference for more polar steroids than cholesterol, including its own product, 27-hydroxycholesterol. This enzyme is important for the enzymatic degradation of the steroid side-chain of cholesterol to give a C24-bile acid [6]. Thus, 7α-hydroxylated C27-steroids are the most important substrates for the CYP27 enzyme in connection with formation of bile acids in the liver. A minor pathway to bile acids, the "acid pathway" starts however with 27-hydroxylation of cholesterol in the liver or extrahepatically. It has been estimated that 5–10% of the total conversion of cholesterol into bile acids starts with an intra- or extrahepatic 27-hydroxylation in man [6].

27-Hydroxycholesterol is the dominating oxysterol in human circulation. Most of it is esterified. Based on arteriovenous concentration differences it has been shown that there is a significant uptake of 27-hydroxycholesterol by two organs, the brain and the liver [10, 11]. Figure 1 shows the measured uptake by the brain and the liver. In both cases, the uptake is likely to be dependent upon the metabolism, which causes a concentration gradient from the circulation into the tissues. In case of the brain, it is known that this organ contains very high levels of the 27OH-metabolizing enzyme CYP7B1 [12]. In patients with a mutation in the CYP7B1 gene, the metabolism is reduced and the driving force for the flux of 27OH into the brain is likely to be absent. Under normal conditions most of the 27OH present in brain and CSF originates from the circulation. It has been shown that the flux of 27OH from the circulation into the brain increases in patients with a disrupted blood–brain barrier [13].

The flux of 27OH and its peripheral metabolites cholestenoic acid and its 7 alpha hydroxylated metabolites from peripheral tissues to the liver is analogous to the classical HDL-mediated reversed cholesterol transport. Given the high level of CYP27 in macrophages, in particular lung macrophages [14], it is likely that this mechanism is important for removal of excess cholesterol. It has been shown in vitro that inhibition of the sterol 27-hydroxylase in lung macrophages leads to a substantial accumulation of cholesterol [11].

Thus the CYP27 catalyzed removal of cholesterol from macrophages may be regarded as an antiatherogenic mechanism [15]. The fact that patients with cerebrotendinous xanthomatosis (CTX), lacking CYP27, often develop premature atherosclerosis in spite of normal levels of cholesterol in the circulation is in accord with this hypothesis. There is an accumulation of 27-hydroxycholesterol in human atheromas [16], possibly reflecting a defense mechanism. Since most of the 27OH present in the atheromas is esterified, part of the explanation for the accumulation could be that it is secondary to a high activity of the acyl CoA-cholesterol acyltransferase. The activity of this esterase is upregulated by cholesterol and oxysterols are better substrates than cholesterol itself for the enzyme. Thus, there may be a continuous "trapping" of the esterified form of 27OH in the atheromas.

The antiatherogenic capacity of CYP27 has been studied with use of a mouse model deficient in ApoE and Cyp27 [17]. When $ApoE^{-/-}$ mice heterozygous with respect to Cyp27 were treated with western diet more atheromas were developed

than in ApoE$^{-/-}$ mice on the same diet. Double knockout mice (ApoE$^{-/-}$, Cyp27$^{-/-}$) developed less atheromas on the western diet due to the marked upregulation of Cyp7a1 which is associated with an antiatherogenic effect.

Regulation of CYP27 Activity

Since 27-hydroxylation of cholesterol is the first step in the "acid pathway" to bile acids, one would perhaps expect a negative feedback regulation of this enzyme by bile acids, similar to the situation in the classical pathway to bile acids starting with 7 alpha hydroxylation of cholesterol. There seems to be very little such regulation however, at least in man [18]. Under in vitro conditions thyroxin and phorbol 12-myristate-13 acetate decreased transcriptional activity whereas dexamethasone, GH, and IGF-1 increased the activity. Substrate availability for the mitochondrial enzyme seems to be a limiting factor under most conditions and overexpression of the STAR protein, which leads to higher influx of cholesterol into the mitochondria, is known to increase the sterol 27-hydroxylase activity [8]. Reflecting the importance of substrate availability the levels of 27-hydroxycholesterol in the circulation are significantly correlated with the levels of cholesterol [19]. As a consequence, increased levels of cholesterol in the circulation can be expected to be associated with increased flux of 27-hydroxy cholesterol into the brain.

Regulatory Effects of 27-Hydroxycholesterol on Cholesterol Homeostasis

Similar to the other two side-chain oxidized oxysterols, 27-hydroxycholesterol is an efficient inhibitor of cholesterol synthesis in vitro. Early work by Kandutsch et al. showed that side-chain oxidized oxysterols have an inhibitory effect on cholesterol synthesis in vitro that is orders of magnitudes higher than that of cholesterol itself [20]. Based on this it was suggested that the inhibitory effect of cholesterol on its own synthesis may be mediated by oxysterols. Cholesterol synthesis in mammals is known to be controlled by a regulated transport of SREBP from the endoplasmic reticulum to the Golgi, where the transcription factors are processed proteolytically to release active fragments. The SREBP-escort binding protein Scap and the anchor protein Insig are key proteins in this mechanism [21]. Cholesterol acts by binding to Scap which leads to binding of this protein to the "anchor" protein Insig. In contrast, side-chain oxidized oxysterols bind to Insig leading to a subsequent binding to Scap, Fig. 2. Thus, the binding of oxysterols to Insig has been suggested to be important for the ability of oxysterols to inhibit cholesterol synthesis in mammalian cells [22]. Given the very high levels of cholesterol in relation to the levels of oxysterols under in vivo conditions, the relevance of this mechanism in vivo has been questioned [23]. Another mechanism by which side-chain oxidized oxysterols may affect cholesterol synthesis is by stimulating a proteolytic inactivation of the rate-limiting enzyme in cholesterol synthesis, HMG CoA reductase. Formation of an

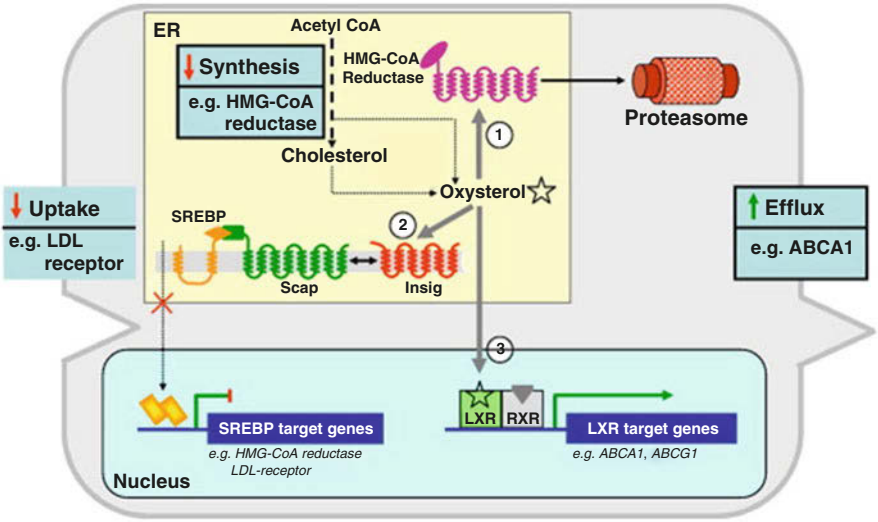

Fig. 2 Mechanism of regulation of cholesterol homeostasis by oxysterols. The different mechanism by which side-chain oxysterols reduce cholesterol levels in the cell by several mechanisms. 1. Accelerated degradation of HMG CoA reductase; 2. Suppression of SREBP activation. 3. Increased cholesterol efflux by activation of LXR-mediated gene transcription. Reproduced from Ref. [4] by permission

HMG–Insig complex may precede this inactivation and the possibility has been discussed that an oxysterol-bound form of Insig can form a complex with HMG CoA reductase just as it can with Insig [22]. Also in this case the relevance of this mechanism under normal in vivo conditions has been questioned [23].

It should be mentioned that mice with high levels of 27-hydroxycholesterol due to an overexpression of CYP27 enzyme or knockout of the CYP7B1 enzyme that is responsible for metabolism of 27-hydroxycholesterol have a normal rate of cholesterol synthesis in the liver [24, 25]. Mice with a lack of 27-hydroxycholesterol due to a knockout of CYP27 also have a normal rate of cholesterol synthesis in the liver, provided that the mice are treated with cholic acid in order to compensate for the reduced formation of bile acids [26].

By treatment of mice with cholesterol deuterated in 27-position and utilizing the fact that 27-hydroxylation of such cholesterol is associated with an isotope effect, evidence was obtained that 27-hydroxylation is not involved in the cholesterol-induced downregulation of HMG CoA reductase in mouse liver [27].

Under normal conditions, 27-hydroxycholesterol does not seem to be important in the regulation of cholesterol homeostasis. Under some specific conditions, cultured fibroblasts from Tangier patients were found to accumulate 27-hydroxycholesterol to levels associated with reduced cholesterol synthesis [28]. It was concluded that side-chain oxidized oxysterols are suppressors of cholesterol biosynthesis under some specific pathological conditions. In addition to this, the flux of 27-hydroxycholesterol into the brain may have some suppressive effect on cholesterol synthesis in this organ (see below).

Regulatory Effects of 27-Hydroxycholesterol on LXR-Signaling

27-Hydroxycholesterol is a weak agonist to LXR [29]. In cultured cells, 27-hydroxycholestrol seems to be important for the activity of the LXR-target gene ABCA1 which is involved in cholesterol efflux from the cells [30]. The importance of this mechanism under in vivo conditions is difficult to evaluate, however.

Mice with an overexpression of CYP27A1 and high levels of 27OH in the circulation and liver have normal levels of LXR target genes in the liver, suggesting that 27OH is not important for regulation of LXR-targeted genes in this mouse model [31].

Treatment of mice with a high dose of cholesterol in the diet results in activation of a number of LXR target genes in the liver. In view of the fact that side-chain oxidized cholesterol species, inclusive 27-hydroxycholesterol, are LXR agonists, the possibility must be considered that the cholesterol-induced effects are mediated by side-chain oxidized oxysterols. In order to test this possibility, a transgenic mouse model was developed with a knockout of the cholesterol 24-hydroxylase (Cyp46), the cholesterol 25-hydroxylase, and the sterol 27-hydroxylase (Cyp27) [32]. As expected, the cholesterol feeding induced five established LXR target genes in wild-type mice (Cyp7a1, Srebp1c, Abcg5, Abcg8, lipoprotein lipase). Three of these genes, lipoprotein lipase, Abcg5, and Abcg8, were not induced in the triple-knockout mice. One target gene, Srebp1c responded partially to cholesterol feeding in the triple-knockout mice and another gene Cyp7a1 responded normally. This experiment was later repeated with Cyp27 knockout mice replacing the triple-knockout mouse model [31]. Similar effects were obtained at the gene level as those obtained with the triple-knockout model. Most of the effects could however not be confirmed at the protein level or at the activity level (lipoprotein lipase). It was concluded that 27-hydroxcholesterol may be of some importance for activation of some LXR target genes and some specific conditions. The importance of this effect under normal conditions is difficult to evaluate, however. It should be emphasized that the high dose of cholesterol needed to induce the LXR target genes is highly unphysiologic.

Role of 27-Hydroxycholesterol in the Brain

In spite of the quantitatively important flux of 27-hydroxycholesterol into the brain [10], the levels of this oxysterol in the mammalian brain is very low, only 1–20% of that of 24S-hydroxycholesterol [33–35]. The protein level of Cyp27 is about 1/3 of that of Cyp46 in the human brain [35]. The most important reason for the low levels of 27-hydroxycholesterol in the brain is likely to be the rapid metabolism in this organ. 27-Hydroxycholesterol is metabolized by the cytochrome P-450 enzyme Cyp7b1 which is abundant in the brain [12]. The importance of this enzyme for the metabolism is evident from the finding that a knockout of this enzyme increases

the levels of 27-hydroxycholesterol in the mouse brain about tenfold (Ali et al. unpublished observation).

The flux of 27-hydroxycholesterol into the brain is likely to be secondary to the high metabolism in this organ, creating a concentration difference between the brain and the circulation. A reduced metabolism of this oxysterol in the brain could then be expected to reduce its flux into the brain. Evidence for this has been obtained in patients with very high levels of 27-hydroxycholesterol due to a mutation in the CYP7B1 gene (see below).

Mice deficient in Cyp27 without circulating levels of 27-hydroxycholesterol have a slightly upregulated rate of synthesis of cholesterol in the brain, consistent with the possibility that the flux of 27-hydroxycholesterol into the brain may be of some importance for cholesterol homeostasis in the brain [34].

The flux of 27-hydroxycholesterol into the brain appears to have a number of negative consequences. Since cholesterol itself does not pass the blood–brain barrier, and since there is a close relation between cholesterol and 27-hydroxycholesterol in the circulation [36], hypercholesterolemia is likely to lead to increased flux of 27-hydroxycholesterol into the brain. Thus a number of negative effects of hypercholesterolemia have suggested to be mediated by 27-hydroxycholesterol [37].

Treatment of mice with dietary cholesterol is known to have negative effects on the memory function in mice [38, 39]. Treatment of wild-type mice with dietary cholesterol thus results in a negative effect on the spatial memory as evaluated by the Morris water test. In accordance with the hypothesis that this effect is mediated by 27-hydroxycholesterol, feeding of mice with a deficiency of Cyp27 with cholesterol did not result in this negative effect [39]. The treatment also resulted in reduced levels of the "memory protein" Arc (activity regulated cytoskeleton associate protein) in hippocampus in wild-type mice but not in $Cyp27^{-/-}$ mice. In accordance with this treatment of primary neuronal cells with 27-hydroxycholesterol resulted in reduced production of Arc as well as reduced levels of NMDAR [40]. It should be mentioned that the authors also demonstrated reduced levels of Arc in the cortex of brain from patients who had died with the Alzheimer's disease (AD).

The negative effects of 27-hydroxycholesterol have also been demonstrated in a mouse model with high levels of 27-hydroxycholesterol due to an overexpression of CYP27 [41]. In accordance with above these mice have a reduced expression of Arc in hippocampus and a reduced spatial memory as evaluated by the Morris water test.

Part of the above negative effects on the memory function by 27-hydroxycholesterol may be due to a reduced uptake of glucose by the brain. Using PET technique it was recently shown that mice with high levels of 27-hydroxycholesterol due to overexpression of CYP27 have a reduced uptake of glucose in the brain [41]. In a clinical PET study a significant correlation was observed between uptake of glucose in the brain and the level of 27-hydroxycholesterol in cerebrospinal fluid. The mechanism behind the inhibitory effect of 27-hydroxycholesterol on glucose uptake by the brain was elucidated by in vitro experiments with cultured neuronal cells as well as use of mice with overexpressed or lack of sterol 27-hydroxlase. Furthermore the role of 27-hydroxycholesterol was also confirmed by intracerebral injections of this oxysterol in wild-type mice [41]. The inhibitory effect of 27-hydroxycholesterol on glucose

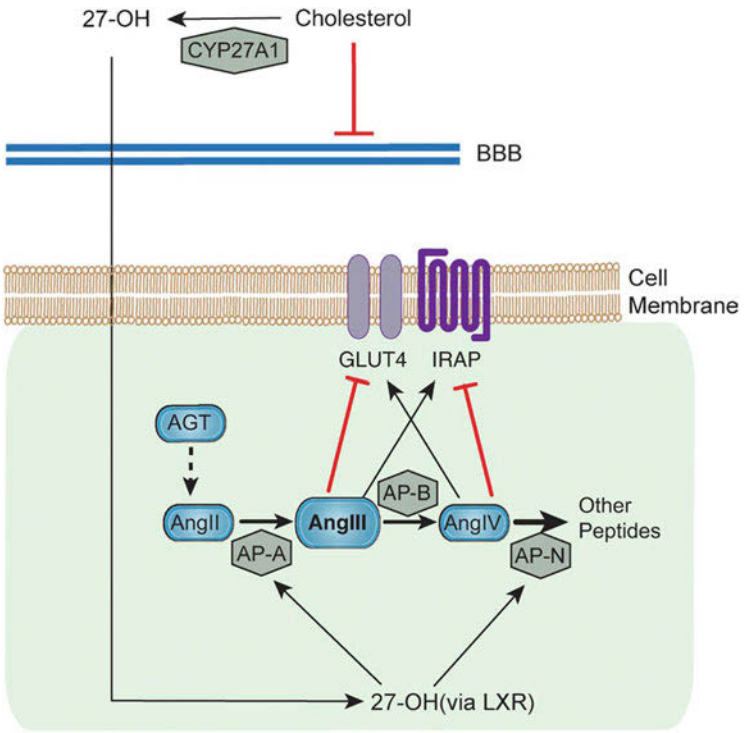

Fig. 3 Mechanism of regulation of glucose uptake by the brain. 27-Hydroxycholesterol is able to pass the blood–brain barrier and activates aminopeptidase A (AP-A) and aminopeptidase N (AP-N) by an LXR-mediated mechanism. AP-A stimulates conversion of Angiotensin II (AngII) into Angiotensin III (AngIII). AngIII is converted into Angiotensin IV (AngIV) by Aminopeptidase B (AP-B). AngIII stimulates production of insulin-regulated aminopeptidase (IRAP) and inhibits production of the glucose transporter GLUT4. AngIV stimulates production of GLUT4 and inhibits production of IRAP. The stimulation of AP-N by 27OH leads to degradation of AngIV leading to increased IRAP and decreased GLUT4. Reproduced from Ref. [41] by permission

uptake was shown to be associated with a decrease of the glucose transporter GLUT4 and an increase of the insulin regulated aminotransferase (IRAP). In addition, 27-hydroxycholesterol reduced the IRAP antagonist angiotensin IV mediated glucose uptake and increased the levels of the AngIV degrading enzyme aminopeptidase N (ApoN). These effects were found to be mediated by LXR signaling. The mechanism behind the inhibitory effect on glucose uptake by 27-hydroxycholesterol is summarized in Fig. 3.

In addition to the above effect on glucose uptake by the brain, 27-hydroxycholesterol seems to be important for the regulation of the renin–angiotensin system in the brain. Brains of mice on a cholesterol-enriched diet showed upregulated angiotensin converting enzyme (ACE), angiotensinogen (ATG) and increased JAK/STAT activity [42]. The possibility was tested that these effects are mediated by 27-hydroxycholesterol. In accordance with this hypothesis the same effects as above were obtained in primary neurons and astrocytes exposed to

27-hydroxycholesterol or 24-hydroxycholesterol. These effects were shown to be partially dependent on LXR signaling. Mice lacking the sterol 27-hydroxlase without production of 27-hydroxycholesterol had reduced RAS activity in the brain. In accordance with this, patients with elevated levels of 27-hydroxycholesterol due to a mutation in the CYP7B1 gene were shown to have elevated markers of RAS in cerebrospinal fluid. It was suggested that 27-hydroxycholesterol could be a link between high plasma cholesterol levels and hypertension [42].

In addition to the above effects on different functions in the brain, 27-hydroxycholesterol may be important in neurodegeneration. Hypercholesterolemia in mid-age is a risk factor for Alzheimer's disease [43] and given the fact that cholesterol itself does not pass the blood–brain barrier, it has been suggested that 27-hydroxcholesterol may be the link between hypercholesterolemia and neurodegeneration [37]. As discussed below, the oxysterol 24S-hydroxycholesterol increases the activity of alpha secretase in human neuroblastoma cells and reduces the activity of beta secretase [44]. These effects can be expected to lead to reduced production of amyloid from amyloid precursor protein. Under the same in vitro conditions, 27-hydroxycholesterol had an inhibitory effect on the effects induced by 24S-hydroxycholesterol. In another in vitro study, 27-hydroxycholesterol was confirmed to have a stimulatory effect on the beta secretase activity [45]. If the above effects are important also under in vivo conditions, increased flux of 27-hydroxycholesterol into the brain can be expected to stimulate generation of amyloid.

Consistent with this it has been shown that there is a substantial accumulation of 27-hydroxycholesterol in the brain of patients who have died with Alzheimer's disease [46]. It should be pointed out, however, that this accumulation could be secondary to the reduced number of neuronal cells containing the Cyp7b1 enzyme responsible for metabolism of 27-hydroxycholesterol.

27-Hydroxycholesterol as a Modifier of Estrogen Receptor Signaling: Consequences for Vascular Diseases, Breast Cancer, Osteoporosis, and the Parkinson's Disease

It has been shown that 27-hydroxycholesterol is a selective estrogen receptor modulator. This means that it can show agonist as well as antagonist activities in a cell and promoter-dependent manner [47, 48]. It was shown that 27-hydroxycholesterol induces a unique conformational change of both the alpha and beta estrogen receptor. The oxysterol was found to inhibit both transcription-mediated and non-transcription-mediated estrogen dependent production of nitric oxide by vascular cells. Furthermore estrogen-induced vasorelaxation of rat aortic rings were reduced by 27-hydroxycholesterol. Evidence was presented that this effect is of importance also under in vivo conditions. Mice with high levels of 27-hydroxycholesterol were thus found to have decreased estrogen-dependent expression of vascular nitric oxide synthase and repressed carotid artery reendothelialization [47]. It was suggested that 27-hydroxycholesterol could contribute to a loss of estrogen protection from vascular disease.

Hypercholesterolemia is a risk factor for breast cancer in postmenopausal women. Evidence has been presented that at least part of this effect may be mediated by 27-hydroxycholesterol. Thus, 27-hydroxycholesterol was shown to increase estrogen-dependent growth and LXR-dependent metastasis in a mouse model of breast cancer [49]. Furthermore it was shown that mice with a deficiency of the sterol 27-hydroxylase had an impaired growth of mammary tumors—an effect that could be reversed by daily injections of 27-hydroxycholesterol. The development of tumors was faster in ApoE mice on a cholesterol-enriched diet compared to the same mice fed a control diet. The tumor growth was found to be attenuated by treatment with an inhibitor of the sterol 27-hydroxylase. The most aggressive human breast cancers were found to express the highest levels of the sterol 27-hydroxylase, suggesting that 27-hydroxycholesterol produced within tumors may contribute to tumorigenesis. CYP7B1 is the most important enzyme involved in the metabolism of 27-hydroxycholesterol. In accordance with above increased expression of CYP7B1 mRNA in human breast cancer was associated with significantly better survival outcome than cancer with a low expression of this gene.

Evidence has been presented that 27-hydroxycholesterol also may contribute to ER-positive breast cancer progression by disrupting constitutive P53 signaling [50].

In contrast to the effects on the primary tumors in the mouse models, the actions of 27-hydroxycholesterol on metastasis did not appear to involve estrogen receptor [49]. Thus treatment with estrogen did not increase metastasis. Treatment with a synthetic LXR agonist increased however metastasis in this mouse model albeit less efficiently than 27-hydroxycholesterol. It was suggested that LXR activation by 27-hydroxycholesterol increases tumor metastasis and that this process occurs independently of estrogen receptor.

It is known that decreased bone density is often associated with high cholesterol and patients on statins have increased bone mineral densities, consistent with cholesterol as a negative regulator of bone homeostasis. 27-Hydroxycholesterol could be the mediator of this effect and it was shown that this oxysterol decreases osteoblast differentiation and enhances osteoclastogenesis resulting in increased bone resorption in mice [51, 52]. This effect was mediated by the effect of 27-hydroxycholesterol on both estrogen receptors and LXR-receptors. Increased concentrations of 27-hydroxycholesterol both by genetic and pharmacological means thus led to decreased bone mineral density that was associated with decreased bone formation and increased bone resorption. Upon manipulation of endogenous estrogen levels, the responses to elevated levels of the oxysterol implicated the estrogen receptor as a likely mediator. The possibility was discussed that the new mechanism can be the basis for new therapeutic strategies [51].

It has been shown that 27-hydroxycholesterol reduces the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and increases α-synuclein levels in vitro in neuroblastoma cells [53]. The regulation of the above systems was found to involve both estrogen receptor and LXR receptors. It was specifically shown that inhibition of ERβ mediates 27-hydroxycholesterol-dependent decrease in TH expression and that this effect could be reversed by the addition of the ER agonist estradiol. It was concluded that concomitant activation of ERβ and

inhibition of LXR signaling prevents the effect of 27-hydroxycholesterol and may therefore reduce the progression of Parkinson's disease by precluding TH reduction and accumulation of α-synuclein. The importance of the above effects in vivo is however difficult to evaluate. It is of interest that a subpopulation of PD patients exhibit amyloid plaques and some AD patients exhibit synuclein aggregates. It was suggested that 27-hydroxycholesterol contribute both to AD, PD, and AD/PD overlap [54]. Convincing evidence for this is still lacking, however.

Possible Role of 27-Hydroxycholesterol in Adipocytes

It has been shown that human and rat adipocytes contain sterol 27-hydroxylase able to convert cholesterol into 27OH. Inhibition of this enzyme increased the rate of differentiation compared to mock-transfected controls [55]. It was suggested that local synthesis of in adipocytes may act as a protective mechanism by limiting the potential of adipocyte differentiation. The relevance of this for the situation in humans is difficult to assess, however, and obesity is not a characteristic feature in patients lacking CYP27A1 (cf. below).

Clinical Consequences of Decreased or Increased Levels of 27-Hydroxycholesterol

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive disease caused by a defective sterol 27-hydroxylase enzyme, due to a mutation in the CYP27A1 gene (For a review, see [56] and references therein). At present, several hundred cases have been documented. The most common clinical findings in CTX include tendon and brain xanthomas, juvenile cataracts, progressive dementia with cerebellopyramidal signs, chronic diarrhea, osteoporosis, and premature atherosclerosis. The preferential site of the xanthomas in the brain is the white matter in cerebellum. In spite of the xanthomas CTX patients are usually normocholesterolemic. Plasma sterol analyses reveal low levels of 27-hydroxycholesterol, elevated cholestanol, and increased levels of some bile alcohols that are normally present in trace amounts only.

The clinical signs of CTX develop gradually during life. Some patients may be mentally degraded already in childhood whereas some patients do not get any neurological symptoms even at high age and the only sign of the disease may be Achilles tendon xanthomas. In some rare cases, the disease is associated with cholestasis in the neonatal stage.

The disease process in CTX is linked to generation of cholestanol. Most probably the formation of cholesterol and cholestanol-containing xanthomas is secondary to this increased production. The marked (often more than 100-fold) accumulation of the cholestanol precursor 7α-hydroxy-4-cholesten-3-one is a consequence of its increased production and its decreased metabolism into bile acids. It has been shown that 7α-hydroxy-4-cholesten-3-one is able to pass the blood–brain barrier and converted into cholestanol [56].

The dominating pathway for conversion of cholesterol into bile acids in humans involves cholesterol 7α-hydroxylase (CYP7A1) as a rate-limiting step. Since this enzyme is subject to a negative feedback regulation by bile acids, the reduced formation of bile acids in CTX, in particular of chenodeoxycholic acid, leads to a marked upregulation of the cholesterol 7α-hydroxylase, CYP7A1. The activity of this enzyme may be increased more than 20-fold in the liver of CTX patients. In addition to the increased formation of 7α-hydroxy-4-cholesten-3-one and cholestanol, the accumulation of bile acid precursors leads to formation of 25-hydroxylated bile alcohols. Such bile alcohols may be excreted in gram amounts in feces. Figure 4 summarizes the mechanism behind accumulation of cholestanol in patients with CTX.

Administration of bile acids to patients with CTX reverses the disease process and leads to normal levels of cholestanol and a reduction of the xanthomas. Even the size of the xanthomas in the brain may be reduced as a result of such treatment. The normalization of the biochemical changes by treatment with bile acid supports that the driving force in the disease process is the markedly increased activity of the cholesterol 7α-hydroxylase rather than the lack of the CYP27-mediated elimination of cholesterol from different cells.

Knocking out Cyp27 in mice give the same biochemical changes as in CTX except that no xanthomas are formed and that the stimulatory effect on Cyp7a1 is less than the effect on the same enzyme in patients with CTX. Also in $Cyp27^{-/-}$ mice all the biochemical changes are normalized after feeding with bile acids.

A specific subgroup of patients with hereditary spastic paresis, called SPG5, has been shown to have mutations in the gene coding for CYP7B1 [57]. The patients are characterized by a progressive spastic paraplegia with variable age at onset. The disease may be complicated by cerebellar ataxia and optic atrophy. As can be expected these patients have markedly increased levels of 27-hydroxycholesterol in plasma and cerebrospinal fluid. The plasma levels of 25-hydroxycholesterol are increased even more, about 100-fold. A metabolite of 27-hydroxycholesterol, cholestenoic acid (3β-hydroxy-5-cholestenoic acid), is also markedly increased in plasma and cerebrospinal fluid. Given the neurotoxic effect of 27-hydroxycholesterol it was suggested that the symptoms in patients with SPG5 are a consequence of the high levels of this oxysterol [57]. Another possibility is that it is the metabolite of 27-hydroxycholesterol, cholestenoic acid, that is causing the symptoms [58]. In accordance with the latter possibility cholestenoic acid was found to cause a loss of neuronal cells under different in vitro and in vivo conditions, a loss that could be prevented by the CYP7B1 metabolite of cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid (3β,7α-diHCA). The latter metabolite is lacking in patients with SPG5 disease. The neuroprotective effect of 3β,7α-dihydroxy-5-cholestenoic acid was found to be dependent upon LXR activation. It is known that adult mice with a knockout of LXR show a progressive accumulation of lipids in the brain and loss of spinal cord neurons, suggestive of a role of LXRs and their ligands on adult motor neurons [59].

In addition to cholesterol, dehydroepiandrosterone is a substrate for CYP7B1. At present the possibility cannot be excluded that other steroids than

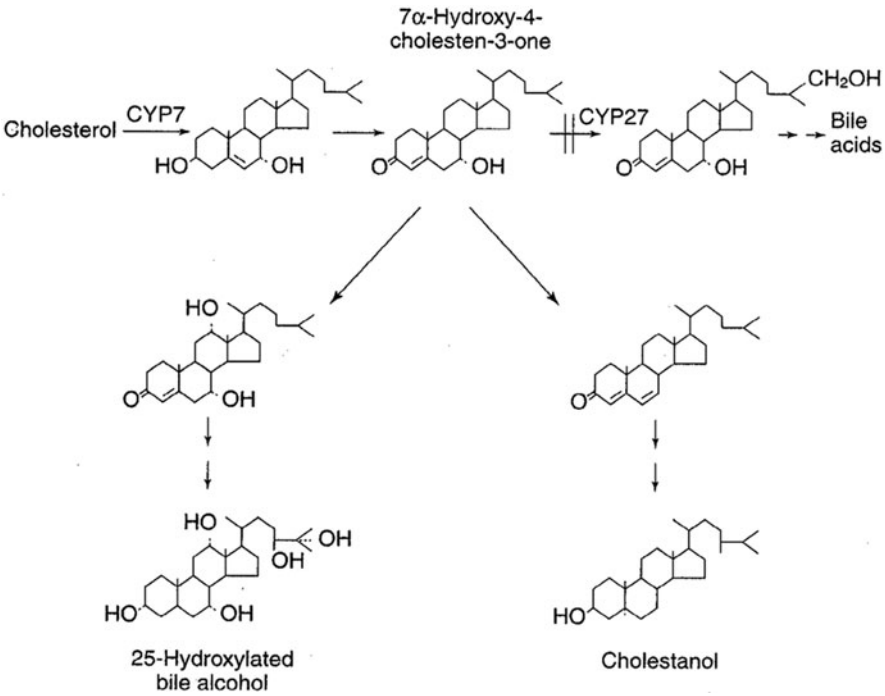

Fig. 4 Metabolic consequences of a lack of the sterol 27-hydroxylase in Cerebrotendinous Xanthomatosis (CTX). The reduced formation of bile acids, in particular chenodeoxycholic acid, leads to increased activity of the cholesterol 7 alpha hydroxylase (CYP7A1) in the liver leading to a marked accumulation of 7 alpha hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis. The latter intermediate is converted into 25-hydroxylated bile alcohols which are excreted in high amounts in bile and feces. The accumulation of cholestanol leads to formation of xanthomas in the brain and in tendons

27-hydroxycholesterol or cholestenoic acid are responsible for the loss of upper neuronal cells in the SPG5 disease.

Mice with a knockout of Cyp7b1 do not get neurological symptoms. The accumulation of 27-hydroxycholesterol and cholestenoic acid in these mice is however lower than that in patients with the SPG5 disease.

Given the high levels of 27-hydroxycholesterol in patients with the SPG5 disease, one would expect them to have increased risk to develop neurodegeneration, high blood pressure, osteoporosis, vascular disease, and breast cancer. Investigations thus far on a very limited number of patients have failed to show a general neurodegeneration that can be detected by cerebrospinal markers [57]. Evidence has however been presented that the renin–angiotensinogen system is activated in the brain of these patients [42].

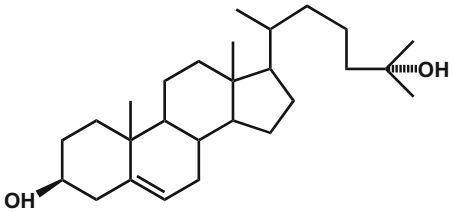

Fig. 5 Structure of 25-hydroxycholesterol

25-Hydroxycholesterol

Structure of 25-Hydroxycholesterol

The structure of 25-hydroxycholesterol (5-Cholesten-3β,25-diol) is shown in Fig. 5.

Mechanisms of Formation

25-Hydroxycholesterol is a far less abundant oxysterol than 24- and 27-hydroxycholesterol. It is present in the circulation at low nanomolar concentrations [60]. Studies on autoxidation of crystalline cholesterol in air showed an increasing formation of 25-hydroxycholesterol with time [61]. The conversion of cholesterol to 25-hydroxycholesterol was shown to proceed via a cholesterol 25-hydroperoxide intermediate which was formed by ground-state biradical triplet oxygen attack on cholesterol [62]. Thermal decomposition of the hydroperoxide gave 25-hydroxycholesterol as the major product [63]. Recently it was demonstrated that recombinant cytochrome P450 enzymes, CYP27A1 and CYP11A1, can mediate the reduction of cholesterol 25-hydroperoxide to 25-hydroxycholesterol. The authors suggest that this reaction can be of potential significance in cholesterol rich tissues exposed to oxidative stress [64]. The quantitative importance of autoxidation for 25-hydroxycholesterol formation in humans is however still unknown [65]. In an in vivo study in rats it was shown that the 25-hydroxycholesterol formed in the liver following a dietary cholesterol challenge was endogenously produced and not formed by cholesterol autoxidation [66].

Cholesterol can be enzymatically converted into 25-hydroxycholesterol by the hydrophobic multi-transmembrane ER protein cholesterol 25 hydroxylase (CH25H). The cDNA cloning of the mouse and human enzymes was reported in 1998 by Lund et al. [67]. In contrast to many other cholesterol metabolizing enzymes, CH25H is not a cytochrome P450 enzyme, but is related to a small group of enzymes utilizing di-iron cofactors for catalyzing hydroxylation of their substrates. The enzyme contains three histidine boxes which are important for the catalytic activity and have been conserved in all vertebrate species examined [67, 68]. The cholesterol 25 hydroxylase gene is intron-less and located on chromosome 10q23 where it is found in tandem with the gene for cholesterol ester lipase [67, 68]. When nine

different sterols were screened for inhibitory action on the CH25H, desmosterol was found to be the most potent inhibitor [67].

The production of 25-hydroxycholesterol as a by-product in other enzymatic reactions have been reported for a number of cytochrome P450 enzymes. The potential importance of these reactions has recently been reviewed [65].

25-Hydroxycholesterol and Foam Cell Formation

Studies by Gold et al. [69] suggested that the transcription factor ATF3 may be involved in the regulation of macrophage foam cell formation. The authors showed that ATF3 binds to the promoter of Ch25h which leads to repression of transcription. Macrophages from Atf3 knockout mice showed highly elevated levels of Ch25h mRNA and 25-hydroxycholesterol. It was hypothesized that Aft3 controls lipid body formation in macrophages. Stimulation of macrophages from wild-type mice with 25-hydroxycholesterol resulted in increased lipid body formation. When Atf3 was knocked out in apoE knockout mice, there was an increased severity of atherosclerosis following 8 weeks of high fat diet [69].

Macrophages from mice lacking the orphan nuclear receptor retinoid-related orphan receptor alpha (Rorα) have enhanced lipid uptake and form larger lipid droplets than wild-type mice [70]. Ch25h protein is reduced approximately 90% in these mice. When bone marrow-derived macrophages from the Rorα knockout mice were treated with 100 nM 25-hydroxycholesterol there was a significantly reduced accumulation of lipid in lipid droplets. Furthermore, when Ch25h was silenced by a specific siRNA causing a 50% reduction in Ch25h RNA, there was a significant increase in lipid droplets. These experiments suggest that 25-hydroxycholesterol and Rorα are important factors for lipid uptake, lipid droplet formation, and eventually foam cell formation.

25-Hydroxycholesterol as a Modulator of the Immune System

Bone marrow-derived macrophages from mice were screened for upregulated genes following lipopolysaccharide (LPS) treatment. Cells exposed to LPS (10 ng/ml) for 2 h showed a highly increased expression of Ch25h, while the expression of Cyp7a1, Cyp27a1, Cyp7b1, and Hmg-CoA reductase were unchanged by the treatment [71]. LPS interacts with Toll-like receptor 4 (TLR4). When macrophages from Tlr4 knockout mice were treated with LPS, no induction in Ch25h expression was seen. The downstream signaling was investigated using macrophages from Myeloid differentiation protein 88 (MyD88) knockout mice. Macrophages from this mouse model were treated with LPS and the expression of Ch25h was induced to the same extent as in macrophages from wild-type mice. This suggests that the MyD88-dependent pathway is not critical for the downstream signaling and that the alternative MyD88-independent pathway involving TRIF is the major signaling pathway. Upon treatment of bone marrow-derived macrophages with LPS, 25-hydroxycholesterol was produced and

was released to the culture medium. In addition to the experiments in mice, US Standard Reference *E. coli* endotoxin (4 ng/kg body weight) was infused in eight healthy volunteers and resulted in a significant increase in plasma 25-hydroxycholesterol after 4 h [71].

Studies on mouse macrophages showed that not only TLR4 agonists could stimulate the production of 25-hydroxycholesterol but also specific agonists of other TLRs [72]. It was shown that TLR activation resulted in increases in Ch25h mRNA and protein and a stimulated production of 25-hydroxycholesterol. Intraperitoneal injection of a TLR4-specific agonist in wild-type C57BL/6J mice resulted in a general induction of Ch25h mRNA and increased levels of Ch25h protein and 25-hydroxycholesterol in various tissues demonstrating induction of Ch25h also in vivo. No induction of Ch25h was seen in Ch25h knockout mice.

A very interesting observation was reported by Bauman et al. using two mouse models with either low levels of 25-hydroxycholesterol (Ch25h knockout mice) or high levels of 25-hydroxycholesterol (Cyp7b1 knockout mice) [72]. Significantly elevated levels of immunoglobulin A (IgA) was determined in sera, bronchial alveolar lavage, and intestinal mucosa from Ch25h knockout mice while mice with high circulatory levels of 25-hydroxycholesterol (Cyp7b1 knockout mice) had significantly reduced IgA levels in sera, lungs, and intestinal mucosa. The effect of 25-hydroxycholesterol on IgA production was confirmed in vitro using splenic B220+ cells isolated from wild-type C57BL/6J mice [72]. This study showed that 25-hydroxycholesterol is an important regulator of the immune system.

It has previously been reported that 25-hydroxycholesterol induces IL-8 production, a chemotactic factor for T-lymphocytes, in human monocytes and macrophages [73, 74].

TLR-induced upregulation of Ch25h transcription is not limited to macrophages, but occurs also in dendritic cells, as shown by Park and Scott [75]. The induction of Ch25h was mainly due to TLR3 and TLR4 signaling. It was shown that the TLR-mediated induction of Ch25h was dependent on the adaptor molecule TRIF and subsequent Type I interferon production and signaling through the JAK/STAT1 pathway, thus identifying Ch25h as an interferon-responsive gene [75].

7α,25-Dihydroxycholesterol and EBI2

Further evidence for the importance of 25-hydroxycholesterol for immune function came when two research groups reported on the identification of the natural ligand for the orphan receptor Epstein–Barr-virus-induced gene 2 (EBI2) [76, 77]. EBI2, which is highly expressed in the spleen, is induced by viral infection, and is required for normal B cell migration. Both research groups identified 7α,25-dihydroxycholesterol as the natural ligand and activator for EBI2. The ligand is a metabolite of 25-hydroxycholesterol, formed by hydroxylation of 25-hydroxycholesterol at the 7-position by the cytochrome P-450 enzyme CYP7B1 (oxysterol 7α-hydroxylase) [12, 78]. Another dihydroxycholesterol, 7α,27-dihydroxycholesterol also activated EBI2, but was less potent than 7α,25-dihydroxycholesterol [76, 77]. Furthermore,

LPS activation of macrophages and dendritic cells led to upregulation of EBI2, but also of CH25H and CYP7B1. In contrast, CYP27A1, responsible for the production of 27-hydroxycholesterol, was downregulated, suggesting that 7α,25-hydroxycholesterol rather than 7α,27-dihydroxycholesterol is the natural ligand for EBI2 [77]. Several recent reviews summarize the role of 25-hydroxycholesterol in innate and adaptive immunity [79–81].

25-Hydroxycholesterol as an Antiviral Factor

In 2013 two independent research groups identified 25-hydroxycholesterol as an antiviral agent [82, 83]. Liu et al. showed that 25-hydroxycholesterol exhibited antiviral activity against several enveloped viruses, but not against non-enveloped viruses. They also showed that 25-hydroxycholesterol reduced HIV infection in a mouse model. The authors suggested that 25-hydroxycholesterol acted through modification of the cell membrane to inhibit viral entry [82].

Blanc et al. [83] found a specific increase in 25-hydroxycholesterol due to an upregulated Ch25h following interferon treatment or virus infection of macrophages. No other oxysterols increased in response to the treatment. It was shown that 25-hydroxycholesterol had a potent antiviral activity against a broad spectrum of viruses. The antiviral effect was shown to be LXR independent. These authors suggested that the mechanism did not involve viral entry into the cell, but rather downregulation of genes via the SREBP pathway, although this mechanism may not account for all the antiviral activity [83].

The effect of 25-hydroxycholesterol treatment on bone marrow-derived macrophages (BMDM) was investigated and it was found that incubation of BMDMs with 5 μM 25-hydroxycholesterol resulted in significant increases in cholesterol esters and ceramide and decreases in sphingolipids [84]. In addition, it was found that 25-hydroxycholesterol treatment induced stress response genes in a concentration- and time-dependent manner. The 25-hydroxycholesterol-mediated induction of stress response genes was independent of LXR activation and SREBP processing. When BMDM were infected with MCMV, the production of 25-hydroxycholesterol was stimulated leading to induction of integrated stress response transcription. Further experiments showed that the most important inducer of the stress response was the kinase GCN2. The authors suggest that the two major effects of 25-hydroxycholesterol, remodeling of the plasma membrane and induction of the integrated stress response, may both contribute to the antiviral activity of 25-hydroxycholesterol.

Still another antiviral mechanism for 25-hydroxycholesterol was proposed by a research group studying Lassa Virus [85]. These authors found that treatment of cells with 5 or 10 μM 25-hydroxycholesterol or overexpressing CH25H led to reduced production of Lassa Virus with reduced infectivity and knocking down CH25H expression increased the production of Lassa Virus. When they examined viral proteins after 25-hydroxycholesterol treatment, they found aberrant glycosylation of the viral protein GP1, important for virus attachment to host cell receptors. The

aberrantly glycosylated GP1 protein was shown to be incorporated into the virus particles.

In experiments in cultivated cells it was shown that 25-hydroxycholesterol in low micromolar concentrations exhibited a marked antiviral activity against some non-enveloped viruses, human papillomavirus-16, human rotavirus and human rhinovirus [86].

Ikegami et al. [87] showed that 25-hydroxycholesterol was significantly increased in serum from patients with hepatitis C infection. When the patients were treated with PEGylated interferon and ribavirin for 3 months, the elevated levels of oxysterols were decreased significantly.

When liver biopsies from patients with chronic hepatitis C were analyzed it was shown that the expression of CH25H was significantly upregulated compared to hepatitis C negative liver biopsies [88]. It was also shown that CH25H was upregulated in primary human hepatocytes infected with hepatitis C virus. Furthermore, it was shown that 25-hydroxycholesterol was a potent inhibitor of hepatitis C virus replication, blocking the membraneous web formation.

Xiang et al. [89] showed that 25-hydroxycholesterol inhibits hepatitis C infection in human cells. However, they report that, unlike in mice, CH25H is not an interferon-inducible gene in human cells and is not dependent on STAT1. Instead, the upregulation of CH25H is a direct host response to viral infection acting to antagonize SREBP function and thus host lipid metabolism.

Zika virus has recently attracted much attention. This virus uses a mosquito vector and Zika infection can lead to serious neurological problems and in some cases microcephaly. Li and coworkers [90] reported that Zika virus infection leads to induction of CH25H and that 25-hydroxycholesterol is an important mediator of host defense against the infection. This was shown using a human alveolar epithelial cell line. Zika virus infection induced the CH25H in a dose-dependent manner. In a cell line where CH25H was knocked out significantly enhanced replication of the virus was observed and overexpression of CH25H resulted in significantly reduced virus replication. It was also shown that 25-hydroxycholesterol acted by interfering with viral entry into the cell. Experiments in BALB/c mice showed that treatment of the animals with 25-hydroxycholesterol significantly reduced viremia upon Zika infection compared to vehicle treatment. Treatment of Rhesus monkeys with 25-hydroxycholesterol protected these animals from Zika infection. Further experiments showed a protective effect of 25-hydroxycholesterol at low micromolar concentrations in Zika-infected human cortical organoids. Finally it was demonstrated that treatment of pregnant mice with 25-hydroxycholesterol prevented Zika infection and reduced the incidence of microcephaly. The authors suggest that 25-hydroxycholesterol may be used as an antiviral agent against many different viruses.

25-Hydroxycholesterol and Inflammation

It has been reported that LPS-stimulated macrophages from Ch25h knockout mice overproduce inflammatory interleukin-1 (IL-1) [91]. When LPS-stimulated macrophages from the knockout animals were incubated with 25-hydroxycholesterol, IL-1 mRNA and protein decreased and a suppressed inflammasome activity was observed. In vivo experiments in mice showed that LPS treatment resulted in higher IL-1 concentrations in serum in Ch25h knockout mice than in wild-type mice. The authors suggested that 25-hydroxycholesterol acts by antagonizing SREBP processing and conclude that 25-hydroxycholesterol acts as a repressor of IL-1 induced inflammation [91]. In contrast, another research group reported that 25-hydroxycholesterol acts as an amplifier of inflammation [92]. Furthermore, they showed that Ch25h knockout mice were partly protected from influenza infection and analysis of lung tissue after influenza infection showed less tissue damage compared to wild-type mice. These experiments suggest that the viral induction of Ch25h resulting in increased production of 25-hydroxycholesterol leads not only to resistance to viral infection but also to induction of inflammatory mediators and subsequent tissue damage [92].

25-Hydroxycholesterol and Obstructive Pulmonary Disease

25-Hydroxycholesterol has been reported to induce IL-1 and IL-8 production in different human cells (Rosklint, Bai). Sugiura et al. investigated whether 25-hydroxycholesterol has a role in chronic obstructive pulmonary disease (COPD) [93]. They found that the expression of CH25H was significantly elevated in lung tissue from patients with COPD and that sputum from these patients contained higher concentrations of 25-hydroxycholesterol than sputum from healthy control subjects. There was, however, no difference in serum 25-hydroxycholesterol between COPD patients and healthy controls. There was a significant correlation between 25-hydroxycholesterol and IL-8 in sputum, and there was also a negative correlation between 25-hydroxycholesterol and pulmonary function. The authors showed a strong immunoreactivity for CH25H in alveolar macrophages and pneumocytes. It was suggested that 25-hydroxycholesterol may be associated with neutrophilic inflammation in COPD.

It has been reported that 25-hydroxycholesterol promotes cell migration and invasion of lung adenocarcinoma cells [94]. Treatment of adenocarcinoma cells with 100 nM 25-hydroxycholesterol enhanced the migratory ability 1.3-fold while the same treatment of cocultured adenocarcinoma cells and THP1-derived macrophages increased the mobility 2.2- to 2.6-fold.

Cerebral Inflammation in X-Linked Adrenoleukodystrophy

The inherited neurodegenerative disorder X-linked adrenoleukodystrophy (X-ALD) is due to mutations in the ABCD1 gene. This gene codes for a transporter important for the transfer of very long chain fatty acids (VLCFA) from the cytosol to peroxisomes, and a defective gene results in accumulation of VLCFA in multiple tissues but also an induced CH25H expression. Patients with X-ALD, especially those with the severe phenotype childhood cerebral ALD, show symptoms of cerebral inflammation. It has recently been suggested that 25-hydroxycholesterol contributes to this inflammation by activating the NLRP3 inflammasome [95]. However, data supporting this hypothesis were generated partly in cell systems where highly unphysiological concentrations of 25-hydroxycholesterol were used. It is therefore not possible to draw any conclusions of the importance of 25-hydroxycholesterol for X-ALD from these experiments.

25-Hydroxycholesterol and Glioblastoma Cell Lines

Expression of CH25H was investigated in two human glioblastoma cell lines, U87MG and GM133 [96]. Addition of TNFα resulted in a significant upregulation of CH25H expression while IL-1 had a somewhat smaller effect. Cytokine addition led to increased protein levels of CH25H and increased levels of 25-hydroxycholesterol both intracellularly and in the culture medium. It was also shown that low nanomolar concentrations of 25-hydroxycholesterol stimulated chemotactic migration of human THP-1 monocytes and primary human monocytes. When the G protein-coupled receptor EBI2 was silenced with SiRNA migration of THP-1 cells by 25-hydroxycholesterol was reduced. The authors hypothesize that production of 25-hydroxycholesterol by glioblastoma cells may recruit monocytes/macrophages to the tumor affecting the tumor development.

25-Hydroxycholesterol and Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease affecting the central nervous system. Kim et al. [97] hypothesized that side-chain oxidized oxysterols may contribute to the disease process in ALS. Determination of 24-, 25-, and 27-hydroxycholesterol in cerebrospinal fluid and serum showed that the levels of 25-hydroxycholesterol were higher in untreated ALS patients than in controls. It was also found that serum 25-hydroxycholesterol concentrations were significantly associated with disease severity and progression. The authors conclude that 25-hydroxycholesterol may be a mediator of ALS pathogenesis involving GSK-3β activation and neuronal apoptosis.

25-Hydroxycholesterol and Angiotensin II-Induced Muscle Wasting

Cardiac cachexia is a frequent finding in patients with congestive heart failure. These patients have increased serum concentrations of angiotensin II and TNFα. Shen [98] hypothesized that angiotensin II-induced muscle wasting is mediated by TNFα. These authors found that infusion of angiotensin II in wild-type mice resulted in skeletal muscle wasting while the same treatment in TNFα-receptor1 knockout mice did not lead to muscle wasting, demonstrating the importance of TNFα signaling in angiotensin II-induced muscle wasting. They further investigated downstream targets for TNFα and found that CH25H was strongly upregulated by TNF-α-treatment. Furthermore, intraperitoneal injection of 25-hydroycholesterol led to decreases in muscle weight as well as body weight. Further studies suggested that 25-hydroxycholesterol acted through reducing Akt signaling resulting in glycogen synthase kinase-3β (GSK-3β) activation.

25-Hydroxycholesterol Is Required for Interferon γ: Induced Proteasomal Degradation of HMG-CoA Reductase in Macrophages

Lu et al. [99] treated macrophages with interferon-γ and found a rapid induction of Ch25h expression which was reduced after 2 h concomitant with the induction of the inflammatory repressor AFT3. It was shown that Toll-like receptor induction of AFT3 was absolutely dependent on interferon signaling. Interferon treatment led to a rapid reduction in HMGCoA reductase protein, due to proteasomal degradation, and this degradation was strictly dependent on synthesis of 25-hydroxycholesterol.

25-Hydroxycholesterol Promotes Osteoclastogenesis

In a recent report from Zhang et al. [100] it was demonstrated in mouse bone marrow derived macrophages that 25-hydroxycholesterol (100 nM) inhibited miR139-5p expression. Experiments suggested that 25-hydroxycholesterol treatment led to increased formation of NFATc/Sp1 complex, which contributed to downregulate the transcription of miR-139-5p and thereby promote osteoclastogenesis.

25-Hydroxycholesterol and Insulin Sensitivity

When C57BL/6 mice were fed a high-fat diet, it was observed that there was a significant decrease in hepatic Ch25h mRNA and protein compared to controls [101]. It was also shown that a subgroup of obese mice with insulin resistance had lower levels of hepatic Ch25h than obese mice with a normal response to glucose and insulin tolerance test. Overexpression of hepatic Ch25h in mice resulted in

significantly elevated levels of mRNA and 25-hydroxycholesterol compared to control mice. The authors also report that in humans hepatic CH25H levels are lower in obese patients than in lean subjects. They suggest that low levels of Ch25h in the liver may be considered a risk factor for obesity with insulin resistance.

25-Hydroxycholesterol in Human Semen

An investigation of human semen samples revealed that 25-hydroxycholesterol was the quantitatively dominating oxysterol in normozoospermic individuals and the concentration was positively correlated to the number of spermatozoa [102]. Patients with low sperm count, reduced sperm motility, or varicocele had significantly lower concentrations of 25-hydroxycholesterol in semen than normozoospermic individuals. It was shown that the enzyme CH25H was present in human spermatozoa and also localized to the seminiferous tubules in the testes. Incubation of spermatozoa with 25-hydroxycholesterol led to calcium and cholesterol depletion in the acrosome region. The authors suggest that 25-hydroxycholesterol has a role in sperm function.

24S-Hydroxycholesterol

Excellent more detailed reviews concerning the cholesterol 24S-hydroxylase and the regulation of this enzyme have been published previously [103, 104].

Structure

The structure of 24S-hydroxycholesterol (24OH) is shown in Fig. 6. It should be noted that the stereoisomer 24S- is the dominating one in the brain and in the circulation of mammals. Low levels of the 24R-isomer are present, however, in the circulation of mice [105].

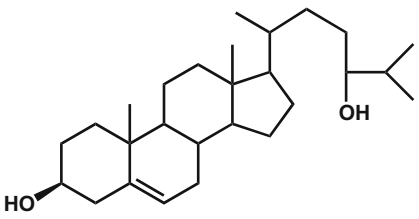

Fig. 6 Structure of 24S-hydroxycholesterol

Biosynthesis and Flux of 24S-Hydroxycholesterol from the Brain

24OH is the major oxysterol in the brain and one of the major oxysterols in the circulation. The steroid was first isolated from horse brain in the 1950s and was initially called "cerebrosterol" [106].

In 1973, Dhar et al. [107] reported that cortical microsomes are able to 24-hydroxylate radiolabelled cholesterol in a very low yield. The reaction required oxygen and NADPH, consistent with involvement of a species of cytochrome P-450. This was later confirmed with use of a more sensitive technique based on incorporation of ^{18}O in the product [108]. In the search for a mechanism responsible for elimination of excess cholesterol from the brain, different cholesterol metabolites were measured in the internal jugular vein and in an artery of human volunteer [33]. It was shown that the levels of 24OH are significantly higher in the internal vein than in the artery. From the arteriovenous difference observed it was calculated that there is a flux of about 6 mg 24OH/24 h from the brain into the circulation in man [33, 109]. A similar flux of 24OH was also demonstrated from the brain of rats and it was shown that the rate of this elimination of cholesterol corresponds to about 2/3 of the rate of cholesterol synthesis [108]. That the cholesterol 24S-hydroxylase system is responsible for about 2/3 of the elimination of excess cholesterol from the brain was later confirmed also in mice [110].

In a general screening for oxysterol biosynthetic enzymes, a full-length cDNA specifying cholesterol 24-hydroxylase was isolated from mouse liver that was used to isolate the human ortholog from a fetal brain cDNA library by cross-hybridization [111]. It was shown that the 24-hydroxylase was a cytochrome P-450 species and it was called CYP46. The enzyme protein as well as the corresponding mRNA were shown to be expressed predominantly in the brain. Although low levels of mRNA were present in the liver and in the testis, no corresponding protein was found in these organs. There was a wide expression of the enzyme in different areas of the brain with the highest levels in areas rich in gray matter. The enzyme appeared to be present in neuronal cells with almost no expression in glial cells [111]. In the brain of patients who have died with the Alzheimer's disease, an abnormal induction of CYP46 was found in glial cells, however [112].

Characteristics of the Cholesterol 24S-Hydroxylase (CYP46A1) in the Brain

Reconstituted human CYP46 was found to have broad substrate specificity and was active toward several C27- and C21-steroids. In addition the enzyme was found to metabolize a number of xenobiotics [113]. Thus the enzyme may participate in metabolism of neurosteroids and drugs that are able to cross the blood–brain barrier. Under in vitro conditions excess enzyme was found to further metabolize 24OH producing 24,25- as well as 24,27-hydroxycholesterols. Only trace amounts of the latter two products are found in the brain, however.

In 2008 the group of Piukuleva described the crystal structure of human CYP46 with and without a high-affinity substrate (cholesterol 3-sulfate) [114]. The enzyme was found to have a banana-shaped hydrophobic active site cavity. There were substantial substrate-induced conformational changes in the structure. Several strong inhibitors and modest coactivators of CYP46A1 were identified. One of the strong inhibitors was the antifungal drug Voriconazole which also was shown to inhibit the cholesterol 24S-hydroxylase in vivo in mice [115]. One of the side effects of Voriconazole is visual disturbances and the human retina contains high levels of the cholesterol 24S-hydroxylase [116]. The possibility was discussed that the inhibition of CYP46A1 by Voriconazole contributes to the above visual disturbances.

Given the importance of CYP46A1 for the cholesterol homeostasis in the brain, one would expect the enzyme to be sensitive to different regulatory systems. Surprisingly, however, a broad spectrum of regulatory axes using a variety of promoter constructs did not result in a significant transcriptional regulation [117]. The CYP46A1 promoter has no canonical TATA or CAAT boxes and a high GC content, a feature often found in housekeeping genes. It has been shown that the Sp family of transcription factors is important for the basal transcriptional control of the human CYP46A1 gene (for a review, see [104]). It has been suggested that a shift in the ratio of different Sp factors during neuronal differentiation might account for the neuronal specific expression of CYP46A1.

CYP46A1 has been shown to be sensitive for epigenetic regulation both in vivo and in vitro [104, 118]. The importance of this type of regulation under in vivo conditions is not known, however.

One of the few factors affecting the transcriptional activity is oxidative stress. The ectopic expression of CYP46 in glial cells in the brain of patients with Alzheimer's disease may thus be a consequence of the oxidative stress in this condition.

During the first 2 weeks of life of the wild-type mouse, however, there is a significant increase in Cyp46a1 mRNA levels in parallel with an increase in 24OH level and a reduction of cholesterol synthesis. The regulator factors behind this increase are not known. Under normal conditions substrate availability may be the most critical factor for the regulation of the activity of the enzyme. Substrate availability is however not of regulatory importance for the transcriptional regulation of the activity [117].

The absence of transcriptional regulation of CYP46A1 in the brain is in marked contrast to the sophisticated transcriptional regulation of CYP7A1, the rate-limiting enzyme for the removal of cholesterol as bile acids in the liver. In the brain regulation cholesterol synthesis thus seems to be more important for maintenance of cholesterol homeostasis than metabolism.

Importance of 24S-Hydroxycholesterol and the Cholesterol 24S-Hydroxylase for Cholesterol Homeostasis in the Brain

In similarity with the other side-chain oxidized oxysterols, 24OH is a potent inhibitor of cholesterol synthesis under in vitro conditions [119]. It was recently shown that

this is of importance also under in vivo conditions. Thus a reduced level of 24OH in the brain due to increased metabolism [34] or due to increased leakage across a defect blood–brain barrier [105] results in an increased rate of cholesterol synthesis. Overexpression of CYP46A1 in mouse brain results in increased consumption of cholesterol and a compensatory increase of cholesterol synthesis [120, 121]. The effects on cholesterol synthesis and metabolism are however well balanced and the levels of cholesterol are not significantly changed under the above conditions. Knocking out Cyp46a1 in mice results in a decrease in cholesterol synthesis by about 40% [111]. Also in this case, the levels of cholesterol in the brain are not changed.

In similarity with cholesterol, more than 90% of 24OH is in the free form in the brain and very small amounts are esterified. In spite of this, inhibition or knockout of the esterifying enzyme ACAT1 (acyl-CoA:cholesterol acyltransferase 1) leads to increased levels of 24OH in the brain of mice (increase by about 30%) [122]. In accordance with above, the increased levels of 24OH were associated with reduced levels of HMG CoA reductase protein.

Importance of 24S-Hydroxycholesterol and the Cholesterol 24S-Hydroxlase for LXR-Targeted Genes

Given the fact that 24S-hydroxycholesterol is an efficient LXR agonist, overexpression of the cholesterol 24S-hydroxlase would be expected to lead to stimulation of LXR-targeted genes. This was not observer, however, neither in the brain [120, 121] nor in the liver [120]. It was recently shown, however, that the lack of effect in the brain may be due to the increased rate of cholesterol synthesis in this organ as a result of the increased consumption of cholesterol [123]. Thus it was shown that overexpression of CYP46A1 in rat cortical neuronal cells leads to an increase in membrane levels of RhoA activation. This increase was found to be due to geranylgeranylation as treatment with a geranylgeranyl transferase inhibitor or a statin abolished the CYP46A1 effect. The increased activation of RhoA was associated with a decrease in the LXR transcriptional activity and in the mRNA levels of LXR target genes. This effect was also demonstrated in vivo in mice with an overexpression of CYP46A1.

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 suggested 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 result in transcription of the LXR-target genes ABCA1 and APOE in glial cells with subsequent increase in cholesterol efflux [124]. This contention is supported by another in vitro experiment demonstrating that 24OH induces expression of APOE and APOE-mediated efflux of cholesterol via an LXR pathway [125]. This mechanism is illustrated in Fig. 7. It should be emphasized, however, that the importance of this attractive self-regulating mechanism in vivo has not yet been clarified. If the

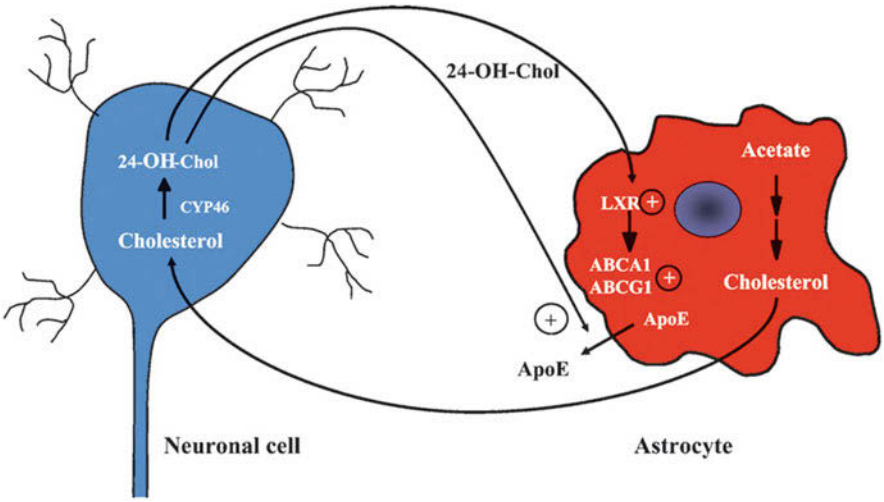

Fig. 7 Theoretical model for regulation of cholesterol homeostasis in neuronal and glial cells. Neuronal cells have a considerably lower capacity for cholesterol synthesis than glia cells and there is an ApoE-dependent flux of cholesterol from the glia cells to neuronal cells. In the neuronal cells part of the cholesterol is converted into 24S-hydroxycholesterol which fluxes from the neuronal cells to the glia cells. 24S-Hydroxycholesterol is an efficient activator of LXR and LXR target genes such as ABCAI. The latter activation leads to increased ABCA1-mediated flux of cholesterol from the neuronal cells. In addition this activation leads to increased synthesis of ApoE. Reproduced from Ref. [23] with permission

mechanism is important in vivo, a correlation between ApoE and 24OH would be expected in cerebrospinal fluid. Such a correlation was observed in patients with Alzheimer's disease and in patients with mild cognitive impairment but not in control subjects [126].

Metabolism of 24S-Hydroxycholesterol and Levels in the Circulation

24S-Hydroxycholesterol is converted into bile acids in human hepatocytes at a rate about 40% of that of the normal intermediate in bile acid synthesis, 7-α-hydroxycholesterol [127]. The oxysterol 7α-hydroxylase CYP7B1 is not involved in the 7α-hydroxylation of 24OH; instead there is a specific 7α-hydroxylase, CYP39A1, with a high specificity toward this oxysteroid [128]. In addition to CYP39A1 also CYP7A1 is able to 7α-hydroxylate 24OH [129]. The relative importance of the two cytochrome P-450 species is not known. In addition to bile acids 24OH is sulfated and glucuronidated and also converted into the corresponding derivatives of 5-cholestene-3β,24,27-triol in the human liver [127]. The total excretion of the latter steroids was estimated to be about 50% of the total flux of 24OH from the brain into the circulation. The less efficient metabolism of 24OH may explain the relatively high levels of this oxysterol in the circulation.

There is a marked effect of age on the levels of 24OH in the circulation. Thus the levels decline by a factor of about five during the first two decades of life. The concentration of the CYP46 enzyme in the brain is however about constant from the first year of life. It was shown that the plasma levels of 24OH closely follows the changes in the ratio between estimated brain weight and estimated liver volume [130]. Liver volume is highly correlated to body surface and in accord with this the absolute as well as cholesterol-related levels of 24OH in the circulation are highly inversely correlated to body surface. Thus the balance between cerebral production and hepatic metabolism appears to be a critical determinant for the circulating levels of 24OH. After the first two decades of life the levels of 24OH are about constant.

Role of the Cholesterol 24S-Hydroxlase for Memory Function

Knocking out the Cyp46A1 gene in mice resulted in a learning deficiency as evaluated by the Morris water test [131]. It was also shown that the long-term potential was impaired in hippocampal slices from these mice. A similar effect was obtained when treating hippocampal slices from wild-type mice with statin. Thus it was concluded that the negative effects of the knockout on the memory function was related to the reduced rate of cholesterol synthesis in the brain of the Cyp46a1$^{-/-}$ mice. The reduced long-term potential could be normalized in hippocampal slices from Cyp46$^{-/-}$ mice and in hippocampal slices treated with statin by addition of geranylgeraniol. The latter steroid is an intermediate in cholesterol synthesis and may be utilized for geranylation of critical proteins of importance for the memory function. The authors suggested that CYP46A1 mediated turnover of cholesterol in the brain is necessary for the normal memory function. Theoretically a higher rate of cholesterol synthesis in the brain could be expected to improve the memory function. In accord with this, old female mice with overexpressed CYP46A1 and increased cholesterol synthesis in the brain showed better spatial memory than control mice [132].

Neuroprotective Effects of 24S-Hydroxycholesterol

Under in vitro conditions 24OH increases the activity of the alpha secretase in human neuroblastoma cells and reduces the activity of the beta secretase [44]. These effects can be expected to lead to reduced production of beta-amyloid from the amyloid precursor protein. In accord with this overexpression of CYP46A1 in the hippocampus and cortex of an AD model, APP23 mice, was found to markedly reduce brain levels of Aβ peptides as well as amyloid deposits. Furthermore the overexpression also improved spatial memory [121]. Using a similar approach, overexpression of CYP46A1 again demonstrated positive effects in another AD model expressing Tau pathology and in a model of Huntington's disease [133, 134].

In the above studies the levels of 24OH in the brain were increased by overexpression of CYP46A1 using an adeno-associated virus-mediated delivery. Another strategy to increase levels of 24OH in the brain is to disrupt the gene coding for acyl-CoA:cholesterol acyltransferase 1 (ACAT1) in mice [122]. Such disruption caused an increase by about 30% in the level of 24OH in the brain in parallel with a decrease in HMG CoA reductase and cholesterol synthesis rate. ACAT 1 disruption in a mouse model for AD caused a reduction in APP and reduced the cognitive decline. Given the fact that a very small fraction only of the sterols in the brain is esterified, the effect of ACAT1 disruption on the levels of free 24OH is surprising.

Furthermore, 24S-OH has been shown to be a potent allosteric modulator of N-methyl-D-aspartate receptors (NMDAR) that plays a critical role in regulating synaptic plasticity [135]. It was shown that 24OH reverses long-term potential deficits induced by the NMDAR channel blocker ketamine.

Given the above neuroprotective effects of 24OH one would expect an opposite effect in mice lacking Cyp46a1. This was not seen in a study utilizing Cyp46a1 deficient mutant APP mice [136]. Surprisingly the lack of Cyp46a1 increased the survival time of the mice.

At the present state of knowledge it seems as if both upregulation of 24OH signaling and downregulation/antagonism may have a therapeutic potential [137].

Association between polymorphisms in the CYP46A1 gene and susceptibility to AD has been demonstrated in a number of studies. However, this association has not yet been unambiguously proven and is still under investigation [138, 139].

Diagnostic Value of 24S-Hydroxcholesterol in Plasma and Cerebrospinal Fluid

Given the neuronal origin of 24OH in the circulation, neurodegeneration with loss of neuronal cells would be expected to reduce the circulating levels of this oxysterol. In accordance with this, patients with advanced AD have reduced levels of plasma 24OH [130, 140]. The level of 24S-OH is also reduced in chronic multiple sclerosis patients [141, 142]. The reduction in the levels of 24OH in patients with neurodegeneration is however relatively modest, with some overlapping with a control population. In the case of Alzheimer's disease the modest degree of reduction of 24OH in the circulation may in part be due to the ectopic expression of CYP46A1 in glial cells that may compensate for the reduced expression in neuronal cells [112].

Neurodegeneration leads to increased rather than decreased levels of 24OH in cerebrospinal fluid (CSF). The reason for this is likely a release of 24OH from dying neuronal cells. The diagnostic potential of 24OH in cerebrospinal fluid seems to be higher than that of 24OH in plasma [143, 144]. The possibility has been discussed that levels of 24OH in CSF can be used as a marker of "brain health" [144].

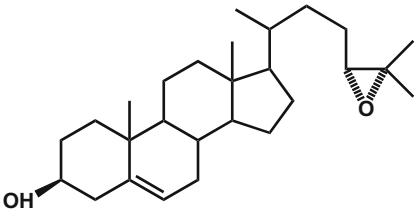

Fig. 8 Structure of 24S,25-epoxycholesterol

24(S),25-Epoxycholesterol

The structure of 24(S),25-epoxycholesterol (24,25EC) is shown in Fig. 8. This oxysterols is a metabolite of mevalonate [145] and a very efficient LXR-agonist [29]. 24,25EC was first identified in fibroblasts [146] and human liver [147]. The very high levels found in the liver were consistent with a suppressive effect on cholesterol synthesis. In later studies, however, the levels in mouse liver were reported to be about two orders of magnitude lower [148].

In a study on the effect of statins on cholesterol homeostasis in human macrophages, a surprising atherogenic effect of the statin treatment was observed. Thus the treatment led to a reduced cholesterol efflux and a downregulation of the LXR target genes ABCA1 and ABCG1 [149]. It was shown that the downregulation of the LXR target genes was an effect of reduced formation of 24,25EC. A selective loss of 24,25EC markedly increased cholesterol synthesis and HMG CoA reductase expression [150]. It was suggested that 24,25EC fine-tunes acute control of cellular cholesterol homeostasis [4].

Interestingly, it was observed that 24,25EC tended to be produced in a constant ratio to cholesterol, across a number of conditions in which cholesterol synthesis rate was altered, suggesting that 24,25EC serves as a measure of cholesterol synthesis [4].

Primary human astrocytes have been shown to synthesize and secrete 24,25EC [151]. It was shown that 24,25EC synthesized in astrocytes can be taken up by neurons and exert downstream effects on gene regulation. The importance of this effect under in vivo conditions is however difficult to evaluate. While 24,25E is formed in a shunt of the mevalonate pathway that produces cholesterol, it is possible that at least part of this oxysterol formed in the brain may be dependent upon the cholesterol 24S-hydroxylase. Thus it was shown that knocking out of the latter enzyme in mice led to a marked decrease in the levels of 24,25EC [152].

LXR receptors have been shown to be critical for midbrain neurogenesis in vivo. Using LC/MS and functional assays, 24,25EC was found to be the most potent and abundant LXR ligand in the developing mouse midbrain [153]. In the embryonic ventral midbrain of mice, the levels of 24,25EC were considerably higher than those of 24OH. It was shown that 24,25EC promoted dopaminergic neurogenesis in an LXR-dependent manner in zebrafish in vivo. Furthermore 24,25EC promoted dopaminergic differentiation of embryonic stem cells.

General Conclusions

The role of the side-chain oxidized oxysterols as intermediates in excretion pathways for cholesterol and as transport forms of cholesterol is well established. It is evident from the present review that production of 24S-hydroxycholesterol in the brain and the flux of this oxysterol into the circulation is important for cholesterol homeostasis and normal function of this organ. The flux of 27-hydroxycholesterol from extrahepatic sources, e.g., macrophages, is likely to be antiatherogenic. The flux of 27-hydroxycholesterol from the circulation into the brain is associated with negative effects on brain function and may be of importance in the development of neurodegenerative diseases. The two side-chain oxidized oxysterols have been shown to have a regulatory role on cholesterol homeostasis in the brain but under normal conditions not in the liver. The reason for this is likely to be that the degree of esterification is much higher in the circulation and liver than in the brain. Thus it is generally believed that it is only the free form of the oxysterol that is able to interact with the appropriate receptors. The ratio between an oxysterol and cholesterol may also be important in connection with the interaction between the oxysterol and the receptor. The ratio between cholesterol and free oxysterol is about 500 in the brain but about 50,000 in the liver and in the circulation. It has been shown that under in vitro conditions a high cholesterol excess can prevent binding of an oxysterol to the binding site of the nuclear receptor LXR [23, 154]. The relevance of such in vitro results for the situation in vivo is difficult to evaluate, however.

It is evident from the present review that the levels and production of 27-hydroxycholesterol is of some importance for development of vascular diseases, breast cancer, osteoporosis, different neurodegenerative diseases. A selective loss of the sterol 27-hydroxylase system and the oxysterol metabolizing system CYP7B1 causes a specific neurodegenerative disease. A loss of the cholesterol 24S-hydroxylating system in the brain due to a mutation in the CYP46A1 gene has not been described yet in humans.

During the last decade a great number of studies have shown that 25-hydroxycholesterol is an important modulator both in innate and adaptive immunity. It is also an antiviral factor. Interestingly some of the effects of 25-hydroxycholesterol are mediated by the 7α-hydroxylated metabolite, 7α,25-dihydroxycholesterol. A knockout of the 25-hydroxylating system in mice results in elevated levels of immunoglobulin A (IgA).

It seems likely that further studies on oxysterols and oxysterol-producing enzymes will identify new drug targets that will help to develop new therapeutic strategies in connection with neurodegenerative diseases, inflammation and viral infections.

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Bile Acids and TGR5 (Gpbar1) Signaling

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Bile Acid Receptors and Bile Acid Sensing Molecules

Bile acid (BA) effects are mediated through different types of BA receptors and sensing molecules, which allow for a cell type- and BA-specific signaling (Fig. 1) [1–5]. Nuclear BA receptors are ligand-activated transcription factors and comprise the farnesoid X receptor (FXR, NR1H4) [6–11], the pregnane X receptor (PXR, NR1I2) [12, 13], and the vitamin D receptor (VDR, NR1I1) [14–16]. FXR is the master regulator of BA homeostasis and is activated by the primary BA chenodeoxycholic acid (CDCA) and its conjugates with an EC_{50} of approximately 5–20 μM [6, 8, 10, 11, 17, 18]. The secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) are also FXR ligands, however less efficient than CDCA [8, 10, 17, 19]. In contrast, only the secondary BA LCA acts as ligand for PXR and VDR [12, 13, 16].

Besides activation of intracellular nuclear receptors, BAs can modulate the signaling of several G protein-coupled receptors (GPCRs) at the cell surface, such as different types of muscarinic (acetylcholine) receptors (e.g., M2 and M3 receptors) [20–23], as well as formyl peptide receptors (FPR) [5, 24, 25]. Furthermore, taurine-conjugated BAs are ligands for the sphingosine-1-phosphate receptor 2 (S1PR2), which is expressed in liver parenchymal cells (hepatocytes) where it regulates sterol and lipid metabolism as well as in cholangiocytes, where its activation triggers cell proliferation [26–31]. TGR5 (Gpbar1, M-BAR) is a GPCR that predominately couples to a stimulatory G protein and is activated by both conjugated and unconjugated primary and secondary BAs [32–34].

Integrins ($\alpha_5\beta_1$) also serve as BA sensing molecules in hepatocytes for taurine-conjugated ursodeoxycholic acid (TUDCA) [35–37]. Uptake of BAs across the plasma membrane is a prerequisite for BA-mediated $\alpha_5\beta_1$ integrin activation since

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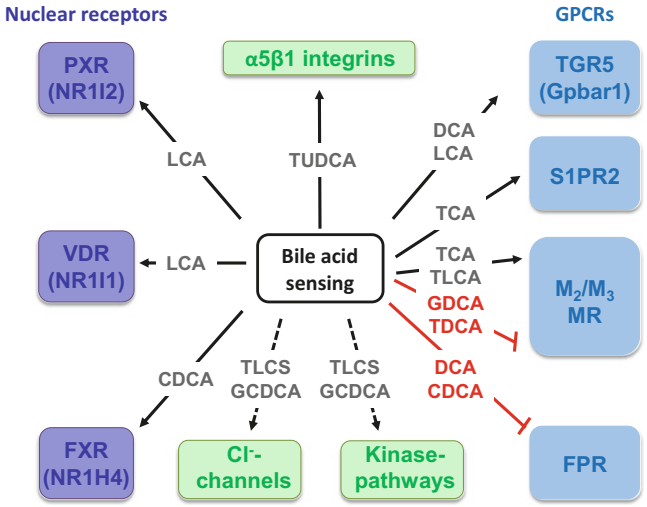

Fig. 1 Bile acid sensing molecules. Several BA responsive receptors and molecules have been identified. Three nuclear receptors (NR) have been demonstrated to be activated by BAs: the pregnane X receptor (PXR), the vitamin D receptor (VDR) and the farnesoid X receptor (FXR) (purple boxes). Moreover, multiple G protein-coupled receptors (GPCRs) are either directly activated or modulated in their activity by different BAs (blue boxes). While TGR5 (Gpbar-1) and the sphingosine-1-phosphate receptor 2 (S1PR2) are activated by various BAs, other GPCRs, such as the formyl peptide receptor (FPR) and the muscarinic acetylcholine receptors M_2 and M_3 can be inhibited in their signaling by BAs. Furthermore, $\alpha_5\beta_1$-integrins, chloride channels, and several kinase pathways are activated by various BAs (green boxes). Dashed arrows indicate potentially indirect signaling, black arrows indicate a stimulatory effect, while inhibitory effects are depicted in red. CDCA chenodeoxycholic acid, GCDCA glycochenodeoxycholic acid, TCA taurocholic acid, TLCA taurolithocholic acid, TLCS taurolithocholylsulfate, LCA lithocholic acid, DCA deoxycholic acid, TUDCA tauroursodeoxycholic acid. Modified after [1]

$\alpha_5\beta_1$ is found on intracellular endomembranes. Activation of $\alpha_5\beta_1$ by TUDCA results in increased bile secretion (choleresis), cell proliferation, and also protects the cells from death receptor-mediated apoptosis [35–37].

Further BA sensors include ion channels and kinase signaling pathways; however, the precise mechanism by which BAs modulate these signaling molecules remains elusive [1, 38–43].

The presence of various nuclear and plasma membrane-bound receptors for BAs not only allow for a cell type- and BA-specific signaling but also help to explain the pleiotropic effects of BAs in the organism.

TGR5, a G Protein-Coupled Receptor for Bile Acids

TGR5 was discovered and characterized as a G protein-coupled receptor for both primary and secondary BAs by Maruyama et al. in 2002 and Kawamata et al. in 2003 [32, 33].

The gene encoding human TGR5 is located in the chromosomal region 2q35 and consists of two exons [44]. The coding region is entirely located in exon 2, encompasses 993 base pairs (bp), and translates into 330 amino acids [33, 44]. The coding regions of rat and mouse TGR5 contain 990 bp and encode for 329 amino acids each. There is a high sequence conservation between human, bovine, rabbit, rat, and mouse TGR5 with amino acid identities ranging from 82 to 91% [32, 33]. TGR5 belongs to the class A of GPCRs (rhodopsin-like GPCRs) and shows the highest amino acid identity to different sphingosine-1-phosphate receptors (S1PR) [32, 33], which is below 30%, however.

TGR5-Dependent Intracellular Signaling Pathways

Heterotrimeric G proteins are formed from an α-subunit, which binds and hydrolyses guanosine triphosphate (GTP), and a complex of a β- and a γ-subunit [45–47]. Four different classes of α-subunits are distinguished: $G\alpha_s$ promotes activation of adenylate cyclase, $G\alpha_{i/o}$ leads to inhibition of adenylate cyclase, $G\alpha_{q/11}$ triggers activation of phospholipase Cβ, and $G\alpha_{12/13}$ are associated with stimulation of Rho guanine-nucleotide exchange factors (GEFs) [45–47]. BA binding to TGR5 leads to an activation of the receptor and association with a G protein consisting of the GDP-bound α-subunit and a βγ-complex [45]. Following the interaction of the GPCR with the G protein, GDP is released and replaced by GTP, which in turn triggers a conformational change of the α-subunit and the subsequent dissociation of the α-subunit from the βγ-complex [45]. Further downstream signaling is then initiated by the α-subunit and the βγ-complex, respectively [45]. In most cell types studied to date, TGR5 will associate with a $G\alpha_s/\beta\gamma$ heterotrimer and thus trigger the activation of adenylate cyclase resulting in an elevation of intracellular cyclic AMP (cAMP) levels [32, 33]. Downstream signaling activated by TGR5 comprise protein kinase A (PKA)-, protein kinase B (AKT)-, mammalian target of rapamycin complex 1 (mTORC1)- and extracellular-signal regulated kinase (ERK)-pathways [32, 48–52]. Furthermore, stimulation of TGR5 results in inhibition of nuclear factor kappa B (NFκB) signaling, elevation of intracellular calcium levels, and activation of different ion channels and modification of gene expression [48, 50, 53–60].

Similar to the S1PR2, TGR5 can couple to different G proteins [27, 32, 33, 49, 61]. It was demonstrated that the receptor can couple to both $G\alpha_s$ as well as to $G\alpha_i$ in biliary epithelial cells depending on the subcellular localization of TGR5 [49]. TGR5 located in the primary cilia of cholangiocytes coupled to $G\alpha_i$ and attenuated cell proliferation, while TGR5 located on the apical plasma membrane associated with a $G\alpha_s$ protein upon ligand binding and triggered cell proliferation [49]. In the FLO cell line, which is derived from human esophageal Barrett's adenocarcinoma, co-immunoprecipitation experiments demonstrated that TGR5 could interact with both $G\alpha_q$ and $G\alpha_{i3}$; however, signal transduction after ligand binding was mediated only by $G\alpha_q$ [61]. Thus, cell type and subcellular localization seem to determine the interaction of TGR5 with a specific G_α protein. Whether

posttranslational modifications also contribute to the G_α protein subclass selectivity of TGR5 is unknown.

TGR5 Ligand Binding and Selectivity

TGR5 recognizes a wide spectrum of ligands, ranging from BAs and neurosteroids as natural TGR5 agonists to synthetic BAs and agonists with a nonsteroidal core (Fig. 2) [34, 62]. Particularly, several nonsteroidal intestine-specific TGR5 agonists are known [63, 64]. The specificity is achieved by the presence of quaternary ammonium groups or by a considerable ligand size; for the latter, two TGR5 agonists are coupled via a linker region (**15c** in Fig. 2). In contrast to other BA receptors, TGR5 is activated by all known BAs, regardless of their substitution pattern and state of conjugation (un-, taurine-, or glycine-conjugated), although with varying levels of potency ranging from 0.29 to 36.7 μM [34]. Generally, the agonistic potential of BAs toward TGR5 increases with the hydrophobicity of the cholane scaffold. The most potent natural agonist of TGR5 with an EC_{50} of 0.29 μM is the secondary BA taurolithocholic acid (TLCA), which is hydroxylated in position 3 of the cholane scaffold only (Fig. 2). Additional hydroxylation of position 12 in the secondary BA deoxycholic acid (DCA) or position 7 in the primary BA chenodeoxycholic acid (CDCA) increases the EC_{50} 4-fold and 23-fold compared to TLCA, respectively (Fig. 2). The stereochemical configuration of the hydroxyl group in position 7 of the cholane scaffold has a large impact on TGR5 activation: The epimers CDCA and ursodeoxycholic acid (UDCA) show a fivefold difference in their efficacy as TGR5 agonists, with CDCA being more potent. This epimeric selectivity has been explained by a hydrogen bond formation of CDCA's 7-α-hydroxyl group to Y89 in transmembrane helix 3 (TM3) of TGR5 (Fig. 3a) [65]. In contrast, due to the β-configuration, UDCA cannot form such a hydrogen bond with its 7-hydroxyl group (Fig. 3b).

The BAs' conjugation is another factor influencing their efficacy toward TGR5. BAs with a free acid moiety and the respective glycine-conjugated derivatives generally exhibit a similar potency, as seen in lithocholic acid (LCA; EC_{50} 0.58 μM) and glycolithocholic acid (GLCA; EC_{50} 0.54 μM). However, taurine-conjugated derivatives are more potent than their related BAs, e.g., TLCA with an EC_{50} of 0.29 μM compared to LCA. Taurine conjugation increases the size of a BA more than glycine conjugation, which allows the bridging of the residues R79 (EL1) and Y240 (TM 6) in TGR5 (Fig. 3c). The salt-bridge interaction between the negatively charged sulfonic acid moiety and the positively charged R79 likely increases the affinity of those BAs toward TGR5 [65]. All BAs employ the 3-hydroxyl groups of their cholane scaffold to form a hydrogen bond to Y240, and this interaction is further stabilized by a hydrogen bond to E169 (TM5) (Fig. 3b). The interaction with Y240 is crucial for the activation of TGR5, as mutation of this residue to alanine or phenylalanine abrogates TGR5 signaling [65]. Agonistic neurosteroids such as pregnanediol (Fig. 2) also utilize their hydroxyl or carbonyl groups to interact with Y240 in TGR5. Lacking acidic groups, they mainly form

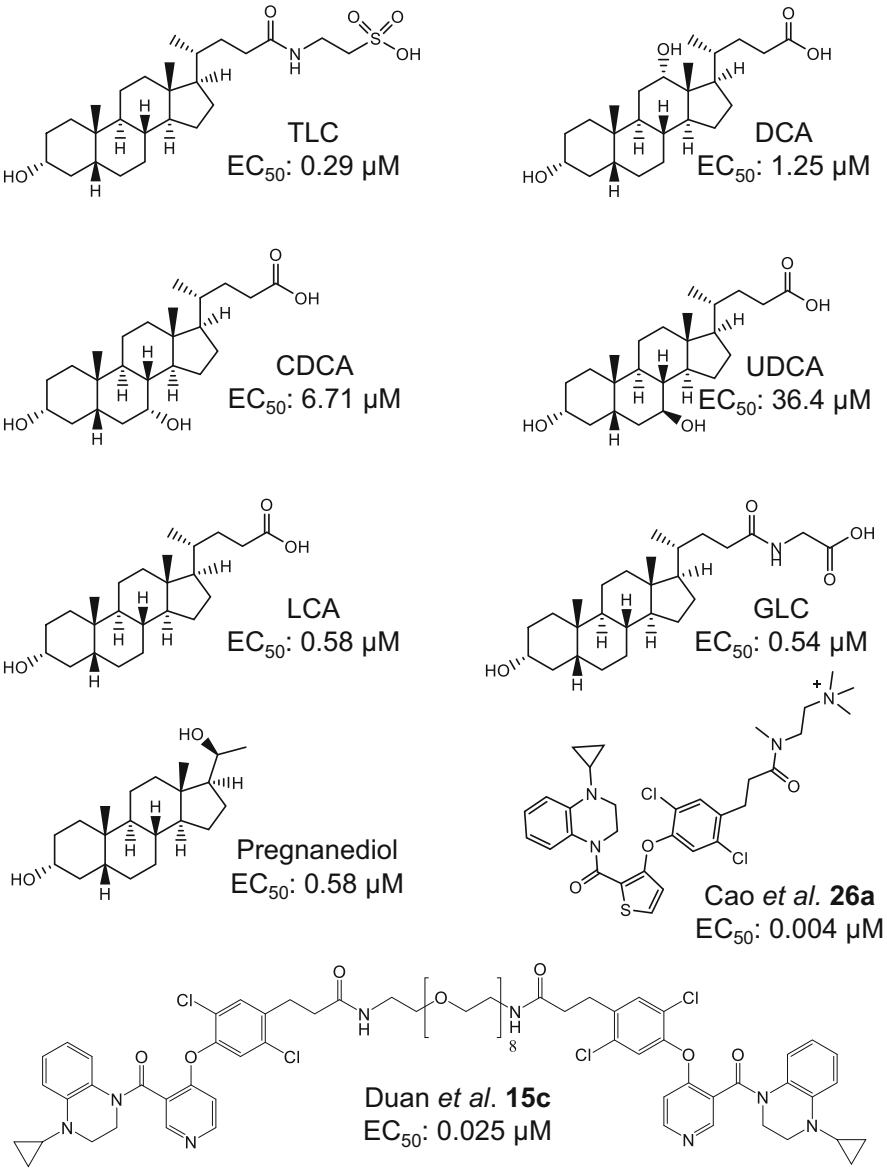

Fig. 2 Bile acid agonists and their EC_{50} values toward TGR5 as reported in Ref. [34, 63]. Primary bile acids: CDCA and UDCA. Secondary bile acids: DCA, LCA, GLC, and TLC. Intestine-specific nonsteroidal TGR5-specific agonists **26a** from Ref. [63] and **15c** from Ref. [64]. The primary bile acids are generally less effective TGR5 agonists than the secondary bile acids. The configuration of the hydroxyl group in position seven (if present) strongly influences the activity: The α-configuration as present in CDCA is more favorable than the β-configuration in UDCA. Conjugation of the acid moiety with glycine increases the activity toward TGR5 only slightly, while taurine conjugation increases the activity markedly

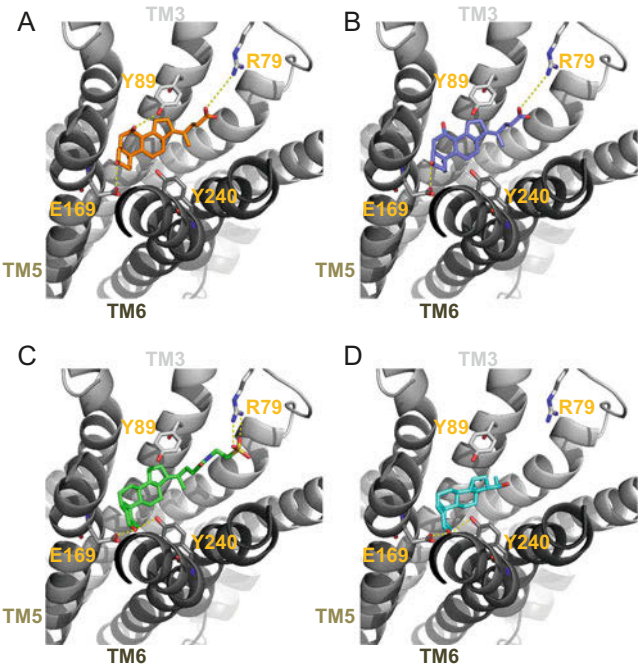

Fig. 3 Binding modes of bile acids and neurosteroids as reported in Ref. [65]. (**a**) Binding mode of CDCA in TGR5. CDCA forms a hydrogen bond to E169 in TM5 (yellow dotted line) and a weak hydrogen bond to Y240 in TM6. Additionally, the 7α-hydroxyl group of CDCA forms a hydrogen bond to Y89 in TM3 (yellow dotted line), and a salt bridge with R79 (yellow dotted line). (**b**) Binding mode of UDCA in TGR5. UDCA forms a hydrogen bond to E169 in TM5 (yellow dotted line) and a weak hydrogen bond to Y240 in TM6. Unlike CDCA, UDCA is unable to form a hydrogen bond to Y89 due to the β-configuration of its 7-hydroxyl group, resulting in a lower efficacy compared to CDCA. (**c**) Binding mode of TLC in TGR5. TLC forms hydrogen bonds to E169 in TM5 and to Y240 in TM6 (yellow dotted lines). With its sulfonic acid moiety, it forms a salt bridge to R79 (yellow dotted lines). These interactions may explain why TLC is the most potent natural bile acid toward TGR5. (**d**) Binding mode of pregnanediol in TGR5. Pregnanediol forms hydrogen bonds to E169 in TM5 and to Y240 in TM6 (yellow dotted line). Lacking an acid group, it mainly forms hydrophobic contacts with Y89 in TM3

additional hydrophobic contacts with Y89 in TM3 to bind to and activate TGR5 (Fig. 3d) at a reasonable EC_{50} [e.g., pregnanediol (Figs. 2 and 3d), EC_{50} 0.58 μM], allowing them to activate TGR5 in the brain [34, 54].

TGR5-specific agonists with a nonsteroidal core mimic BAs through the presence of an acid or amide moiety, which is linked to a system of three to four variably interconnected aromatic and aliphatic rings. The ring furthest from the acid or amide moiety always contains a heteroatom (e.g., **26a**, **15c** in Fig. 2). Although the binding mode of nonsteroidal TGR5 agonists is unknown, it is possible that the heteroatom is necessary to form a hydrogen bond to Y240 (TM6), which is crucial for the activation of TGR5. Finally, as TGR5 binds ligands of various shapes and sizes, it is surprising that to date no antagonist of TGR5 is known. All the more because it is

often easier to develop ligands that bind to GPCRs but do not activate them, as such ligands do not need to bridge TMs 3 and 6 in a specific manner to induce the movement of TM6 leading to GPCR activation [66].

TGR5 Tissue Distribution

TGR5 mRNA was detected almost ubiquitously in human and rodent tissues [32, 67, 68]. In mice, the strongest signal for TGR5 expression was detected in the gallbladder, followed by high expression levels in the spleen, lung, placenta as well as ileum and colon [67, 68]. In human tissues, a similar expression pattern was found with high TGR5 mRNA levels in gallbladder, placenta, spleen, lung, liver, stomach, small intestine, uterus, and mammary gland [32, 53, 69]. On the protein level, TGR5 has been detected in CD14-positive monocytes and tissue-resident macrophages in both humans and rodents, different nonparenchymal cells of the liver, gallbladder epithelial cells and smooth muscle cells, astrocytes, neurons and microglia of the central as well as astrocytes and neurons of the enteric and peripheral nervous system [4, 48–50, 53–58, 70–73]. Furthermore, TGR5 has been localized in intestinal epithelial cells, enteroendocrine L-cells, human kidney proximal tubule cells and podocytes, murine brown adipocytes, human skeletal muscle cells, and pancreatic β cells [56, 59, 74–79].

In rodent and human liver, TGR5 is localized in sinusoidal endothelial cells (LSEC), in liver resident macrophages (Kupffer cells, KC), and cholangiocytes [4, 52, 57, 58, 71, 80–82]. While quiescent hepatic stellate cells (HSC) do not express TGR5, the receptor is upregulated during culture of isolated HSC and can also be detected in activated, myofibroblast-like HSC in vivo [57, 83, 84]. Using immunofluorescence staining of rat and human liver cryosection, TGR5 has not been detected in hepatocytes, indicating that expression levels are much lower as compared to the TGR5-expressing nonparenchymal liver cells [52, 57, 82].

Regulation of TGR5 Expression, Localization, and Function

Very little is known on the regulation of TGR5 expression, localization, and function to date. An upregulation of TGR5 mRNA has been observed in the frontal cortex of mice following acute liver failure, which was induced by intraperitoneal injection of azoxymethane [85]. In contrast, a downregulation of the receptor has been demonstrated in isolated rat astrocytes following stimulation with ammonia (NH_4Cl; 0.5–5 mM, 72 h) both on the mRNA and protein level. In line with this finding, reduced levels of TGR5 mRNA were detected in cortical brain tissue from patients with hepatic encephalopathy as compared to samples from control subjects [54].

Stimulation of either rat astrocytes or human macrophages with the TGR5 agonistic progesterone metabolites 5β-pregnan-3α-ol-20-one or 5α-pregnan-

3α-ol-20-one and 5α-pregnan-3β-ol-20-one triggered a significant downregulation of TGR5 mRNA levels [54, 69].

Thus, downregulation of TGR5 mRNA expression may represent a mechanism of receptor desensitization in response to continuous stimulation [54, 69]. This may be highly relevant since TGR5 unlike many other GPCRs does not interact with β-arrestins 1 and 2 or G protein-coupled receptor kinases 2, 5, or 6 and therefore does not traffic from the plasma membrane to endosomes in response to activation [86]. Ligand binding to TGR5 in the plasma membrane induced a sustained cAMP response, indicating that TGR5 does not desensitize to repetitive stimulation [86].

TGR5 Functions in Liver in Health and Disease

Role of TGR5 for Bile Acid Homeostasis and Bile Secretion Under Physiological and Cholestatic Conditions

Targeted deletion of TGR5 in mice is not associated with an obvious phenotype or the spontaneous development of liver disease [67, 68]. However, TGR5 knockout mice have a smaller BA pool size, despite unchanged expression levels of the rate-limiting enzyme of BA synthesis Cyp7a1 and similar fecal excretion rates of BAs as wild-type littermates [4, 5, 67, 72]. Bile acid pool composition is also altered in absence of TGR5 with a relative increase in taurocholic acid (TCA) and taurodeoxycholic acid (TDCA) and a decrease of tauro-β-muricholic acid (TβMCA), which may be attributed to lower Cyp7b1 expression [72, 87].

In cholangiocytes and gallbladder epithelial cells, TGR5 is localized in the primary cilia, which extend from the plasma membrane into the bile duct or gallbladder lumen, as well as on the apical plasma membrane [49, 53, 71]. Ligand binding to TGR5 on biliary epithelial cells triggers elevation of intracellular cAMP, which in turn promotes CFTR (ABCC7)-dependent chloride secretion [53, 80, 88]. Subsequently, chloride is exchanged across the apical plasma membrane against bicarbonate by the anion exchanger 2 (AE2, SLC4A2), thereby promoting formation of a protective bicarbonate film/bicarbonate umbrella as well as bicarbonate-rich biliary bile flow (choleresis) [53, 72, 80–82, 88–92]. Since not only transport activity but also surface expression of CFTR and AE2 is regulated by cAMP, stimulation of TGR5 increases chloride and bicarbonate secretion directly and also indirectly through enhanced insertion of CFTR and AE2 into the apical plasma membrane from intracellular vesicles [53, 80, 88]. The bicarbonate umbrella together with the glycocalix creates an alkaline microenvironment, which hampers the protonation of hydrophobic glycine-conjugated BAs, inhibits diffusion of protonated apolar BAs across the apical membrane of biliary epithelial cells, and thus protects the cells from BA toxicity [88, 90–93]. Therefore, it is not surprising that cholangiocytes from TGR5 knockout mice are more susceptible toward BA-induced cell damage [52]. Besides maintenance of the bicarbonate umbrella, TGR5 exerts antiapoptotic effects in biliary epithelial cells via serine phosphorylation of the CD95 death receptor [52]. Activation of TGR5 also triggers cholangiocyte proliferation through

elevation of reactive oxygen species, subsequent activation of Src kinase, matrix-metalloproteinase-dependent shedding of epidermal growth factor (EGF), transactivation of the epidermal growth factor receptor (EGFR), and subsequent phosphorylation of mitogen-activated kinases (MAPK) ERK1/2 [52]. Cholangiocyte proliferation in response to BA feeding (CA, LCA) or common bile duct ligation (CBDL) is impaired in TGR5 knockout mice in vivo [52]. Besides a reduced cholangiocyte proliferative response, TGR5 knockout mice are more susceptible toward bile acid-mediated, cholestatic liver injury [52, 94, 95]. Cholic acid (0.5% for 7 days or 1% for 5 days) feeding and CBDL for up to 7 days resulted in a more pronounced liver injury in the absence of TGR5 as demonstrated by higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and/or more pronounced liver cell necrosis on histology [52, 94, 95]. Livers from TGR5 knockout mice not only displayed significantly decreased cholangiocyte but also significantly reduced hepatocyte proliferation [52]. The mechanisms underlying reduced hepatocyte proliferation in TGR5 knockout mice remain elusive to date, since TGR5 protein levels are below the detection level in hepatocytes [57]. The impaired proliferative response of hepatocytes in mice with targeted deletion of TGR5 has also been observed after partial hepatectomy (PHx) [94]. Following PHx, concentrations of hepatic BAs were elevated and biliary BA composition was more hydrophobic in the absence of TGR5 [94]. Treatment with the BA binding resin cholestyramine (2%) alleviated liver injury in TGR5 knockout mice, suggesting that the higher hepatic BA levels as well as the altered composition of the BA pool contribute to the observed phenotype [94, 95].

Gallbladder volume was decreased in TGR5 knockout mice as compared to wild-type animals both on chow as well as on BA (CA, 0.2%)-enriched diet [48, 72, 95], which was attributed to reduced TGR5-dependent biliary secretion but also to impaired smooth muscle cell relaxation in the absence of TGR5 [48, 72]. In contrast, gallbladder size of wild-type mice increased up to 230% following administration of different synthetic TGR5 agonists (6α-ethyl-23(S)-methyl-cholic acid (INT-777), a 4-phenoxypyrimidine-5-carboxamide derivative (compound 18) or a 4-phenoxynicotinamide derivative (compound 23 g)) [72, 96, 97]. Although gallbladder hypomotility, as observed in TGR5 knockout mice, is associated with increased risk of cholesterol gallstone formation [72, 98], mice with targeted deletion of TGR5 did not develop cholesterol gallstones when fed a lithogenic diet [68].

Immunomodulatory and Metabolic Functions of TGR5 in Liver

In macrophages, stimulation of TGR5 suppresses inflammatory cytokine and chemokine expression and secretion, inhibits phagocytosis and migration, and induces an anti-inflammatory macrophage phenotype, characterized by maintained expression of interleukin (IL) 10 despite downregulation of pro-inflammatory cytokines [50, 55, 57, 99–102]. The mechanism underlying reduced inflammatory cytokine expression comprises TGR5-dependent elevation of cAMP and subsequent inhibition of I kappa B kinase (IKK), which in turn prevents phosphorylation of the

inhibitor of nuclear factor-κB (IκB) and thus hampers the nuclear translocation of NF-κB-p65 resulting in reduced transcriptional activity of NF-κB [55, 103]. The signaling pathway resulting in decreased chemokine secretion is dependent on an AKT-mediated activation of the mTOR complex-1 (mTORC-1), which increases the relative expression and protein levels of the dominant-negative CCAAT/enhancer binding protein β (C/EBPβ) isoform liver inhibitory protein (LIP) thereby suppressing expression of chemokines such as Ccl2, Ccl3, and Ccl4 [50, 103].

In vivo, intraperitoneal injection of lipopolysaccharide (LPS) resulted in a more severe phenotype as well as liver injury in TGR5 knockout mice as compared to wild-type animals, which was characterized by significantly increased mortality (Reich, Häussinger, Keitel unpublished), elevated levels for alanine (ALT) and aspartate (AST) aminotransferases, enhanced inflammatory infiltrates in liver tissue, and increased hepatocyte apoptosis [100]. TGR5 knockout mice were also more susceptible to infection with *Listeria monocytogenes* (8×10^4 CFU/ml) as demonstrated by a significantly higher mortality rate, increased listeria titers in liver and spleen as well as a more aggravated liver inflammation and damage (Reich, Häussinger, Keitel, unpublished) (Fig. 4).

Activation of TGR5 has been shown not only to exert anti-inflammatory effects in liver and adipose tissue but also to improve various aspects of the metabolic syndrome, such as obesity, insulin resistance, and atherosclerosis [5, 55, 56, 59]. Treatment of wild-type mice fed a high fat diet (HFD) with the TGR5 agonist INT-777 attenuated obesity, reduced fat mass, and improved glucose tolerance through increased intestinal glucagon-like peptide-1 (GLP-1) secretion [56]. Furthermore, administration of INT-777 lowered liver fatty acid and triglyceride concentrations resulting in decreased hepatic steatosis and improved serum ALT and AST levels as compared to the HFD-fed control animals [56]. The beneficial effects of TGR5 agonist on steatohepatitis may be attributed to reduced hepatic and adipose tissue inflammation, to an increase in TGR5-mediated energy expenditure and an improved insulin sensitivity due to enhanced intestinal GLP-1 secretion [50, 56, 59, 103]. Whether direct effects of TGR5 agonists on hepatocytes also contribute to the attenuation or improvement of steatohepatitis in mice on HFD or obese mice remains unknown. Treatment of obese db/db mice with a dual FXR/TGR5 agonist (6α-ethyl-24-nor-5β-cholane-3α,7α,23-trio-23-sulfate sodium salt, INT-767) for 6 weeks increased the proportion of intrahepatic $Ly6C^{low}$ anti-inflammatory macrophages and ameliorated steatohepatitis as assessed by histology [104]. This is in line with results from human macrophages, where bile acid treatment in a TGR5-dependent way promoted the differentiation of an anti-inflammatory macrophage phenotype, characterized by an increased IL10/IL12 ratio [99].

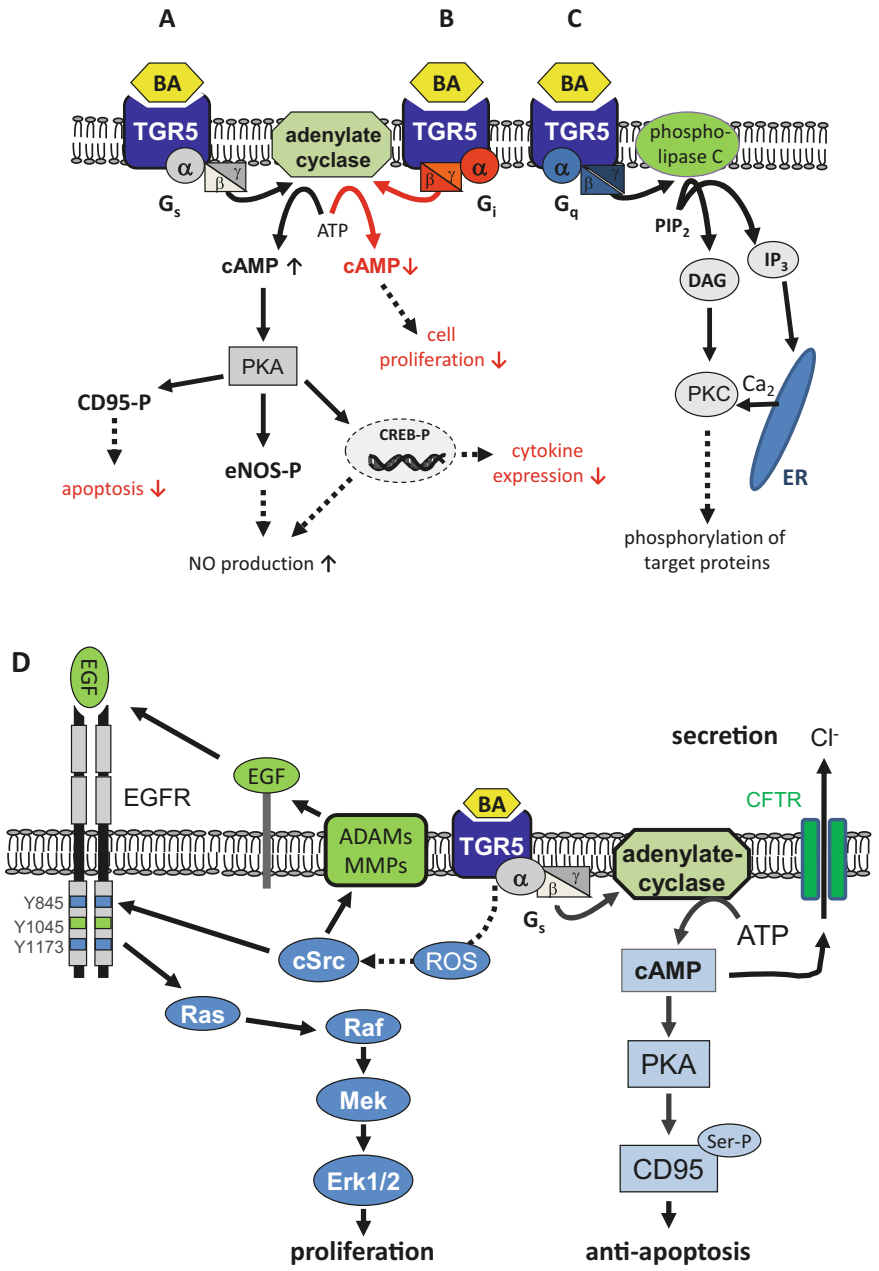

Fig. 4 TGR5-dependent bile acid signaling. (**a**) In most cell types TGR5 is associated with a G_S-protein, therefore, ligand binding triggers and activation of adenylate cyclase inducing an increase of the ATP-dependent cAMP production and an activation of the protein kinase A (PKA), which in turn may trigger several different signaling pathways. (**b**) Furthermore, interaction of TGR5 with G_i-proteins inhibiting adenylate cyclase activity has been demonstrated for ciliated

Role of TGR5 in Sinusoidal Endothelial Cells and Hepatic Stellate Cells

Liver sinusoidal endothelial cells (LSECs) are exposed to varying concentrations of nutrients, including BAs. After food intake, BA levels rise in portal venous blood and reach concentrations between 14 and 43 μM [1, 105–107]. Ligand binding to TGR5 on LSECs triggered not only increased expression of endothelial NO synthase (eNOS) but also stimulated phosphorylation of eNOS at serine 1177 via activation of protein kinase A (PKA), resulting in increased NO production in rat liver slices [58]. Similar results were obtained after TLCA treatment of endothelial cells from bovine aorta or human umbilical vein [108]. Furthermore, TLCA inhibited LPS-mediated upregulation of vascular cell adhesion molecule-1 (VCAM-1) and subsequent monocyte [108]. LSECs are an important NO donor in the hepatic sinusoids. Decreased NO production in LSECs is one hallmark of portal hypertension [1, 109–112]. Thus, stimulation of TGR5 and activation of the cAMP-PKA-eNOS-NO downstream signaling pathway may be beneficial in portal hypertension [1, 4]. In carbon tetrachloride treated mice simultaneous administration of a TGR5 agonist (6β-ethyl-3α,7β-dihydroxy-5β-cholan-24-ol (BAR501) 15 mg/kg/day) did not protect the animals from development of liver fibrosis, however inhibited the development of endothelial dysfunction and portal hypertension [113]. This beneficial effect was associated with reduced expression of endothelin-1 and increased expression of cystathionine-y-lyase (CSE), an enzyme responsible for the generation of the vasodilatory agent hydrogen sulfide [113].

TGR5 mRNA expression was below the detection level in freshly isolated HSCs but increased significantly within days in culture [57, 83, 84]. In activated, myofibroblast-like HSC elevation of cAMP led to an internalization of the endothelin-A (ET-A) receptor thereby attenuating the contractile response of the cells toward endothelin-1 [114]. Stimulation of TGR5 on activated HSCs may trigger cAMP-mediated ET-A receptor desensitization and thereby contribute to reduced portal pressure.

While activation of the TGR5-cAMP-PKA-eNOS-NO signaling pathway in LSECs may allow for adaption of sinusoidal blood flow in response to nutrient intake thereby promoting hepatic metabolism under physiological conditions, stimulation of TGR5 on LSECs and activated HSCs may attenuate portal hypertension development after liver damage [1, 84].

Fig. 4 (continued) cholangiocytes, where activation of TGR5 inhibited cell proliferation [49]. (**c**) Coupling of TGR5 with G_q-proteins has been observed in the oesophageal adenocarcinoma cell line (FLO) and triggered expression of NADPH oxidase NOX-5 and cell proliferation [61]. G_q-proteins may signal through activation of phospholipase C which in turn triggers the synthesis of diacylglyerol (DAG) and inositol trisphosphate (IP_3). By induction of proteinkinase C (PKC) activity and release of intracellular Ca_2 from the endoplasmatic reticulum (ER) these two second messenger proteins are causing the phosphorylation of different target proteins. (**d**) TGR5-dependent signaling in cholangiocytes. Modified after [52, 81]

TGR5 in Human Liver Disease

In contrast to mice, little is known on the role of TGR5 for the pathogenesis of human liver diseases [95]. In line with the high expression of TGR5 in cholangiocytes, TGR5 expression, localization, and function have been studied in biliary diseases. TGR5 protein levels as measured by relative quantification of TGR5 immunofluorescence staining in relation to cytokeratin 7 staining were significantly higher in human cholangiocarcinoma (CCA) tissue as compared to cholangiocytes from the nontumorous resection margins [52, 88]. Using CCA-derived cell lines (EGI-1 and TFK-1), it was demonstrated that activation of TGR5 triggers cell proliferation using the same ROS-cSrc-MMP-EGFR-ERK1/2 signaling pathway as in cultured murine cholangiocytes [52]. Furthermore, TGR5 stimulation induced apoptosis resistance and promoted cell migration and invasiveness. Thus, the receptor may contribute to CCA progression [81, 115].

An overexpression of TGR5 has also been described in cystic cholangiocytes of polycystic liver disease (PLD) [116, 117]. Stimulation of TGR5 in rodent cystic cholangiocytes promotes a rise in intracellular cAMP, which triggers proliferation and cyst growth, while deletion of TGR5 in a rodent model of PLD attenuates cyst formation [81, 82, 116, 117].

In contrast to CCA- and PLD-derived biliary cells, which are characterized by high TGR5 expression levels, a reduction in TGR5 immunofluorescence staining intensity has been observed in cholangiocytes of livers from patients with primary sclerosing cholangitis (PSC) as well as in livers from Abcb4 (Mdr2) knockout mice, which serve as an animal model for PSC [52, 88, 95, 116]. The mechanisms as well as the timing (early or late) of the TGR5 downregulation in the disease course of PSC is yet unclear [88, 95]; however, the reduced TGR5 expression may render cholangiocytes more susceptible toward BA-mediated cytotoxicity and thus accelerate disease progression [95].

Conclusion

Bile acids are signaling molecules with pleiotropic endocrine and paracrine functions, which are mediated by multiple BA sensing molecules, thus enabling a BA- and cell type-specific response. BAs regulate bile acid, glucose, lipid and energy homeostasis, modulate the immune response and affect cell survival and cell proliferation. Therefore, BAs and BA sensors have emerged as attractive targets for the treatment of metabolic diseases such as steatohepatitis, obesity, diabetes, and atherosclerosis. TGR5 (Gpbar1, M-Bar) is a G protein-coupled receptor highly responsive to primary and secondary BAs as well as to various progesterone metabolites. The receptor is almost ubiquitously expressed and has been detected in tissues participating in BA synthesis and secretion such as the liver, intestine, and kidney. However, TGR5 is also found in placenta, adrenal glands, and brain, where the receptor may primarily serve as membrane-bound receptor for steroid hormones. In line with the broad tissue expression, TGR5 has numerous functions including

modulation of the immune response, regulation of glucose and energy homeostasis as well as intestinal motility. In the liver, TGR5 activation can modulate liver microcirculation, promote biliary secretion and proliferation of biliary epithelial cells, induce gallbladder filling, and exert anti-inflammatory effects. Targeted deletion of TGR5 renders mice more susceptible toward inflammatory as well as cholestatic liver injury and impairs liver regeneration. In contrast, pharmacological stimulation of TGR5 improves steatohepatitis.

While TGR5 is overexpressed in cholangiocarcinoma tissue and promotes apoptosis resistance, cell proliferation, cell migration and invasiveness in CCA cell lines, the receptor is downregulated in cholangiocytes of livers from patients with progressive sclerosing cholangitis (PSC) as well as in livers from Mdr2 knockout mice, which serve as an animal model for PSC. Further studies are needed to elucidate the role of TGR5 in human liver disease.

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Bile Acids as Regulatory Signalling Molecules

Madlen Matz-Soja

Introduction

Bile acids (BAs) are a large family of molecules that have a steroidal structure and are synthesized from cholesterol in the liver. BAs are physiological detergents important for the emulsification of dietary fats, drugs, and lipid-soluble vitamins in the intestine, and their subsequent absorption and transport to the liver for metabolism is followed by distribution to other tissues and organs. BAs also act as signalling molecules and are important for the regulation of their own synthesis, uptake, and secretion as well as the control of cholesterol synthesis and the regulation of lipid and glucose metabolism. These processes are accomplished via the direct activation of the nuclear receptor farnesoid X receptor (FXR), TGR5, the pregnane X receptor (PXR), and the vitamin D receptor (VDR) [1]. In addition, other nuclear receptors, such as the constitutive androstane receptor (CAR) and the liver X receptor (LXR), can be indirectly influenced by BA, and these receptors, in turn, influence BA synthesis via feedback mechanisms and have a considerable influence on the metabolic processes of the entire organism. This chapter will focus on BA homeostasis, which is affected by BA synthesis, metabolism, and disposition in the liver and intestine. Furthermore, the roles of BAs as signalling molecules and therapeutic drugs to treat several diseases and metabolic imbalances will be discussed. Since there are cross-species differences in the synthesis and metabolism of BAs, the chapter will focus on humans and mice and will point out differences between these two species [2, 3].

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Bile Acid Homeostasis

Bile Acid Synthesis in the Liver

The synthesis of BAs in the liver is performed by hepatocytes and involves 17 distinct enzymes located in the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes [4]. These enzymes catalyze the oxidation and modifications of cholesterol at its steroid ring and facilitate the oxidative cleavage of three carbons from the cholesterol side chain to form C24 BAs [5]. These modifications can be classified into two distinct pathways—the neutral and the alternative BA synthesis pathways. The neutral pathway (also known as the classic pathway) is the major BA synthesis pathway in the liver. Via the neutral pathway, cholesterol is converted to 7-α-hydroxycholesterol (7α-HOC) by the rate-limiting enzyme cholesterol 7-α-hydroxylase (CYP7A1), which is located in the endoplasmic reticulum. The sterol 12α-hydroxylase (CYP8B1) converts the intermediate 7α-hydroxy-4 cholesten-3-one (C4) to 7α, 12α-dihydroxy-4-cholesten-3-one, resulting in the synthesis of cholic acid (CA). Without 12αhydroxylation by CYP8B1, C4 is converted to chenodeoxycholic acid (CDCA). The mitochondrial sterol 27-hydroxylase (CYP27A1) catalyzes steroid side chain oxidation in both CA and CDCA synthesis.

In the alternative pathway, CYP27A1 converts cholesterol to 3β-hydroxy-5-cholestenoic acid, which is then hydroxylated by oxysterol 7α-hydroxylase (CYP7B1) to form 3β,7α-dihydroxy-5-cholestenoic acid. Finally, CDCA is generated from 27-hydroxycholesterol (27-HOC). In the mouse liver, most CDCA is converted to α- and β-muricholic acid (MCA). MCA is only found in trace amounts in humans [6, 7]. In rodents, the alternative pathway can account for up to 25% of the total BA synthesis, whereas in humans, this route contributes less than 10% of the total amount of BAs. However, recent studies have shown that the alternative pathway is significantly more active in childhood, while in adults, the classical pathway makes a more significant contribution to the composition of BA pool [8] (Fig. 1).

Newly synthesized BAs are conjugated to the amino acids glycine and taurine, secreted through the apical membrane of hepatocytes, and stored in the gallbladder. The conjugation of BAs by only two amino acids is a result of the substrate specificity of pancreatic carboxypeptidases that cleave all other conjugating moieties [9]. Only conjugation with taurine or glycine makes BAs indigestible and unabsorbable in the proximal small intestine where most lipid absorption occurs. Such conjugated BAs are generally less hydrophobic than unconjugated bile acid whereby the hydrophobicity depends on the type of conjugation. In healthy individuals, nearly all BAs are present in their conjugated form [10].

Humans have two primary BAs (CA, CDCA), whereas five primary BAs (CA, CDCA, the muricholic acids α MCA and β MCA, and UDCA) are synthesized in mice [11] (Table 1).

The regulation of BA synthesis is closely linked to the whole-body BA pool, which must be relatively constant. To maintain homeostasis, fine-tuned feedback

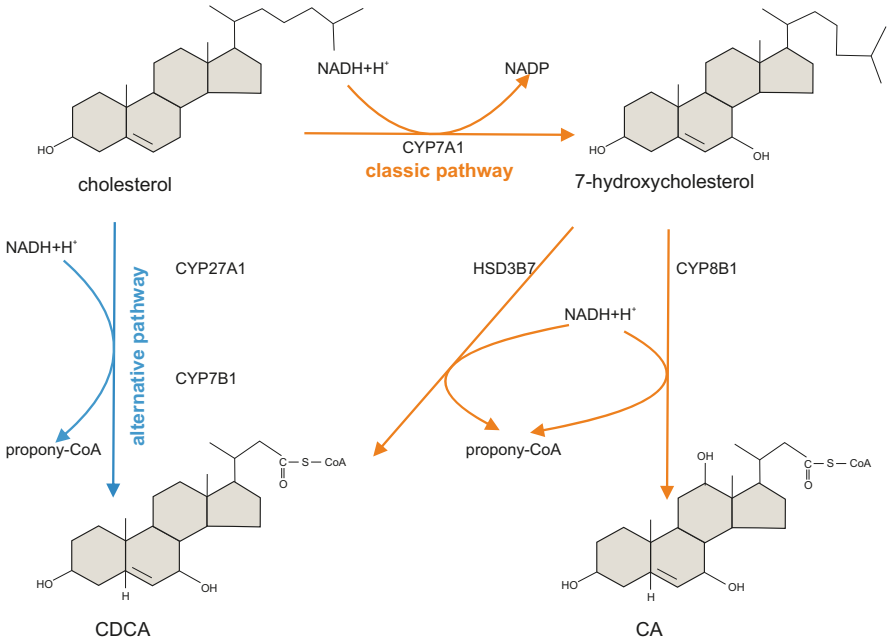

Fig. 1 The "classic pathway" and the "alternative pathway" to synthesize cholic acid (CA) and chenodeoxycholic acid (CDCA). In the "classic pathway" CYP7A1 converted cholesterol into 7-hydroxycholesterol in the endoplasmic reticulum. Via several steps, involving the enzymes Cyp8B1 and HSD3B7, the two primary bile acids CA and CDCA are synthesized. In the "alternative pathway" the enzymes Cyp27A1 and Cyp7B1 are used to synthesize CDCA from cholesterol

mechanisms exist that are mediated via cholesterol intake and several nuclear receptors, which act as both BA and biological sensors [12].

Excess cholesterol consumption negatively regulates cholesterol uptake and synthesis by proteolysis of the sterol regulatory element-binding proteins (SREBPs) which in turn are able to coordinate the synthesis of the two major components of membranes: fatty acids and cholesterol [13]. Since cholesterol is the source for BA synthesis, this mechanism not only reduces the amount of cholesterol, but also reduces the synthesis of BAs.

Another method of regulating the BA level in an organism is by downregulating bile acid biosynthesis via suppression of the key enzyme *Cyp7A1* by several BA receptors [14], which will be discussed in detail in the section "Bile Acids and Their Interaction with Nuclear Receptors."

Bile Acid Metabolism

Hepatocytes secrete conjugated BAs into the bile, which is stored in the gall bladder and then reabsorbed by passive diffusion before being transported from the intestinal

Table 1 Primary and secondary bile acids in mice and humans

Abbreviation	Bile acid name	Bile acid type	Species
CA	Cholic acid	Primary	Human/mouse
CDCA	Chenodeoxycholic acid	Primary	Human/mouse
DCA	Deoxycholic acid	Secondary	Human/mouse
GCA	Glycocholic acid	Glycol-conjungated	Human/mouse
GCDCA	Glycochenodeoxycholic acid	Glycol-conjungated	Human/mouse
GDCA	Glycodeoxycholic acid	Glycol-conjungated	Human/mouse
GLCA	Glyolithocholic acid	Glycol-conjungated	Human/mouse
GLCAS	Glycolithocholic acid sulfate	Glycol-conjungated	Human/mouse
GUDCA	Glycoursodeoxycholic acid	Glycol-conjungated	Human/mouse
HDCA	Hyodeoxycholic acid	Secondary	Human/mouse
LCA	Lithocholic acid	Secondary	Human/mouse
MCAa	alpha-Muricholic acid	Primary	Mouse
MCAb	beta-Muricholic acid	Primary	Mouse
MCAo	omega-Muricholic acid	Primary	Mouse
TCA	Taurocholic acid	Tauro-conjungated	Human/mouse
TCDCA	Taurochenodeoxycholic acid	Tauro-conjungated	Human/mouse
TDCA	Taurodeoxycholic acid	Tauro-conjungated	Human/mouse
TLCA	Taurolithocholic acid	Tauro-conjungated	Human/mouse
TLCAS	Taurolithocholic acid sulfate	Tauro-conjungated	Human/mouse
TMCAa/b	alpha/beta Tauromuricholic acid	Tauro-conjungated	Mouse
TUDCA	Tauroursodeocycholic acid	Tauro-conjungated	Human/mouse
UDCA	Ursodeoxycholic acid	Secondary/primary	Human/mouse

epithelial cells back to the liver following enterohepatic circulation (EHC) (Fig. 2). The process of BA secretion into the bile is accomplished by the polarity of hepatocytes, the mechanisms of which are complex and include intracellular and membrane trafficking systems and components, the cytoskeleton, tight junctions (TJs), and intracellular molecules [15]. In response to the peptide hormone cholecystokinin, the gallbladder releases bile into the duodenum to enable fat solubilization and absorption. The majority of the BAs that reach the distal intestine are reabsorbed during EHC.

In detail, the EHC can be described as the movement of BA molecules from the hepatocytes into canalicular bile, through the biliary tract and into the duodenum. As a result of propulsive small intestinal motility, BAs then flow in the intestines where they are actively absorbed from the distal ileum and passively from the large intestine. In the next step, BAs are transported to the liver via the portal venous blood and are then efficiently taken up by the hepatocytes. To complete the EHC, the BAs enter the hepatocytes and are actively secreted into canalicular bile again [16]. This BA uptake by hepatocytes, like BA synthesis, is a process that does not occur in all hepatocytes in the liver lobe in the same manner. Hydrophilic BAs cross the cell membrane of hepatocytes by diffusion and show no zonal differences in transport velocity. In contrast, hydrophobic BAs transverse the cytosol bound to BA

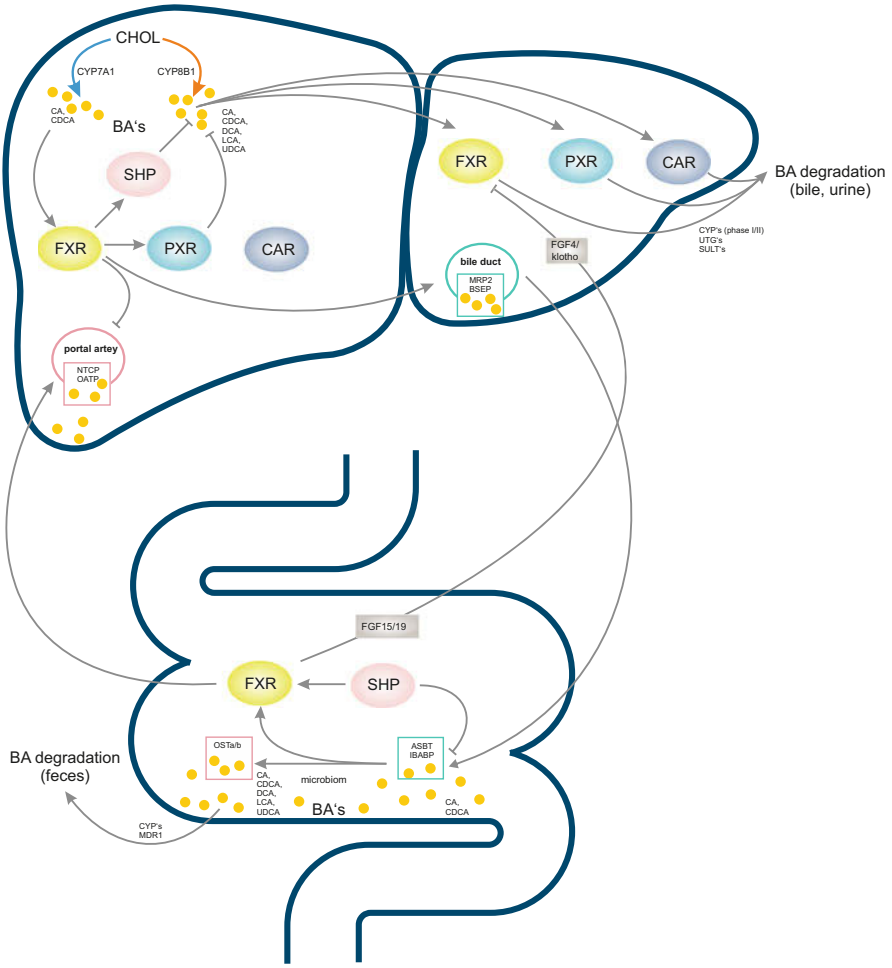

Fig. 2 The enterohepatic circulation of bile acids and its associated nuclear receptors. Cholesterol is degraded in the liver by CYP17A1 and CYP8B1 to the primary BA's cholic acid (CA) and chenodeoxycholic acid (CDCA). Through the two transporters bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) in the canalicular membranes of the hepatocytes, CA and CDCA can be transported into the bile. In the gut, CA and CDCA are metabolized to the secondary bile acids deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA). Most bile acids (primary and secondary) are reabsorbed in the intestine and returned to the liver via the enterohepatic circulation (EHC). The transporters apical sodium-dependent bile acid transporter (ASBT) and organic solute transporter alpha and beta (OSTα/β), which are expressed in the intestine are responsible for the absorption of BAs into the portal blood. At the basolateral membrane of hepatocytes, the transporters sodium/taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) reabsorb the BAs. The nuclear receptors FXR, PXR, and CAR are mainly metabolizing enzymes in the liver for induction Phase I and II to eliminate the excess BAs in the form of bile and urine

transporters or are distributed to organelles. Such transport systems like vesicles, glutathione S-transferase, and 3-α-hydroxysteroid dehydrogenase show a strong periportal distribution which leads to a faster transport in periportal hepatocytes compared to pericentral hepatocytes [17].

In addition to the enterohepatic circulation of BAs, a small proportion of conjugated BAs escapes this active transport and are deconjugated and further modified by the luminal bacteria into secondary BAs. Thereby, bacterial 7-dehydroxylase removes a hydroxyl group from C-7 and converts CA to deoxycholic acid (DCA) and CDCA to lithocholic acid (LCA) [18]. CYP3A1 and epimerases also convert CDCA to secondary BAs, including hyocholic acid (HCA), murideoxycholic acid (MDCA), ω-muricholic acid (ω-MCA), hyodeoxycholic acid (HDCA), and ursodeoxycholic acid (UDCA). Most LCA and ω-MCA are excreted into feces [19] (Fig. 2) (Table 1).

Bile Acids as Signalling Molecules in Metabolic Regulation

In addition to the immensely important function of BAs as physiological detergents for the absorption and transport of nutrients, fats, and vitamins from food, BAs also act as signalling molecules. This property enables BAs to regulate their own synthesis, the uptake, secretion, and synthesis of cholesterol, and the metabolism of lipids, glucose, and energy. This can be realized via the direct activation of the nuclear receptor farnesoid X receptor (FXR), the pregnane X receptor (PXR), the vitamin D receptor (VDR), and the G_s protein-coupled receptor TGR5 [1]. In addition, other nuclear receptors, such as the constitutive androstane receptor (CAR) and the Liver X receptor (LXR), can be indirectly influenced by BAs, which in turn influence BA synthesis via feedback mechanisms and have a considerable influence on global metabolism (Fig. 2).

Bile Acids and Their Interaction with Nuclear Receptors

BAs have the ability to directly and indirectly activate different nuclear receptors such as FXR, PXR, VDR as well as CAR to specifically influence their own biosynthesis and metabolic regulatory processes.

For FXR, two distinct genes (FXRα, NR1H4 and FXRβ, NR1H5) are known that are evolutionarily conserved between humans and rodents, but in contrast to the mouse, FXRβ is a pseudogene in humans [20]. The activation of FXR can only be achieved via binding of CDCA and its conjugates as well as LCA and DCA whereas the other BAs are not able to activate FXR [21] (Fig. 1). The binding of these BAs induces the formation of a heterodimer between FXR and retinoid X receptor (RXRFXR) which is able to bind to the inverted repeats of AGGTCA-like sequences with one nucleotide spacing (IR1) located in the promoters of the FXR target genes. The RXRFXR heterodimer then induces the expression of the negative nuclear receptor SHP, which inhibits the nuclear receptors liver-related homolog-1 and

hepatocyte nuclear factor 4α (HNF4α). This inhibition results in inhibition of the transcription of CYP7A1, the driver enzyme of BA synthesis [6, 22]. In the intestine, activation of FXR by BAs induces the expression of the intestinal hormone fibroblast growth factor 15 (FGF15; or FGF19 in humans), which activates hepatic FGF receptor 4 (FGFR4) signalling to inhibit BA synthesis. In addition, activation of FXR also leads to downregulation of the intestinal BA transporter ASBT and the hepatic uptake transporters NTCP and OATP, which also reduces the BA concentration. This effect of FXR is further enhanced by a simultaneous upregulation of the hepatic efflux transporters BA export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) [23, 24]. However, FXR inhibits not only CYP7A1 but also CYP8B1 and CYP27A1 transcription, as alterations to FXR activation are controlled by complicated mechanisms [25]. In addition to the modulation of BA synthesis, FXR activation also results in an overall decrease in triglyceride levels and modulation of glucose metabolism [26].

Also highly expressed in the liver and the intestine is the pregnane X receptor, PXR, which is also able to regulate CYP7A1 indirectly by inhibiting HNF4α and PGC-1α transactivation of Cyp7a1 gene expression [27]. Only secondary BAs are able to bind to the receptor and induce BA catabolism in this way, whereby the most potent ligand of PXR is LCA [28]. It is unclear whether BA can activate the receptor under physiological conditions when plasma levels are lower than 100 nmol/L, but it is probable that after rupture of the intrahepatic bile duct in cholestasis, PXR increases BAs clearance by CYP3A induction and decreases its biosynthesis by suppressing CYP7A1 expression [29]. After BA binding, PXR is translocated into the nucleus to form a heterodimer with RXR and activates target gene transcription. The range of regulated genes is wide, including many phase I and II enzymes and the uptake and efflux transporters.

Additionally, the vitamin D receptor has a dramatic influence on BA homeostasis. The alteration of BA synthesis via VDR is associated with the HNF4α Cyp7A1 axis, whereby the expression of VRD in the liver is restricted to nonparenchymal liver cells such as Kupffer cells or sinusoidal endothelial cells [30]. Like PXR, VDR can be activated by LCA and its metabolite 3-ketoLCA, while most Bas, including CDCA, CA, DCA, or muricholic acid, do not activate VDR [31]. The function of VDR in BA homeostasis is similar to that of PXR, namely, it exerts a protective role against BA toxicity and protects against bile duct infections. The latter is achieved through the stimulation of VDR by both vitamin D and LCA, which induces the production of the antimicrobial peptide cathelicidin in the bile duct epithelial cells [32]. In addition, LCA binding also increases the expression of CYP3A4, leading to elevated BA clearance, while the expression of CYP7A1 can be reduced through VDR activation [33]. In addition to influencing BA homeostasis, the VDR also has other functions such as regulating mineral homeostasis and metabolism, and a broader range of biological functions are connected to its expression such as cell growth, differentiation, antiproliferation, apoptosis, adaptive and innate immune responses [34]. Being widely expressed in various tissues, VDR represents an important therapeutic target in the treatment of diverse disorders that will be discussed later in detail.

Since the regulation of BA homeostasis by targeting nuclear receptors is very similar, it is assumed that FXR, PXR, and VDR coordinately regulate BAs, lipoproteins, drugs, glucose, and energy metabolism (Fig. 2).

Like PXR, the nuclear receptor CAR plays important roles in the regulation of BA metabolism and detoxication by inducing genes involved in BA conjugation and transport in order to maintain homeostasis [35]. Since BAs cannot directly bind to or activate CAR, the regulation occurs through well-known CAR activators such as TCPOBOP and Phenobarbital (PB). Both drugs are able to decrease the total BA level in the mouse liver, mainly by decreasing the amount of Taurine-conjugated CA (T-CA) to inhibit Cyp8b1. In mice, Cyp8b1 activation results in an increase in the proportion of muricholic acid (MCA). Furthermore, TCPOBOP is also able to increase Cyp7a1 expression in the liver to return the BAs to physiological concentrations. CAR activation also increases bile flow by increasing BA-independent flow, but the biliary excretion of BAs is not altered. In addition, CAR is also able to regulate BA homeostasis via the induction of LCA sulfation [35]. However, whether modulations of CAR are promising tools to regulate BA homeostasis in humans is questionable because of the differences in BA regulation between mice and humans. For example, in contrast to mice, humans have very low levels of MCA in the BA pool. Furthermore, glycine conjugates of BAs are the predominant amino acid conjugate in humans, while the taurine conjugates are predominant in mice.

Bile Acids and Their Interaction with TGR5

TGR5 (Takeda G protein-coupled receptor 5), also known as Gpbar-1 is a G_S protein-coupled receptor that is responsive to various unconjugated and conjugated Bas. Taurine-conjugated lithocholic acid and taurodeoxycholic acid TDCA are the most potent endogenous BA agonists for TGR5 [36, 37]. In addition, secondary BAs, like LCA and DCA, are produced in the intestine by gut bacteria and are able to bind to the receptor [6]. The receptor is located on cholangiocytes, the epithelial surface of the gallbladder and intestinal cells, the basolateral surface of smooth muscle, neural cells, brown adipose tissue, immune cells including dendritic cells and macrophages, and enteroendocrine cells that produce glucagon-like peptide 1 (GLP-1). However, hepatocytes do not express TGR5 [38]. In the liver, BAs activate the phosphorylation of endothelial nitric oxide synthase (NOS) through TGR5 activation and cAMP release, leading to nitric oxide (NO) synthesis in sinusoidal endothelial cells [39]. In gallbladder smooth muscle, TGR5 activation lowers intracellular calcium levels, decreasing the rhythmic discharge of intracellular Ca^{2+} necessary to induce contraction [40]. Another mechanism for BA regulation via TGR5 is by the alteration of the alternative BA synthesis pathway via CYP7B1expression. These results come from recent studies using Tgr5-deficient mice that show a reduced Cyp7b1 expression and a dramatic change in the BA composition in the gallbladder.

In addition to the regulation of BA synthesis, modulation of TGR5 also leads to changes in the hepatic fatty acid uptake and oxidation rate, making TGR5 targeting a potent drug candidate for BA-associated liver diseases [41]. In addition, TGR5 binding is also a liver-specific process that promotes liver regeneration. TGR5 expressed in Kupffer cells (KC), biliary epithelium, and sinusoidal endothelial cells constitutes a permeable barrier between hepatocytes and blood. Recently, it was shown that TGR5 takes control over bile hydrophobicity and cytokine secretion after partial hepatectomy to prevent liver injury in mice [42].

Bile Acids and Diseases

Since BAs have multiple important functions in the body, it is easily imaginable that pathological changes in BA synthesis, secretion, or transformation can lead to many diseases which affect the entire organism.

The main problem caused by an increase in BAs is due to their detergent activity against cell membranes, which can cause cytotoxic effects that lead to mitochondrial and endoplasmic reticulum apoptosis, cell necrosis, and ultimately cancer [3, 43]. Thereby, BA toxicity highly correlates with hydrophobicity, which is ranked: LCA/DCA > CA, UDCA, MCA, and HCA [2]. The consequences of high concentrations of BAs in intra- and extrahepatic systems are manifold and affect various functions of almost all organs.

For example, anomalies in intestinal BAs can induce systemic intestinal infections by disrupting the barrier function of the small intestine and promoting the translocation of bacteria [44]. A strong increase in hydrophobic BAs upregulates the amount of pro-inflammatory cytokines and NF-κB, changes the composition of intestinal microbiota, increases endotoxin levels, aggravates the inflammatory response caused by glucose tolerance and insulin resistance, and augments intestinal permeability [45]. Other bowel diseases can result from high concentrations of intestinal DCA, which enhance the excretion of chloride ions, increase intestinal permeability, and inhibit mucosal healing [46].

In the liver, the most common diseases caused by alterations in BA homeostasis are cholestatic liver diseases. Cholestasis is mainly caused by a disruption of bile flow, which leads to a lack of bile in the intestine and an accumulation of toxic BAs in the liver. In addition, in cholestatic patients, a dramatically increased BA concentration can be found in systemic circulation (up to 100-fold in humans) [31, 47]. In addition to disruption of bile flow, impediment of the bile ducts by tumor or stones, mutations in genes that encode BA transporters, and dysregulation of the bile transport system by drugs, pregnancy, and pathophysiological conditions can cause disease [31, 48]. Cholestasis can be classified into primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), which are the two most common chronic cholestatic liver diseases in adults. The development of PBC is an immune-mediated injury of intrahepatic biliary epithelial cells leading to cholestasis, fibrosis, and biliary cirrhosis [49].

In addition, a high concentration of BAs accelerates the senescence of hepatic secretory cells, facilitates the generation of tumor-promoting factors, and induces the progression of nonalcoholic steatohepatitis (NASH) and liver cancer [50]. Additionally, the hepatic control of xenobiotic and drug metabolism is closely associated with the regulatory network of BA homeostasis.

The fact that the characteristic changes of BAs in blood and tissues can indirectly indicate disease states has led to BAs being used as biomarkers for many diseases [50].

Bile Acids as Therapeutic Agents

BAs have an immense potential as therapeutic agents to produce beneficial effects in cases of primary biliary cirrhosis (PBC), primary sclerosing cholangitis, gallstones, digestive tract diseases, cystic fibrosis, and cancer [8]. Because BAs also influence metabolic-associated diseases such as nonalcoholic liver disease and diabetes, clinical studies have started to investigate these possibilities.

Bile Acids as Therapeutic Agents for Liver Diseases

BAs are being used as therapeutics, especially in patients with cholestatic liver diseases such as PCB. Thereby the administration of UDCA is the accepted therapy to treat PCB. This treatment inhibits intestinal BA absorption, resulting in an increase in BA secretion rich in bicarbonate to eliminate toxic substances from hepatocytes. As result, the entire BA pool is enriched with less toxic, hydrophilic BAs, which relieves parenchymal necrosis and apoptosis [51]. Unfortunately, up to 20% of PBC patients are UDCA nonresponders and have a reduced prognosis compared to healthy individuals [52]. For those patients, obeticholic acid (OCA, also known as INT-747) has been recently registered as a second-line therapy after demonstrating beneficial effects on liver biochemistry in approximately 50% of patients with an inadequate response to UDCA [49].

The treatment of PSC is much more complicated compared to PCB since several clinical studies showed no survival benefit from treatment with UDCA and other drugs. Therefore, liver transplantation is the only intervention shown to prolong survival of the patients [49].

BAs are also useful in the treatment (dissolution) of gallstones by increasing the concentration of bile acid and lowering cholesterol levels in the bile (resulting in less saturated bile).

Since BAs also have a significant influence on energy metabolism, targeted changes in BA receptors and the BA pool are used to increase glycemic control in diabetes. The main focus of the investigations is on nuclear transcription factors such as FXR in the liver and intestines, and the G protein-coupled receptor TGR5 in enteroendocrine cells and pancreatic β cells, as these interact directly with BAs [53]. In general, due to the many organs in which the signature of BAs is mediated

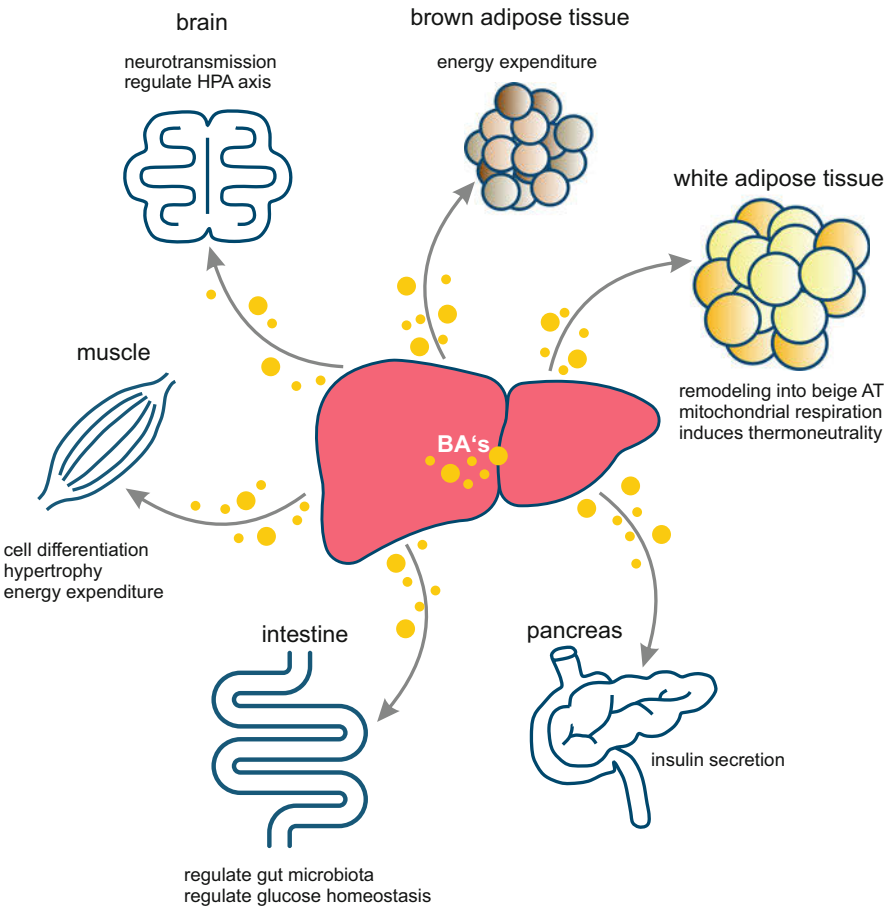

Fig. 3 The role of bile acids in the entire organism

by TGR5 and FXR, the mode of action of such inhibitors is highly complex which will have to be investigated further in the future (Fig. 3).

FXR Agonists for the Treatment of Metabolic Liver Diseases

One once-promising FXR agonist, obeticholic acid (OCA, also known as INT-747) is a semisynthetic derivative of chenodeoxycholic acid and was approved in the United States for the treatment of PBC after meeting the primary endpoint of reduced alkaline phosphatase level in a 2016 phase III clinical trial [54, 55]. This drug has also shown promising results in the treatment of NASH in a phase II trial, [56] demonstrating an improvement in the histological features of NASH, including hepatic steatosis, inflammation, hepatocyte ballooning, and liver fibrosis, and is

currently in a phase III clinical trial in NASH patients [57]. However, recently, the FDA announced a warning about an increased risk of serious liver injury and death associated with OCA in patients with moderate to severe decreases in liver function. In addition, treatment with OCA in humans can lead to an increase in low-density lipoprotein cholesterol and a reduction of high-density lipoprotein cholesterol as well as increased pruritus [54].

In addition to OCA, other FXR agonists such as GS-9674 and Tropifexor (also known as LJN-452) have promising potential as therapeutic agents for cholestatic liver diseases and are currently undergoing phase II trials for PBC [58]. The advantages of these two compounds are that they are non-BA formulations and are thus expected to cause less pruritus and hyperlipidemia compared to OCA.

Recently, the non-bile acid FXR agonist, EDP-305, demonstrated a promising safety and tolerability profile in a phase I study including healthy individuals and patients with presumed NAFLD [59]. The drug is now under evaluation for patients with PBC [23].

TGR5 Agonists for the Treatment of Metabolic Liver Diseases

Targeting the BA receptor TGR5 is also a useful treatment for several metabolic diseases. The activation of TGR5 decreases body weight and forces the secretion of the hormone GLP-1, which promotes insulin release from β cells of the pancreas [60] (Fig. 3).

Furthermore, since TGR5 is highly expressed in monocytes and macrophages, where it modulates immune responses [61], targeting TGR5 lowers the levels of pro-inflammatory cytokines in monocytes. This finding has led to new insights into the modulatory role of BAs in pathology, where inflammatory processes play a central role, including colitis and atheroma development.

A new semisynthetic derivative of cholic acid, 6α-ethyl-23(S)-methyl-3α,7α,12-α-trihydroxy-5β-cholan-24-oic acid (INT-777), is a selective TGR5 agonist that has a protective effect on many inflammatory diseases, such as sepsis, atherosclerosis, diabetic nephropathy, and hepatic steatosis [62].

Conclusion

BAs are extremely interesting compounds whose manifold functions are not yet fully understood. On the one hand, they serve to dissolve food components and are simultaneously secreted and received as signal molecules by various organs (Fig. 3). Since it is known that alterations in BA homeostasis are essentially responsible for various diseases, great efforts have been made to develop pharmaceutical concepts to restore this balance. Unfortunately, it is precisely the regulation of this equilibrium, which involves a wide variety of tissues, that causes the greatest difficulties in the treatment with these drugs. For example, the effects of FXR agonists not only reduce cholesterol metabolism in the liver but also might promote

reverse cholesterol transport out of tissues. Therefore, in patients who are treated with such drugs, cholesterol changes need prospective monitoring and analysis in future studies of these therapies for liver disease [56].

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Oxysterols and Bile Acid Act as Signaling Molecules That Regulate Cholesterol Homeostasis: Nuclear Receptors LXR, FXR, and Fibroblast Growth Factor 15/19

Klementina Fon Tacer

Summary

Organisms constantly cycle between fed and fasted states. Intricate homeostatic mechanisms have evolved that enable metabolic flexibility and maintain stable levels of nutrients despite fluctuation in their availability [1]. At the cellular level, it is now well appreciated that metabolites themselves can serve as indicators and signaling molecules of the metabolic status. Metabolites are recognized by specific sensors, among which are nuclear receptors (NRs), that either activate or inhibit downstream pathways to control the flux through the metabolic pathways [2]. Oxysterols and bile acids, first considered merely as the intermediates and the end products of steroidogenesis and cholesterol catabolism, are now well-recognized signaling molecules that regulate the cholesterol and bile acid homeostasis and integrate it with the other physiological processes, such as the immune response and cell growth. They do that primarily by binding to nuclear receptors: liver X receptors (LXRs) and farnesoid X receptor (FXR). The discovery that oxysterols and bile acids serve as physiological ligands for LXR and FXR opened the gates for uncovering their diverse physiological roles. LXRs promote cholesterol efflux, fatty acid synthesis, and conversion of cholesterol to bile acids for excretion to prevent cholesterol excess toxicity, whereas FXR regulates bile acid homeostasis by suppressing de novo bile acid synthesis in the liver and promoting bile acid reabsorption in the gut. In the gut, FXR also induces the expression of the endocrine hormone fibroblast growth factor FGF15/19. FGF15/19 acts on the FGF receptor in hepatocytes to further fine-tune diverse aspects of the postprandial response, including bile acid homeostasis. Through LXR and FXR, oxysterols and bile acids maintain balanced, finely tuned regulation of the cholesterol and bile acid homeostasis integrated with the fatty acid and glucose

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metabolism. Furthermore, oxysterol-LXR and BA-FXR-FGF15/19 regulated pathways offer exciting opportunities for treating metabolic diseases and related disorders. In this chapter, I will review our current understanding of the metabolic pathways leading to oxysterols and bile acids and functions of oxysterols and bile acids that are mediated by nuclear receptors LXR and FXR.

Introduction to Cholesterol Homeostasis

Cholesterol was first identified as a crystalline component of human gallstones more than 200 years ago and extensive research since then has shown that cholesterol is a vital component to every single cell. As an essential building block of cell membranes and a precursor of several biologically important molecules, such as steroid hormones, vitamin D, and bile acids, cholesterol is required for survival of every cell and organism as a whole [3]. On the other hand, excess cholesterol is toxic and linked to serious health problems, including cardiovascular diseases, cancer, and neurodegeneration [4–8]. To keep cholesterol levels within narrow limits, cells evolved elegant mechanisms to sense changing cholesterol levels and respond to them by fine-tuning the balance between cholesterol uptake, synthesis, and removal [9–14].

Cells supply themselves with cholesterol from two sources, by uptake of cholesterol-rich lipoproteins and by de novo synthesis [6]. While the entire cholesterol structure can be synthesized from acetate, the sterol ring cannot be degraded by mammalian cells [6, 15–21]. Thus, excess cholesterol is either esterified and stored in lipid droplets within cells or excreted from the body in the form of bile acids to keep the cellular pool constant. For removal from the body, cholesterol or its oxidized derivatives, oxysterols, are secreted from the extrahepatic cells and carried by lipoproteins back to the liver in a process, referred to reverse cholesterol transport [22]. In the liver, cholesterol and oxysterols that come from the periphery are further metabolized to bile acids and secreted into bile [6, 23].

Oxysterols attracted first attention with the “oxysterol hypothesis” that proposed that oxysterols rather than cholesterol itself are the main mediators of the cholesterol negative feedback loop [24, 25]. Although it was later shown that cholesterol contributes to inhibition of cholesterol synthesis, oxysterols contribute substantially to cholesterol homeostasis by inhibiting the synthesis and promoting cholesterol removal. Oxysterols inhibit the cholesterol synthesis by accelerating degradation of the rate-limiting enzyme, HMG CoA reductase (HMGCR), and preventing activation of the master transcriptional activator of cholesterol biosynthesis sterol response element-binding protein 2 (SREBP-2). In addition to synthesis inhibition, oxysterols also promote cholesterol export and removal from the body by bile acid synthesis. While the inhibition of cholesterol synthesis is mediated by the direct binding of oxysterols to the target proteins HMGCR and INSIG [26, 27], the activation of cholesterol export and bile acid synthesis is mediated principally by binding to nuclear receptors LXRs (liver X receptors) and activation of several target genes [28]. In addition to LXR, oxysterols were also found to bind and activate other nuclear receptors [29]; however, this is beyond the scope of this review.

The primary way for cholesterol elimination from the body is bile acid synthesis and excretion, accounting for approximately 90% of cholesterol breakdown [6]. Bile acids also play a central role in dietary lipid digestion and absorption as lipid solubilizers [30]. They are generated in hepatocytes, stored in the gall bladder, and after a meal secreted with bile into the duodenum. Bile acids are then absorbed by passive diffusion and actively transported back from the terminal ileum to the liver by the portal vein in a process named enterohepatic circulation [31, 32]. Therefore, bile acid levels fluctuate in the systemic circulation [33], what puts them in a position to function as endocrine signaling molecules that inform peripheral tissues about nutritional and energy status. Indeed, discoveries over last 20 years showed that bile acids as ligands for several nuclear hormone receptors including farnesoid X receptor (FXR; also known as NR1H4) and G-protein-coupled receptors such as TGR5 (also known as GPBAR1, M-BAR, and BG37) regulate their own synthesis, their enterohepatic recirculation, and triglyceride, cholesterol, and glucose homeostasis [34–36].

Cholesterol Catabolism: Synthesis of Oxysterols and Bile Acids

Generation of LXR Ligands: Oxysterol Synthesis

Oxysterols are cholesterol metabolites that have at least one additional oxygenated functional group. They can result from naturally occurring metabolic pathways or, in a much lower extent, by reactive oxidative species-induced chemical oxidation. Naturally occurring oxysterols that are also LXR ligands, such as 24(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 24(*S*),25-epoxycholesterol, are intermediary substrates in the rate-limiting steps of the three major pathways of cholesterol metabolism: bile acid synthesis, steroid hormone synthesis, and cholesterol synthesis itself [28, 29, 37–44] (Fig. 1).

The majority of oxysterols are generated during the initial steps of cholesterol elimination and bile acid synthesis (>90%). Most of the enzymes involved belong to the cytochrome P450 (CYP) family, heme-containing monooxygenases, that add hydroxy group either to the side chain of the molecule or to the sterol ring at the position C7, such as CYP7A1, the rate-limiting enzyme of the classical pathway of bile acid synthesis [30, 45]. Bile acid synthesis takes place in the liver, where CYP7A1 is solely expressed. However, in the extrahepatic tissues cholesterol can get hydroxylated on the side chain by sterol 27-hydroxylase (CYP27A1), cholesterol 24-hydroxylase (CYP46A), or cholesterol 25-hydroxylase (CH25H). Resulting 27-, 24-, and 25-hydroxycholesterols can more readily cross the plasma membrane and are a more suitable form for removal of excess cholesterol from the cells [46, 47].

CYP27A1 is a mitochondrial cytochrome P450, ubiquitously expressed enzyme, that converts cholesterol to 27-hydroxycholesterol, the most abundant oxysterol in human and mouse plasma [48, 49]. CYP27A1 is a promiscuous enzyme that can also catalyze the oxidation of the side chain of 7α-hydroxylated intermediates at the later steps of the bile acid synthesis in the liver and cholesterol at carbons 24 and

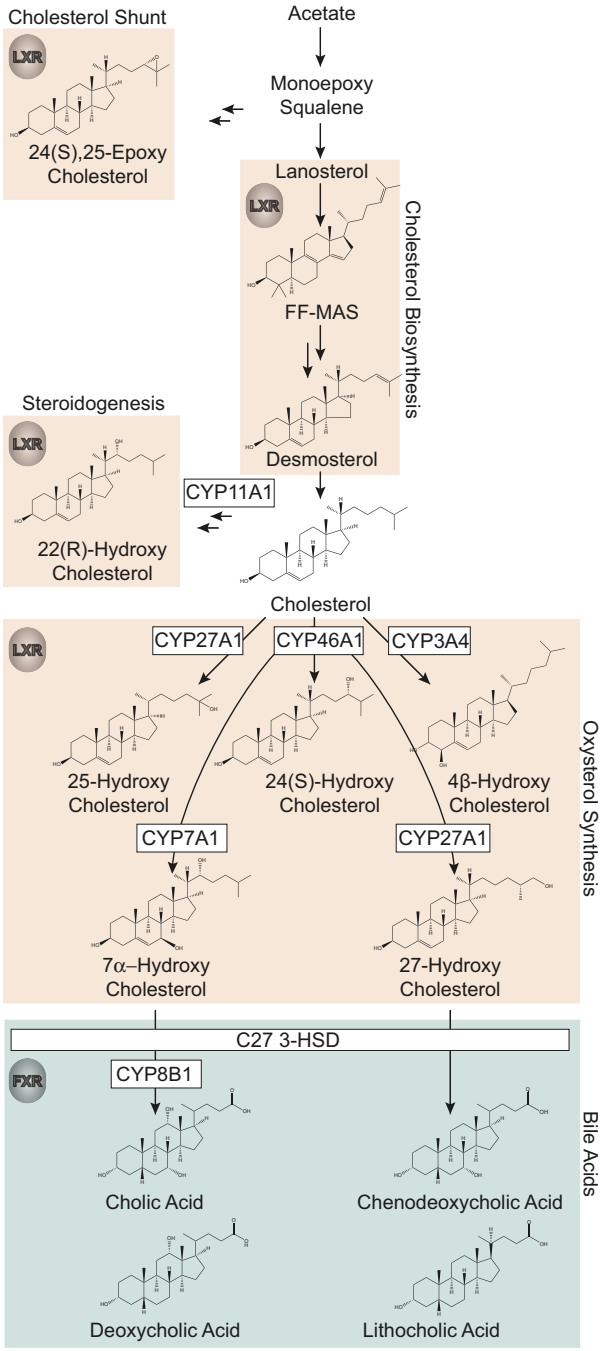

Fig. 1 Cholesterol and bile acid synthesis pathways generate ligands for liver X receptor (LXR) and farnesoid X receptor (FXR)

25 [30, 50]. CYP27A1 initiates the alternative or acidic pathway of bile acid synthesis, which accounts for 25% of the bile acid pool, whereas the CYP7A1-initiated classical pathway is responsible for the rest 75% [30].

Brain-specific cholesterol 24-hydroxylase (CYP46A) converts cholesterol into 24*S*-hydroxycholesterol that can traverse the blood-brain barrier, enter the circulation, and be delivered to the liver for further degradation to bile acids. Thus, the CYP46A1 function is important for the removal of excess cholesterol from the brain and critical for maintaining cholesterol and cholesterol turnover in the brain [51–54]. Cholesterol turnover, about 6–7 mg of cholesterol is converted to 24(*S*)-hydroxycholesterol every day, which is critical for memory and learning [55]. The majority of 24(*S*)-hydroxycholesterol generated enters circulation to be delivered to the liver, a fraction of it is directly converted into cholestanoic acid through the successive intervention by CYP39A1 (24(*S*)-hydroxycholesterol 7α-hydroxylase), CYP27A1 and HSD3B7 [56].

Cholesterol 25-hydroxylase (CH25H) converts cholesterol to 25-hydroxycholesterol. CH25H is not a cytochrome P450, it is expressed in all tissues with the highest level in the lung. It is dispensable for the bile acid synthesis in the mouse, but it may be important in the tissue-specific cholesterol catabolism, as 25-hydroxycholesterol is one of the most potent inhibitors of the SREBP pathway [57]. Drug-metabolizing enzymes CYP3A4 and CYP3A5 (the mouse orthologues are Cyp3a11 and Cyp3a13, respectively) can also convert cholesterol into 4-β-hydroxycholesterol, which is among the most abundant and stable oxysterols in human plasma [6, 29, 30, 58, 59]. Another cytochrome playing a role in the synthesis of oxysterols is the cholesterol side-chain cleavage enzyme known as CYP11A1, a rate-limiting enzyme of steroidogenesis. CYP11A1 is responsible for the production of 22(*R*)-OHC and 20(*S*),22(*R*)-di-OHC in steroidogenic tissues, two intermediates in the pathway leading from cholesterol to pregnenolone [60–62].

In general, most oxysterols are derived from cholesterol. By contrast, 24(*S*),25-epoxycholesterol has a unique origin. Rather than originating from cholesterol, it is produced in a shunt pathway of the post-squalene part of the cholesterol synthesis [63]. Two enzymes that are critical in the synthesis of 24(*S*),25-epoxycholesterol are squalene monooxygenase (SM) and 2,3-oxidosqualene cyclase (OSC), both of which are also part of the cholesterol synthesis. OSC catalyzes the conversion of 2,3-monoepoxysqualene to lanosterol, the dedicated step in the cholesterol biosynthesis. However, OSC also catalyzes cyclizing of 2,3;22,23-diepoxysqualene (DOS) to 24(*S*),25-epoxylanosterol, which is subsequently transformed into 24(*S*),25-epoxycholesterol (24,25-EC) in the shunt pathway, suggesting that the two pathways are taking place side by side. Indeed, like cholesterol, 24,25-EC too can be produced by most cell types, including macrophages and brain cells, however in a 10^3–10^4 lower extent compared to cholesterol [63, 64]. Emerging evidence suggests that endogenous 24,25-EC serves as a measure of cholesterol synthesis and works at several levels to control acute cholesterol homeostasis [63]. Synthesis of 24,25-EC closely parallels that of cholesterol and it is also inhibited by the feedback control when cholesterol accumulates. This suggests that 24,25-EC has a distinct function

from other oxysterols and may specifically protect against the newly synthesized cholesterol, as opposed to cholesterol entering the cell in lipoproteins [63].

In addition to 24,25-EC, the flux through the cholesterol biosynthetic pathway [65] and the cholesterol biosynthesis intermediates, such as FF-MAS [28] and desmosterol [66, 67] have been also shown to activate LXR and may contribute to the cell-specific and target-specific regulation of LXRs, understanding of which will be instrumental for dissociation of diverse LXR functions in designing more selective agonists [68].

Generation of FXR Ligands: Bile Acid Synthesis

Ninety-five percent of the bile acid pool is recycled by the enterohepatic circulation, whereas the rest is secreted in the stool and must be replenished by de novo synthesis. This means that roughly 500 mg of cholesterol is turned into bile acids in adult human liver every day [30]. Bile acid synthesis takes place in the liver, where the reverse cholesterol transport delivers excess cholesterol and oxysterols from the periphery to enter the bile acid synthesis. Severe symptoms presented in the patients with mutations in one of the nine bile acid synthesis genes highlight the physiological importance of the cholesterol catabolism and bile acid synthesis and they range from hypercholesterolemia to liver failure and progressive neuropathy [50, 69].

Seventeen reactions of the bile acid synthesis convert almost insoluble cholesterol into a soluble conjugated bile salt that is readily excreted into the bile. They can occur in two major ways, the classical (neutral) pathway, accounting for generating 75% of the bile acid pool, and the alternative (acidic) pathway that generates the reminder 25% [6, 30]. CYP7A1 initiates and performs the rate-limiting step of the classical pathway, while CYP27A1 initiates the alternative pathway (Fig. 1).

In the classic synthesis pathway, cholesterol is first subjected to 7a-hydroxylation by CYP7A1 [6, 30]. As the rate-limiting step of the bile acid synthesis, CYP7A1-catalyzed conversion of cholesterol to 7α-hydroxycholesterol is critical for the whole-body cholesterol homeostasis. Thus, CYP7A1 gene expression and enzyme activity are highly regulated [70, 71]. Mice deficient in CYP7A1 have a high incidence of postnatal lethality due to liver failure, vitamin deficiencies, and lipid malabsorption [72–74]. About 90% of them die within the first 3 weeks of birth, but animals that survive this period begin synthesizing bile acids using the alternative pathway [73]. However, the bile acid pool size in these animals never exceeds 25% of normal, which is generally contributed by the alternative pathway. The reduction in the bile acid synthesis is not compensated by an increased expression of the other bile acid biosynthetic enzymes, but rather by 95% decrease in the intestinal cholesterol absorption, which results in a 200% increase in hepatic cholesterol synthesis to maintain steady cholesterol levels [75, 76].

In the alternative pathway, 7α-hydroxylation is preceded by the hydroxylation on the side chain of the cholesterol molecule, predominately mediated by CYP27A1 [77]. However, to be converted into bile acids, oxysterols must also undergo

7α-hydroxylation. Two microsomal cytochrome P450 enzymes CYP39A1 and CYP7B1 catalyze these reactions [6, 30, 78]. The CYP39A1 acts on 24(*S*)-hydroxycholesterol and CYP7B1 catalyzes the conversions of 25-hydroxycholesterol and 27-hydroxycholesterol. CYP39A1 is abundantly and constitutively expressed in mouse and human liver and in the nonpigmented epithelium of the eye. CYP7B1 is expressed at high levels in the adult liver and at lower levels in the kidney, brain, and prostate. In mice, hepatic expression of this enzyme is induced during the third week of life and thereafter exhibits a sexually dimorphic expression pattern with higher expression in the male [48, 73]. Mice that lack CYP7B1 have elevated plasma levels of 25-hydroxycholesterol and 27-hydroxycholesterol but not 24-hydroxycholesterol [48, 79]. These animals also have increased levels of cholesterol 7α-hydroxylase, presumably to compensate for the reduced bile acid biosynthetic capacity [48]. This increase (30%) is roughly equal in size to that of the residual bile acid pool in cholesterol 7α-hydroxylase-deficient mice [75]. In humans, only up to 10% of the bile acid pool originates from oxysterols, but the fraction that derives from the CYP7B1 versus the CYP39A1 is unknown.

The 7α-hydroxylated intermediates derived from cholesterol and oxysterols are next converted into their 3-oxo, Δ4 forms by a microsomal 3-hydroxy-Δ5 -C27-steroid oxidoreductase (C27 3-HSD). The products of the C27 3-HSD enzyme take one of two routes in subsequent steps of bile acid synthesis, if intermediated are subjected to sterol 12α-hydroxylation by a microsomal cytochrome P450 (CYP8B1), primary bile acid generated is cholic acid, otherwise chenodeoxycholic acid. Thus, two primary bile acids that are produced by most of the vertebrate species are cholic acid and chenodeoxycholic acid (rat, human, and hamster), muricholic acid (mouse), ursodeoxycholic acid (bear), or hyodeoxycholic acid (pig). The level of CYP8B1 in the liver determines the relative amounts of the two primary bile acids, hydrophobicity of the BA pool, and controls the rate of feedback inhibition [6, 30].

Before active secretion into the canalicular lumen, primary BAs are conjugated with taurine or glycine to form less cytotoxic bile salts. After postprandial stimuli, bile salts are released from the gallbladder into the small intestine. In the distal ileum, 95% of the bile salts are actively reabsorbed and returned back to the liver through the portal circulation to reduce the energy expenditure for de novo BA biosynthesis [7]. In the colon, the primary BAs are transformed to the secondary BAs (lithocholic acid, LCA and deoxycholic acid, DCA) through the intestinal bacteria de-conjugation, then passively absorbed by enterocytes, returned back to the liver where they are re-conjugated. As stated earlier, approximately 5% of the BA pool per day escapes intestinal reabsorption and is excreted into the feces. This loss is accurately compensated by de novo synthesis in the liver to maintain the pool size which represents a major determinant of cholesterol turnover [80].

Despite their beneficial role in solubilizing lipophilic nutrients and facilitating their intestinal absorption, high levels of bile acids are cytotoxic. Increased bile acid concentration is linked to inflammation, DNA damage, increased cell proliferation, and decreased apoptosis, all of which can promote the neoplastic transformation of

hepatocytes and intestinal cells. Therefore, tight regulation of bile acid concentration is essential for both, cholesterol homeostasis and liver and intestinal health [81–83]. Since the late 1960s it was well appreciated that the rate of bile acid synthesis is precisely regulated to prevent bile acid or cholesterol accumulation [84, 85]. By the 1980s it was uncovered that the two rate-limiting enzymes, CYP7A1 and CYP8B1, are regulated on the transcriptional level, inhibited by bile acid accumulation and, in some species, activated by cholesterol accumulation [80]. The discovery that oxysterols and bile acid bind to and activate nuclear receptors gave the first insights into the molecular mechanisms underlying the cholesterol- and bile acid-mediated feedforward and feedback loops. Intensive research over the last couple of decades using nuclear receptors agonists and genetic mouse models uncovered the intensive molecular cross-talk between liver, intestine, and other organs that orchestrates cholesterol and bile acids homeostasis and integrates it with energy metabolism and other physiological pathways.

Nuclear Receptors Are Lipophilic Ligand-Activated Transcription Factors

Nuclear receptors provide an important link between transcriptional regulation and physiology, regulating the expression of genes involved in diverse physiological processes from reproduction and development to metabolism. First recognized as the mediators of steroid hormone signaling [2], the protein family now includes 48 members from the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and vitamins A and D to a large number of nuclear receptors, whose ligands, target genes, and physiological functions were initially unknown, thus classified as orphans [86–88]. The ligands and function for many of these, initially orphans, have been over the last two decades discovered. Unlike the classic endocrine hormone nuclear receptors, many orphan nuclear receptors function as sensors that respond to cellular lipid levels and elicit gene expression programmed to protect cells from the lipid overload and contribute to the whole body metabolic homeostasis [2, 89]. Since the major function of these lipid-derived ligands is to maintain the homeostasis of the ligands themselves, the discovery of the ligand enabled insights into the physiological pathways they regulate, as was the case for sterol-activated liver X receptors (LXRs) and bile acid-activated farnesol X receptor (FXR) [89–91].

Nuclear receptors share very similar structural organization: an N-terminal activating domain, a DNA-binding domain, a ligand-binding domain, and a C-terminal activating domain [92]. As transcription factors, they activate or repress gene expression by binding to the regulatory regions of target genes and act in concert with co-activators and corepressors. Many nuclear receptors induce the transcription of target genes following ligand binding. Ligand binding results in a conformational change to the receptor and release of corepressors. Activated nuclear receptors bind specific DNA sequence, hormone response element, composed of two palindromes of six nucleotides, separated by one to six nucleotides. Unlike the

classic steroid receptors that bind to DNA as homodimers, nonsteroid receptors like LXR and FXR bind mostly as heterodimers in which their common partner is RXR (retinoic X receptor) [2].

In addition to LXR and FXR, this group also includes receptors for fatty acids (the peroxisome proliferator-activated receptors, PPARs), xenobiotics (the pregnane X receptor, PXR), and constitutive androstane receptor (CAR). In contrast to the classic steroid hormone receptors, these receptors bind their lipid ligands with lower affinities, comparable to the physiological concentrations that can be affected by dietary intake (>1–10 μM), suggesting their role as lipid sensors. Ligand binding to each of these receptors activates a metabolic cascade that maintains nutrient lipid homeostasis and prevents lipid toxicity in excess by governing the transcription of a common family of genes involved in lipid metabolism, storage, transport, and elimination [2, 89].

Cytochrome P450s (CYPs), enzymes with unique ability to add molecular oxygen to small molecules, play a key role in the generation and degradation of orphan nuclear receptors' ligands [93], that are involved in vitamin A and D, steroid, cholesterol, and fatty acid metabolism. In addition to the generation of the ligands, CYPs are also very important for ligand inactivation and clearance, a process mostly driven in the liver by the phases I, II, and III of drug metabolism and controlled in large part by the xenobiotic receptors PXR and CAR. By regulating the dynamics between the ligand synthesis and its degradation, CYPs are critical for the responsiveness of the system to the changes in the environment and adaptation of the organism. As described earlier, cytochromes P450 play central roles in the synthesis of oxysterols bile acids [6, 30, 45, 94–96].

Together, nuclear receptors respond to changes in the metabolic environment by inducing the target gene expression. To maintain lipid homeostasis, orphan receptors including LXR and FXR coordinate regulation genes that catalyze a transformation of lipid ligands into inactive metabolites and facilitate their metabolic clearance, the intracellular lipid-binding proteins that buffer and transport hydrophobic ligands; and transporters, which shuttle their lipid ligands and precursors [89]. Furthermore, nuclear receptors also activate the expression of endocrine hormones, like fibroblast growth factor FGF-15/19 by FXR, FGF-21 by PPARs and FGF-23 and by doing that integrate the environmental stimuli with the whole-body physiology [97].

Oxysterols-Activated Liver X Receptors Regulate Cholesterol Homeostasis

Liver X Receptors

The first LXR isotype cloned was LXRα that is highly expressed in the hepatocytes [98, 99], hence the name liver X receptor. There are two LXRs, LXRα (NR1H3) and LXRβ (NR1H2) that share a high degree of homology, but show different expression pattern, suggesting the tissue-specific function of the two isoforms. Consistent with the regulation by oxysterols, LXRα is expressed at the highest level in the

enterohepatic tissues, liver, and gut, but also in the adipose, macrophages, lung, spleen, and kidney, whereas LXRβ is broadly expressed in all tissues [100, 101].

LXRs work as heterodimers in conjunction with retinoid X receptors (RXRs, NR2B1). LXR-RXR heterodimers bind to LXR response elements (LXREs) in the promoters or enhancers of LXR target genes where they are associated with corepressors, such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) [102, 103]. Following ligand binding to LXR or RXR, corepressors are released and co-activators (SRC-1, p300, ACS-2, TRRAP, or PGC-1α) are recruited what results in gene transcription activation [104]. The typical LXRE in the promoters of the bona fide targets contains two replicates of the hexamer AGGTCA separated by four nucleotides (referred to as direct repeat 4, DR4) [99]. LXRs control cholesterol homeostasis mostly by activating gene expression, which is the focus of this paragraph, however, they were shown to also repress gene expression, particularly the expression of inflammatory genes in macrophages [105].

Oxysterols and Cholesterol Synthesis Inhibitors Are the Physiological Ligands of LXRs

Cholesterol and its oxygenated metabolites were long known to autoregulate their own homeostasis [106]. The discovery that naturally occurring sterols activate LXRα and LXRβ helped to uncover the underlying molecular mechanisms [28, 37, 107, 108]. Several cholesterol metabolites were found to be potent LXR ligands in vitro at the concentrations they physiologically occur. For example, side-chain oxysterols 24(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol that are synthesized from cholesterol in the brain and adrenals [28, 37]. In addition, LXR physiological agonists include cholesterol biosynthesis intermediates desmosterol [109] and meiosis activating sterols (follicular fluid meiosis activating sterols, FF-MAS) that normally accumulate in macrophages and gonads [19, 28, 37, 110, 111] and 24(*S*),25-epoxycholesterol, a metabolite from the cholesterol biosynthesis shunt pathway [112, 113]. Oxysterols bind directly to the ligand-binding domain of LXRs what enables recruitment of the co-activator proteins to the receptor and transcriptional activation of the target genes [28, 37, 107, 108, 114].

Intriguingly, the cell uses oxysterols [115] and intermediates of cholesterol synthesis [18, 19, 116, 117], which are present in at least 1000 times lower concentration compared to the cholesterol, to sense and balance the cholesterol level. Similarly, the cholesterol sensing system composed of the sterol responsive element-binding protein (SREBP), SREBP cleavage-activating protein (SCAP), and insulin-induced gene (INSIG) are all localized to the membranes of the endoplasmic reticulum which contains only ∼1% of a cell's total cholesterol, whereas the majority of the cell's cholesterol pool is in the plasma membrane (60–90%) [118, 119]. These further suggest that lower concentration levels of sterols are required for fine-tuning and maintaining cholesterol levels in a narrow range [120].

In addition to confirm the binding of the ligand to LXR and activation of downstream target genes in vitro, even more difficult challenge was to determine whether naturally occurring sterols indeed serve the same LXR-activating function in the animal [115]. So far, several lines of evidence support the physiological function of diverse endogenous sterols as LXR ligands. For example, LXR response is altered by inhibition of the cholesterol synthesis or feeding cholesterol-rich diet to mice. Further, inhibition of 2,3-oxidosqualene: lanosterol cyclase, the enzyme which elevates intracellular levels of 24,25-epoxycholesterol in cultured macrophages, caused LXR activation [112, 121–123]. In the contrary, the expression of cholesterol sulfotransferase (SULT2B1b), which sulfates sterols and prevents them to bind LXR, attenuated LXR signaling [124]. And finally, knockout mice that do not synthesize 24S-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol had compromised LXR response in the liver when fed cholesterol-rich diet [124].

Interestingly though, not all target genes are affected the same way, suggesting that LXRs activate distinct classes of target genes depending on the ligand present [125–128]. Consequently, different LXR ligands may serve distinct physiological roles and indicate different alternations in homeostasis. Whereas oxysterols derived from cholesterol indicate cholesterol accumulation, cholesterol synthesis intermediates serve as a measure of cholesterol synthesis rate [129]. Likewise, cholesterol metabolism is regulated in a tissue-specific manner, affected by the physiological/pathophysiological state of the organism generating the tissue-specific sterol profiles [16, 17, 19, 117]. Together with the tissue-specific expression of NRs, co-activators, and repressors [101], tissue-specific sterol patterns may underlie distinct LXR functions. For example, in the liver of mice without the major three oxysterols, 25-, 25-, and 27-oxysterol, RCT is severely affected; however, the expression of CYP7A1 and SREBP-1c is not affected in the liver in these mice. This suggests that oxysterols activate LXR to induce cholesterol esterification and export, whereas other ligands are responsible for the activation of bile acid and fatty acid synthesis [124]. In line with this, it was recently shown that the flux through the cholesterol biosynthetic pathway is required for the maximal SREBP-1c expression and high rate of fatty acid synthesis in the liver [65]. In contrast, 24,25EC and desmosterol selectively activate RCT in macrophages without triglyceride accumulation in hepatocytes [66, 68, 112, 113, 129]. Together all these data suggest that diverse naturally occurring sterols selectively modulate LXRs to control whole-body cholesterol homeostasis. Understanding the underlying molecular mechanisms of the tissue and/or isotype-specific effects of LXR actions will be essential for successful therapeutic strategies aiming to exert beneficial effects on cholesterol and glucose homeostasis while circumventing the undesired effects on hepatic lipid metabolism [130, 131].

LXRs Promote Reverse Cholesterol Transport

Nonsterol agonists, identified subsequently by the pharmaceutical industry, and genetic mouse models helped to elucidate the physiological role of LXRs in

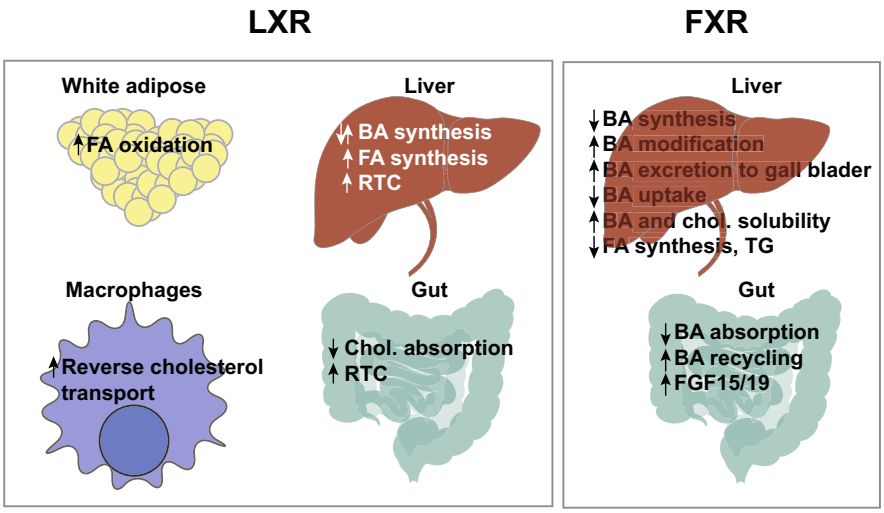

Fig. 2 Activated LXR and FXR regulate cholesterol and bile acid metabolism to enable a proper level of cholesterol and bile acid and prevent their potential toxic effects if in excess

cholesterol and fatty acid metabolism [132, 133]. Although mice lacking LXRα and LXRβ were not overtly affected by the deletion of the LXR genes when kept on regular chow, they showed profound phenotypes when challenged with a high-cholesterol diet. Mice accumulated massive amounts of cholesterol in the liver because of impaired RCT and conversion into bile acids (Fig. 2) [123, 134].

One of the best-characterized effects of LXR is promoting the reverse cholesterol transport (RCT), the process of cholesterol delivery from the periphery to the liver for excretion. To stimulate RCT, LXRs activate the expression of ATP-binding cassette transporter A1 (ABCA1) and other genes (ABCG1, ABCG5, and ABCG8) that export cholesterol from cells to apolipoprotein AI (APOAI) and pre-β high-density lipoprotein (HDL) in plasma. *ABCA1* is one of the most highly regulated LXR target genes in macrophages in other peripheral tissues [135–139]. LXRs also induce expression of apolipoprotein genes (*APOE*, *APOC1*, *APOC2*, and *APOC4*) [140, 141] and lipid remodeling genes, including phospholipid transfer protein (*PLTP*), human cholesterol ester transfer protein (*CETP*), and lipoprotein lipase (*LPL*) [142, 143], all of which facilitate efficient transport of cholesterol from the periphery to the liver.

In the intestine, LXR activation increases RCT while also decreasing intestinal cholesterol absorption, both of which improve lipoprotein profile and prevent hepatic steatosis [144]. In macrophages, high cholesterol conditions cause the accumulation of desmosterol that acts as a specific LXR activator [66]. Desmosterol-activated LXR promotes the expression of the genes responsible for cholesterol export (e.g., *ABCA1*, *ABCG1*), while lipogenic gene expression remains unaffected. The regulated accumulation of desmosterol underlies many of the protective responses, including activation of LXR target genes, inhibition of SREBP target genes, selective reprogramming of fatty acid metabolism, and suppression of

inflammatory genes, observed in macrophage foam cells [66] and could be exploited in selective atherogenic drug development [111, 145].

LXRs Regulate Bile Acid Synthesis in the Liver

One of the first LXR target genes identified was CYP7A1, the rate-limiting enzyme bile acids synthesis [13, 133]. Because of compromised bile acid synthesis, mice lacking LXRα develop a marked accumulation of cholesterol in the liver when fed a high-cholesterol diet [123]. However, LXR-induced regulation of CYP7A1 is not conserved in humans. In human dietary cholesterol and LXR agonists repress CYP7A1 and inhibit BA synthesis. This repression is mediated, at least in part, through induction of the orphan nuclear receptor, short heterodimer partner (SHP), which is induced by bile acids [146].

In general, regulation of CYP7A1 and cholesterol homeostasis varies greatly across species. Cholesterol induces CYP7A1 expression in mice, rats, dogs, and certain nonhuman primates [147–151], species that adapt to high-cholesterol diets with almost no change in plasma cholesterol levels. In contrast, cholesterol has little effect or even suppresses CYP7A1 in other species, including rabbit, hamster, African Green monkey, and human [146, 147, 152, 153]. These species are prone to hypercholesterolemia in response to dietary cholesterol. This suggests that some species as rodents cope with excess cholesterol by stimulating its conversion into the bile acids for excretion from the body, when other species, including humans, evolved an alternate approach, in which cholesterol absorption in the intestine is reduced by decreasing bile acid production.

LXRs Inhibit Cholesterol Uptake and Synthesis

When activated, LXRs not only promote cholesterol efflux but also simultaneously inhibit cholesterol uptake and synthesis in the mouse liver. LXRs maintain cellular and systemic cholesterol homeostasis in coordination with SREBP, sterol response element-binding protein, a master regulator of cholesterol biosynthesis and low-density lipoprotein (LDL) uptake [14]. They do that by activating the expression of negative regulators of SREBP-2 pathway, E3 ubiquitin ligases IDOL, and RNF145 and noncoding RNA LeXiS [154–156].

While LXRs are activated in response to elevated cholesterol levels, SREBPs are activated by low cellular cholesterol levels. In cholesterol depleted conditions, the SREBP-2 transcription factor activates the expression of the LDL receptor and cholesterol biosynthesis genes [157]. In contrast to SREBP-2, LXR negatively regulates the LDL receptor pathway by controlling the expression of IDOL (inducible degrader of LDLR). IDOL is an E3 ligase that targets the LDL receptor for ubiquitination and lysosomal degradation [154]. Interestingly, the activity of the LXR-IDOL-LDL receptor axis is also tissue and species-specific. In mice, LXR and IDOL regulate LDL receptor protein levels in the periphery, but not the liver. In

contrast, in primates, LXR activation induces also hepatic IDOL expression and reduces the LDL receptor protein. Consequently, LXR activation in primates causes increased plasma LDL levels, that can be blunted by IDOL inhibition, whereas in mice plasma LDL levels are not changed by LXR agonists [158].

In addition to IDOL, LXRs activate expression of another E3 ubiquitin ligase RNF145, leading inhibition of the expression of genes involved in cholesterol biosynthesis and reduces plasma cholesterol levels. RNF145 triggers the ubiquitination of SCAP, potentially inhibiting its transport to Golgi and subsequently preventing the processing and activation of SREBP-2 [156].

Under conditions of excess cholesterol, LXRs also activate the expression of the long noncoding RNA LeXis. LeXis interacts with and affects the DNA interactions of RALY, an RNA-binding protein that acts as a transcriptional cofactor for cholesterol biosynthetic genes in the mouse liver [155], further confirming that the hepatic sterol levels are fine-tuned by the reciprocal actions of the SREBP and LXR pathways.

LXRs Regulate Fatty Acid Metabolism

In addition to modulating cholesterol metabolism, LXRs also promote de novo lipogenesis in the liver to prevent excess cholesterol toxicity. Fatty acids are used to generate cholesterol esters that are less toxic than free cholesterol. LXRs stimulate biosynthesis of fatty acids through the induction of transcription factor sterol regulatory element-binding protein 1C (SREBP-1C) and lipogenic genes acetyl CoA carboxylase (ACC), stearoyl CoA desaturase 1 (SCD1), and fatty acid synthase (FAS) expression [114, 123, 136]. These findings severely tempered therapeutic approaches to target LXRs for treating cardiovascular diseases.

Interestingly, in the liver of triple KO mice, CYP46A1/CYP27A1/CH25 KO, the activation of SREBP-1C by LXR is not affected, suggesting that other ligands than 24, 25, or 27-hydroxycholesterol are required as ligands for lipogenesis activation [124]. Furthermore, in liver-specific SREBP-2 KO mice, it was shown that flux through the cholesterol biosynthetic pathway is required for maximal SREBP-1C and fatty acid synthesis activation by LXR [65]. These studies not only demonstrate that the cholesterol and FA synthesis are coupled, but also suggest that in the liver cholesterol biosynthesis or shunt pathway intermediates may serve as LXR ligands to specifically regulate fatty acid synthesis. Understanding the distinct set of target genes and beneficial effects upon selective activation of LXRs will be fundamental in future endeavors to develop therapies by targeting LXR [159].

FXR Regulates Cholesterol and Bile Acid Homeostasis

Farnesoid X Receptor (FXR, NR1H1)

FXR was originally named based on its pharmacological activation by terpenoid, farnesol [160]. Subsequent studies showed that the physiological ligands for FXR are bile acids (BAs), including the primary bile acids cholic acid and chenodeoxycholic acid [34–36]. As LXR and other orphan nuclear receptors, FXR also regulates the expression of target genes by binding to DNA as a heterodimer with RXR. In addition to BAs, the FXR–RXR heterodimer can also respond to RXR ligands. The consensus farnesoid X response elements (FXREs) is composed of two hexamers AGGTCA, organized as an inverted repeat, and separated by a single nucleotide (IR-1) [133, 161]. In addition to inducing gene expression directly, FXR also mediates the repression of a number of genes indirectly through the regulation of another nuclear receptor short heterodimer partner (SHP, NR0B2) expression [162–164]. SHP is an atypical nuclear hormone receptor that has just a ligand-binding domain and no DNA-binding domain, and inhibits the activity of several nuclear receptors.

FXR is expressed predominately in the liver, intestine, kidney, and adrenal glands, with some low expression in the heart, adipose, and vasculature [101, 133, 160]. In the liver and intestine, FXR target genes activate transcriptional feedback and feedforward regulatory loops to suppress bile acid (BA) synthesis, promote covalent modification of BAs into less toxic molecules, and induction of hepatic bile acid efflux [13, 165]. In this way, FXR prevents the hepatic toxicity of BAs and controls BA and cholesterol homeostasis.

Bile Acid Are FXR Ligands

The important role of bile acids (BAs) for the absorption of dietary lipids and lipid-soluble vitamins had been appreciated for a long time; however, BAs were rediscovered in 1999 when they were found as endogenous FXR ligands. Now they are well-appreciated signaling molecules that regulate their own synthesis and enterohepatic circulation integrated with energy metabolism and immune system [34–36]. Bile acids exert their physiological functions by activating a nuclear receptor FXR [34–36], a G protein-coupled receptor TGR5 (Gpbar1/M-BAR) [166, 167] and other nuclear receptors, such as vitamin D receptor (VDR, NR1I1) [168], the constitutive androstane receptor (CAR, NR1I3), and pregnane X receptor (PXR, NR1I2) [169, 170], all of which contribute to the metabolism and excretion of bile acid and confer protection upon bile acid exposure. In this chapter, I will focus on BAs function mediated by activating FXR and its downstream effector, fibroblast growth factor 15/19 (FGF-15/19), and their role in bile acid and cholesterol homeostasis.

FXR Regulates Bile Acid Homeostasis

The physiologic role of FXR in bile acid homeostasis was confirmed in FXR-knockout (KO) mice, which have disrupted increased synthesis and pool size [171] and fail to adapt to dietary cholic acid overload [172]. Owing to their cytotoxic properties, chronic exposure to elevated bile acids causes spontaneous liver tumor development in *FXR-KO* mice [173, 174]. Many FXR target genes have been identified over the years, particularly in the liver and intestine where FXR exerts real-time control over all steps of enterohepatic bile acid circulation. Among the genes regulated by FXR are those encoding enzymes involved in the bile acid synthesis, modification, transport, and enterohepatic circulation (Fig. 2) [13, 133, 162–165, 175–179].

Bile acid synthesis is controlled by a feedback regulatory mechanism whereby activation of FXR represses expression of the CYP7A1, the rate-limiting enzyme in the classic bile acid synthesis pathway, and the CYP8B1, required for the synthesis of cholic acid. FXR activation induces the expression of the atypical nuclear receptor SHP. SHP interacts with nuclear receptors liver receptor homolog 1 (LRH1, NR5A2) and hepatocyte nuclear factor 4α (HNF4α) at the promoters of target genes and recruits various proteins, including the mSin3A-Swi/Snf complex, G9a methyltransferase, and the corepressor subunit GPS2 to repress CYP7A1, CYP8B1, and even SHP itself to ultimately limit this axis, too [162, 163, 180, 181]. By inhibiting CYP8B1, FXR also controls the ratio of cholic acid (CA) over chenodeoxycholic acid (CDCA) that determines the hydrophobicity of the BA pool [182] and negative feedback regulation as CA is critical for BA-mediated inhibition of CYP7A1 [126].

To reduce hepatic toxicity, FXR induces BA modification and conjugation to form bile salts conjugated with taurine or glycine. Several BA modifying enzymes, including sulpho-transferase 2A1 (SULT2A1), UDP-glucuronosyltransferase 2B4 (UGT2B4) and CYP3A4, and conjugation enzymes like acid CoA synthase (BACS) and bile acid CoA–amino acid N-acetyltransferase (BAAT) are positively regulated by FXR [183–185].

To prevent cholestasis, FXR promotes secretion of BA into the gall bladder through bile salt export pump (BSEP, ABCB11) and multidrug resistance proteins 2 and 3 (MDR2 and MDR3). Furthermore, FXR also induces expression of phosphatidylcholine transporter ABCB4, and by doing that enables the proper ratio of BA and phospholipids in the bile, crucial for the solubilization of cholesterol and BAs. Loss-of-function mutations in these genes lead to cholestasis [186, 187] and FXR has protective effects against gall stone formation in mice [188].

Bile is stored and concentrated in gall bladder during fasting and, in response to feeding, released into the small intestine to facilitate the digestion of dietary lipids [189]. In the intestine, BA activates FXR to inhibit BA absorption and promotes BA movement and recycling of BA back to the liver. FXR downregulates the apical sodium-dependent bile salt transporter (ASBT, SLC10A2, or IBAT) by SHP-dependent inhibition of LRH1 in mice [190]. In the enterocytes, FXR promotes the movement of BAs from the apical to the basolateral membrane by upregulating

the ileal bile acid-binding protein (IBABP) [191]. BA is then secreted into the portal blood for return to the liver by FXR-induced expression of the heterodimeric organic solute transporters OSTα and OSTβ [179].

Having reached the liver, bile salts are taken up by sodium taurocholate cotransporting polypeptide (NTCP; also known as SLC10A1) and organic anion transporting polypeptide (OATP; also known as SLCO1A2), both of which are negatively regulated by FXR, thereby limiting the increase in the hepatic bile salt levels. Finally, BAs are resecreted in the bile what closes up the BAs enterohepatic circulation [192]. Together, BA induced FXR regulates various genes that maintain a balance of bile acid homeostasis and at the same time prevent bile acid-induced toxicity.

BAs and FXR Regulates Cholesterol and Lipid Metabolism

Bile acids are critical for fat metabolism because they facilitate postprandial digestion and absorption of dietary lipids and lipid-soluble vitamins in the small intestine. On the other hand, BAs are the major gateway for cholesterol elimination from the body. BAs also regulate lipid metabolism as signaling molecules. FXR-KO mice uncovered the importance of BAs and FXR for triglyceride metabolism. FXR-KO mice have disrupted lipid absorption and altered expression of genes that control lipid metabolism [133, 172, 193–196]. FXRs decrease triglyceride levels by several mechanisms, including reducing lipogenesis and promoting increased uptake of triglycerides and fatty acids.

FXR-deficient mice exhibit marked hypercholesterolemia and hypertriglyceridemia [172, 193–196]. FXR promotes the expression of *APOC2* and inhibits expression of APOC3 [195, 197]. APOC2 and APOC3 are plasma proteins that work in a converse manner, whereas APOC2 activates lipoprotein lipase (LPL), APOC3 inhibits it. LPL interacts with very low-density lipoproteins (VLDLs) and chylomicrons to promote the release of triglycerides and hydrolysis into fatty acids. FXR activation of APOC2 contributes to a reduction in triglyceride levels. Furthermore, induction of *VLDLR*, *PLTP*, and *APOE* expression has also been associated with FXR activation and could further contribute to increased uptake and reduced plasma levels of triglycerides [13].

In the liver, FXR reduces lipogenesis via inhibition of sterol-regulatory element-binding protein 1C (SREBP-1C) and fatty acid synthase (FAS) in an SHP-dependent manner [194, 198].

In human cells, FXR also induces expression of peroxisome proliferator-activated receptor-α (PPARα) and its target genes, and by doing that promotes fatty acid transport and oxidation and another way to decrease triglyceride level [199]. In all, FXRs decrease triglyceride levels by several mechanisms, including reducing lipogenesis and promoting uptake, catabolism, and oxidation of triglycerides and fatty acids. Furthermore, by promoting lipoprotein catabolism and clearance, FXR agonists may also contribute to the hypocholesterolaemia observed after treatment.

Endocrinology of Bile Acids: The BA–FXR–FGF15/19 Pathway

In the gut, FXR is expressed in enterocytes throughout the small intestine and colon [101, 200] where it regulates genes that are involved in the BA enterohepatic circulation and secretion [13, 133]. In addition, in the ileum, FXR also induces the expression of fibroblast growth factor 15 (FGF15) [200–202]. FGF15 and its human orthologue FGF19, hence referred to as FGF15/19, belong to the atypical subfamily of FGFs that evolved to function as endocrine hormones. All three members of this subfamily, FGF15/19, FGF21, and FGF23 are activated by distinct nuclear receptors, FXR, PPARs, and VDR, respectively, to regulate distinct metabolic adaptations. Endocrine FGFs function by binding to the FGF receptor (FGFR) in conjunction with a specific coreceptor from the Klotho family of proteins [97, 203, 204].

In mouse liver, FGF15/19 exerts its function by binding to a receptor complex composed of the FGFR4, which has tyrosine kinase activity, and βKlotho, a single transmembrane protein. FGF15/19, released from the small intestine after postprandial bile acid surge, plays a crucial role in coordinating bile acid homeostasis. FGF15/19 is secreted into the portal circulation, circulates to the liver, where it binds to the FGFR4/βKlotho receptor and inhibits bile acid synthesis by repressing CYP7A1 and causes refilling of the gallbladder via relaxation of the gallbladder smooth muscle [72, 201]. Like FXR, also FGF15/19-mediated inhibition of CYP7A1 requires a small heterodimer partner (SHP) to efficiently repress bile acid synthesis. Mice lacking SHP are refractory to the inhibitory effects of either FXR agonists or FGF15/19 on *Cyp7a1* expression [200, 205]. Both the FGFR4-KO and βKlotho-KO mice phenocopy the FGF15-KO mice and have increased CYP7A1 expression and small gallbladders [206, 207]. The important contribution of the FGF15/19 to the whole body bile acid homeostasis is further supported by finding that farnesoid X receptor-null mice are protected against cholestasis and spontaneous hepatocarcinogenesis by selectively expressing FXR solely in the intestine [208, 209].

Conclusion

Nuclear receptors LXR and FXR are physiological sensors of cholesterol and bile acid levels that act as molecular switches that translate changes in their ligand levels into gene expression to maintain cholesterol and bile acid balance. Activation of LXRs serves to maintain whole-body cholesterol homeostasis by upregulating reverse cholesterol transport from the periphery and activating hepatic catabolism into bile acids. To ensure that bile acid homeostasis and metabolic balance are maintained, activation of FXR and endocrine hormone FGF15/19 in the intestine and liver initiates an important feedback loop to inhibit further bile acid synthesis and reset the digestive system for the next meal. The biological actions of LXR, FXR, and FGF15/19 extend beyond the regulation of cholesterol and bile acid homeostasis. They regulate diverse aspects of the postprandial enterohepatic response, including hepatic glucose, lipid, and protein metabolism and provide several therapeutic opportunities for treating metabolic diseases and bile acid-related disorders.

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Cytochrome P450 Metabolism Leads to Novel Biological Sterols and Other Steroids

F. Peter Guengerich and Francis K. Yoshimoto

Introduction

Cytochrome P450 (P450, CYP) enzymes are of general interest because of their roles in the metabolism of drugs, carcinogens, sterols, and fat-soluble vitamins [1]. Roughly one-fourth of the 57 human P450s are involved mainly in steroid metabolism (Table 1) and deficiencies are often quite debilitating. In classic mammalian cholesterol synthesis (Fig. 1), the only P450-catalyzed step is the removal of the 14α-methyl group, and the two 4-methyl groups are removed by a different oxygenase, a cyanide-sensitive oxygenase that generates carboxylic acids [5], which are cleaved. Similar reactions occur in plants and fungi (Fig. 2). However, a plethora of P450s are involved in the degradation of cholesterol (Fig. 3) and in the conversion of cholesterol to critical hormones and other steroids (Fig. 4).

Although this chapter is focused on mammalian P450s and their roles in the metabolism of sterols and other steroids, structural analogues of cholesterol are present in plants (i.e., campesterol, sitosterol, stigmasterol) and fungi (Fig. 2) and are critical in membrane synthesis. Also, some complex bacteria depend on hosts for sterols and utilize these in membrane biogenesis. P450 51 enzymes in pathogenic fungi and yeasts are important targets for mammalian diseases related to infections [6, 7], and several P450s are essential to *Mycobacterium tuberculosis*, the infectious bacterium causing tuberculosis. P450 51 enzymes are also targets in diseases caused by the parasites *Trypanosoma cruzi* and *Aspergillus* species, causative agents in Chagas disease and chronic pulmonary aspergillosis [8, 9].

The P450s involved in the metabolism of endogenous compounds have generally been considered to be highly specific in the past, due to their critical roles

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Table 1 Classification of human P450s based on major substrate class [2]

Steroids	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1B1[a]	1A1[a]	2J2	4F2	2R1[b]	2A7
7A1[a]	1A2[a]	2U1	4F3	24A1[b]	2S1
7B1	2A6[a]	4A11	4F8	26A1	2W1
8B1	2A13[a]	4B1[b]	5A1	26B1	4A22
11A1[a]	2B6[a]	4F11	8A1[a]	26C1	4X1
11B1	2C8[a]	4F12		27B1	4Z1
11B2[a]	2C9[a]	4F22		27C1	20A1
17A1[a]	2C18	4V2			
19A1[a]	2C19[a]				
21A2[a]	2D6[a]				
27A1	2E1[a]				
39A1	2F1				
46A1[a]	3A4[a]				
51A1[a]	3A5[a]				
	3A7				
	3A43				

[a]X-ray crystal structure(s) reported (for human enzyme) [3]
[b]Rat or rabbit X-ray crystal structure reported

[10, 11]. However, more recent studies have shown that, like the P450s that oxidize many xenobiotics [2], these P450s also have some flexibility and will oxidize whatever is able to bind in such a manner as to expose atoms to the iron–oxygen complex. Even the line between oxidizing endogenous and xenobiotic chemicals can become blurred [12, 13].

P450 7A1 and Production of Oxysterols

P450 7A1 is involved in the 7α-hydroxylation of cholesterol, the first step in bile acid formation (Fig. 3) [14]. This is the major pathway of cholesterol removal in the liver [2, 15]. The enzyme is highly regulated, involving lithocholic acid X receptor (LXRα) and other pathways.

Oxysterols are of interest because of their biological properties. One of these, 7-ketocholesterol, is a strong inhibitor of P450 7A1 (IC_{50} ~1 μM) and also regulates cholesterol homeostasis, cytotoxicity, and apoptosis, as well as the induction of inflammation, growth inhibition, and vascular endothelial growth factor [16, 17]. Much of the literature had attributed the source of 7-ketocholesterol to lipid peroxidation and other radical processes acting on cholesterol [17–19]. A 7-hydroxycholesterol dehydrogenase had been purified from hamsters [20], but the activity is not present in rats or most humans.

7-Dehydrocholesterol is more sensitive to chemical oxidation than any other sterol. Some humans have low Δ7 reductase (DHCR7) activity (Fig. 1) and

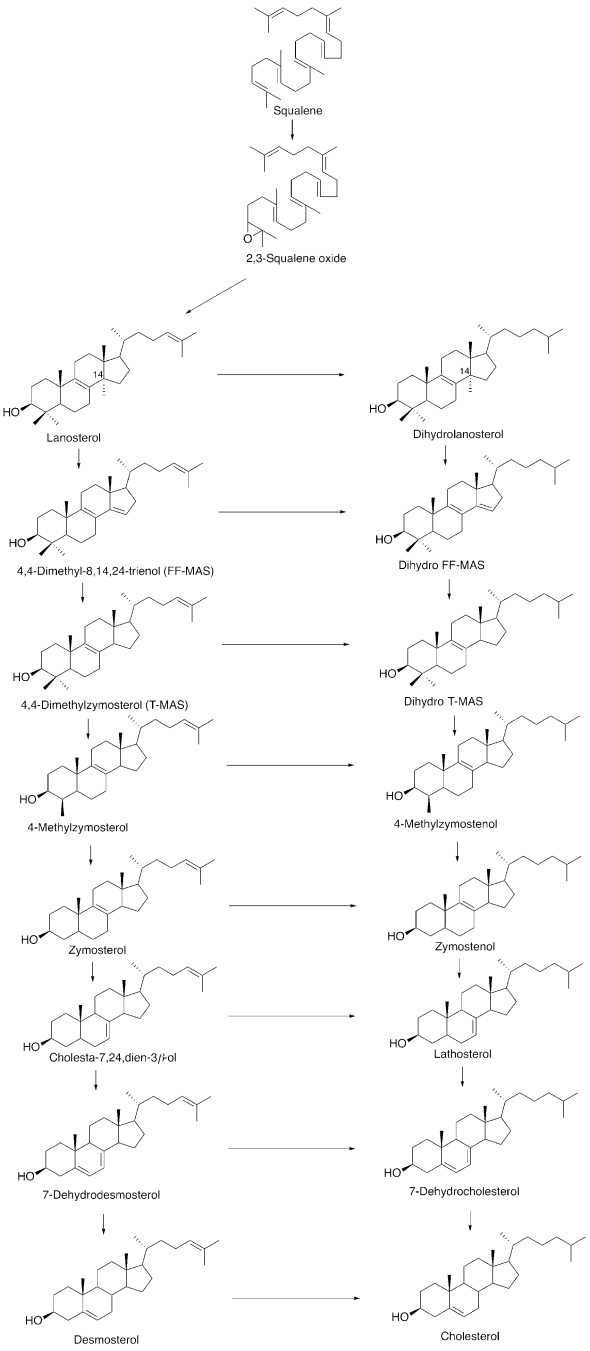

Fig. 1 Classic pathway of cholesterol synthesis, including both Bloch (left) and Kandutsch–Russell (right) branches [4]. The reductase converting the Bloch intermediates is 7-dehydrocholesterol reductase (DHCR7)

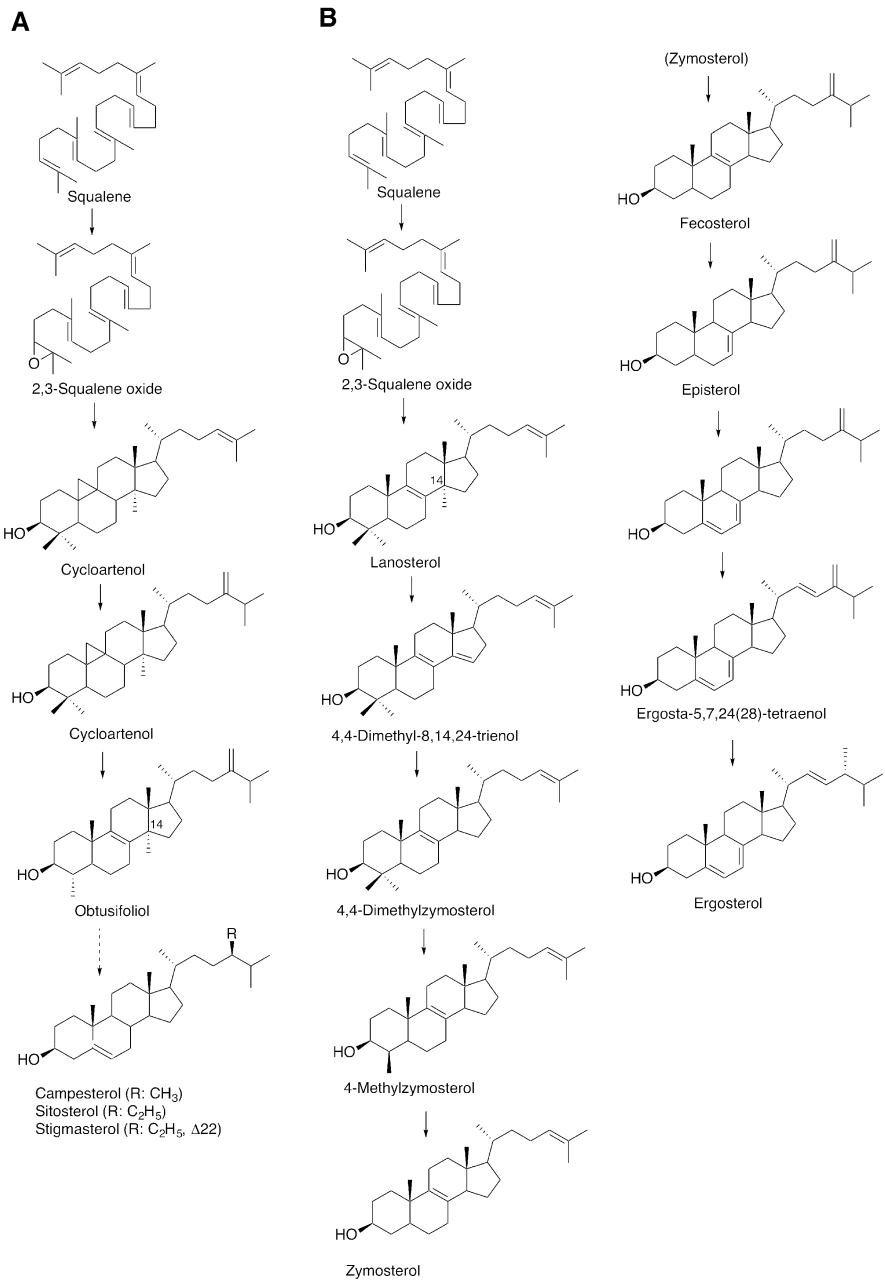

Fig. 2 Sterol synthesis in (**a**) plants and (**b**) fungi/yeast

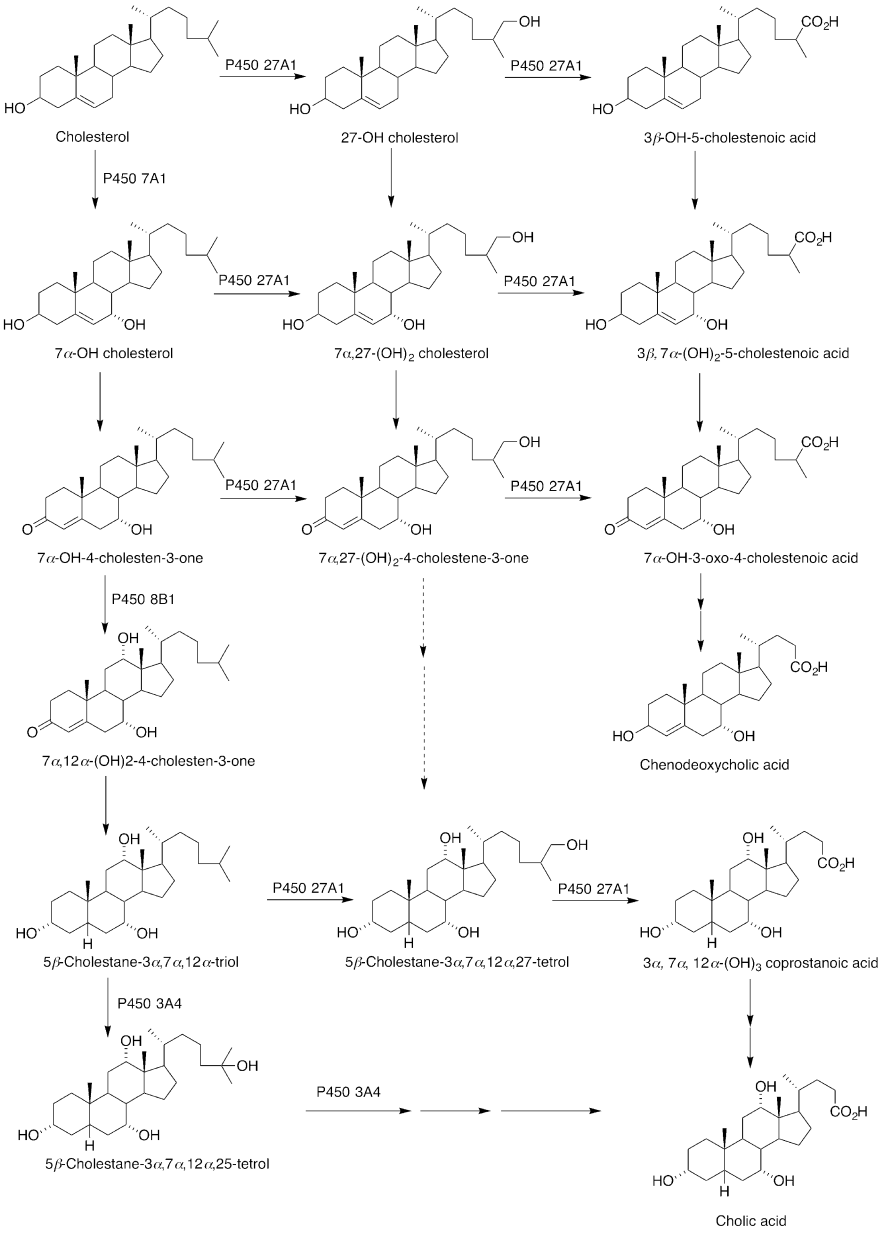

Fig. 3 Classic pathway of nonhormonal degradation of cholesterol [2]

accumulate 7-dehydrocholesterol. Δ7- and Δ8-dehydrocholesterol are in equilibrium (Fig. 5). We hypothesized that 7-ketocholesterol might be formed from the oxidation of 7-dehydrocholesterol, the direct precursor of cholesterol in the

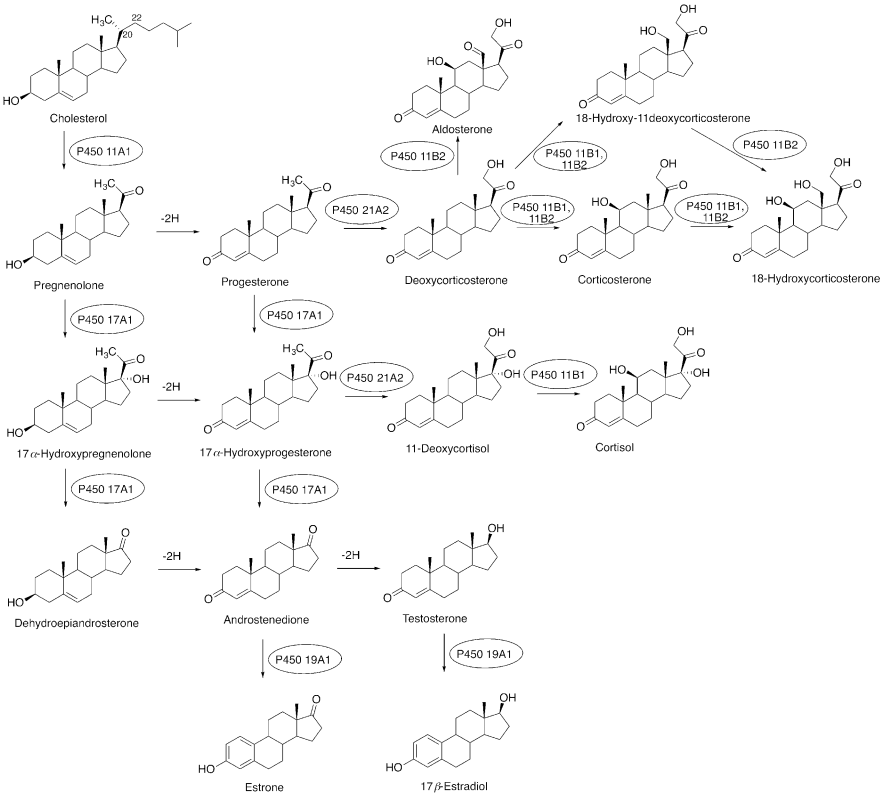

Fig. 4 Classic pathway of conversion of cholesterol to steroids [2]. The reactions labeled "-2H" are catalyzed by dehydrogenases

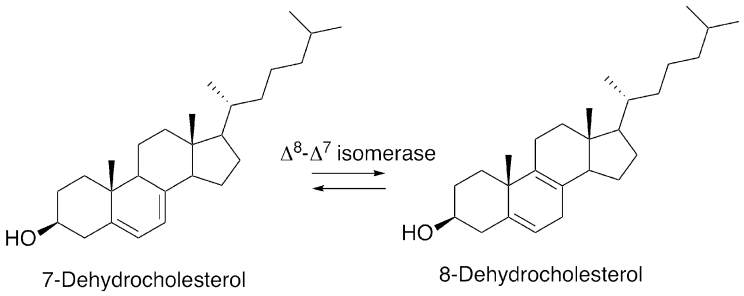

Fig. 5 Interconversion of 7-dehydrocholesterol and 8-dehydrocholesterol by Δ^8–Δ^7 isomerase [21]

Kandutsch–Russell pathway (Fig. 1). Both 7-dehydrocholesterol and lathosterol were oxidized to their 7-keto products by P450 7A1, as well as to the (α-) epoxides. The epoxides are relatively stable, and they were shown not to degrade to the 7-keto

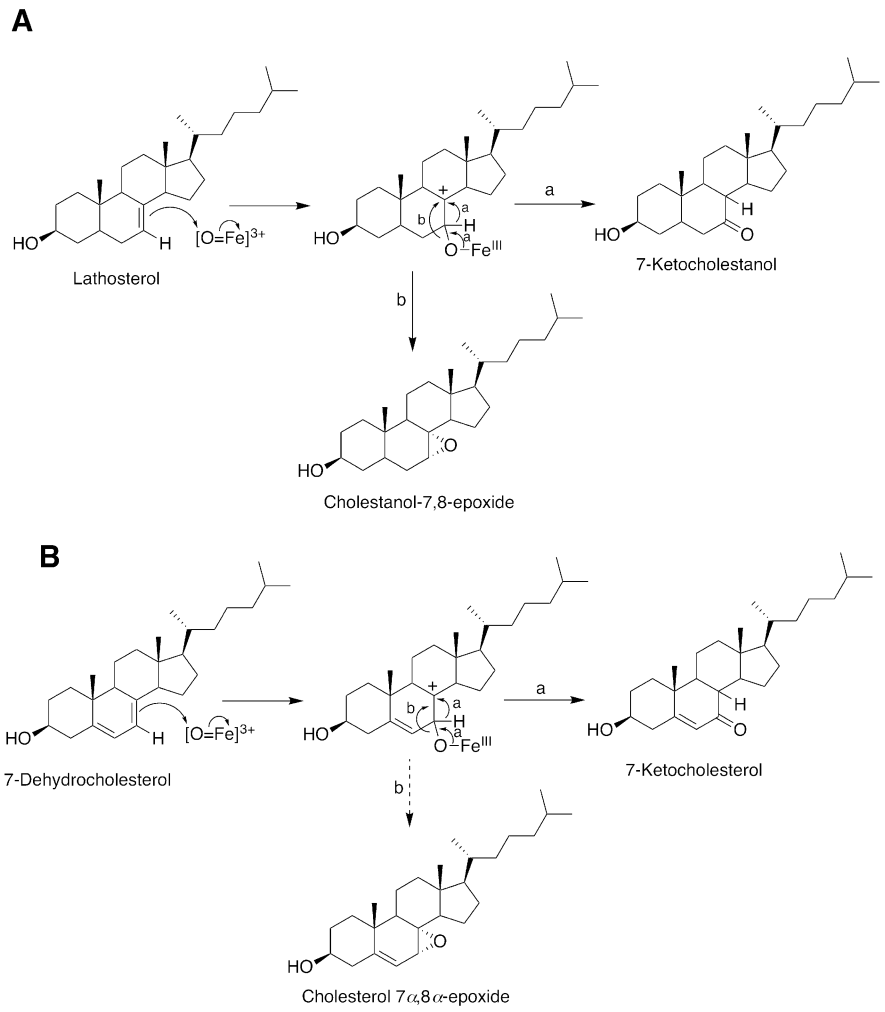

Fig. 6 Oxidation of lathosterol and 7-dehydrocholesterol by P450 7A1 [22]

products under physiological conditions [22]. With lathosterol as the substrate, the product balance was slightly in favor of the epoxides. With 7-dehydrocholesterol, the balance was in favor of the formation of the 7-keto product over epoxide, ~9:1 [22].

The epoxides did not rearrange to the 7-keto products and we proposed a mechanism involving a carbocation intermediate, with a 7,8-hydride shift (Fig. 6). In support of this proposal, deuterium from [7-d_2] 7-dehydrocholesterol was retained in the product 7-ketocholesterol, consistent only with hydrogen migration [22]. Mechanistically, this hydride shift is equivalent to the process reported earlier for the oxidation of vinyl monomers by rat P450 2B1 [23, 24].

Some patients with cerebrotendinous xanthomatosis (CTX) and Smith–Lemli–Opitz syndrome (SLOS) have high levels of 7-dehydrocholesterol, resulting from the DHCR7 enzyme deficiency [25, 26]. Two patients with CTX were found to have elevated levels of 7-dehydrocholesterol and 7-ketocholesterol, as well as significant levels of cholesterol-7,8-epoxide [26]. No epoxide was detected in plasma of healthy volunteers. Treatment with chenodeoxycholic acid (to downregulate P450 7A1) reduced the levels of 7-dehydrocholesterol, 7-ketocholesterol, and the 7,8-epoxide [26]. Patients with SLOS were also found to have elevated levels of 7-ketocholesterol and cholesterol 7,8-epoxide. The results support the view that 7-dehydrocholesterol is the precursor of 7-ketocholesterol and cholesterol 7,8-epoxide in vivo, presumably via the action of P450 7A1. Although causal roles of these oxysterols in CTX and SLOS are not firmly established, these are likely to be some of the problematic oxysterols.

Cholesterol 5α, 6α-Epoxide

Watabe and Sawahata [27] reported that bovine adrenal cortex homogenates oxidized cholesterol to its α-epoxide (5α,6α-epoxychoestan-3β-ol), which is then hydrolyzed by an epoxide hydrolase [27, 28] to cholestane-3β,5α,6β-triol (Fig. 7). The reaction was supported by NADPH and had other characteristics of a P450 reaction, but a specific P450 has not been identified. No β epoxide was detected [27]. Cholesterol epoxides are direct mutagens and are considered to be cytotoxic and potentially carcinogenic [29–31].

P450 46A1 and the Origin of 24- and 25-Hydroxy 7-Dehydrocholesterol

24*S*-Hydroxycholesterol, formed by P450 46A1 in brain, is an important oxysterol that regulates cholesterol homeostasis through interaction with LXRs and the SREBP2 pathway [32]. 24-Hydroxy-7-dehydrocholesterol has been identified in brain tissue of a rat model for SLOS, which is related to a defect in the gene

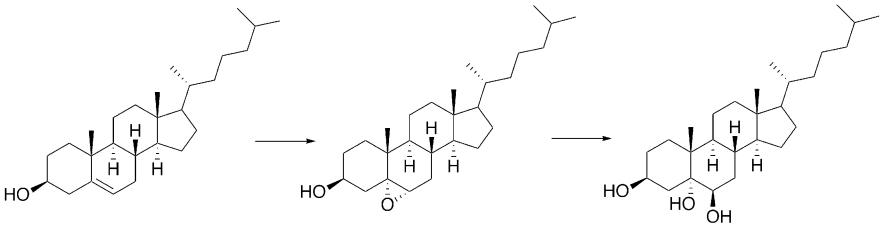

Fig. 7 Oxidation of cholesterol to its α-epoxide, followed by enzymatic hydrolysis [27]

(*DHCR7*) that reduces 7-dehydrocholesterol to cholesterol (Fig. 1). However, previous work with (human) P450 46A1 reported that 7-dehydrocholesterol was not a substrate for recombinant human P450 46A1 [33]. However, it seemed less likely that 24-hydroxy-7-dehydrocholesterol would be formed by the desaturation of 24-hydroxycholesterol, and we reinvestigated the issue [34].

P450 46A1 was found to bind cholesterol, 7-dehydrocholesterol, and desmosterol (Fig. 1) with μM K_d values [34]. P450 46A1 oxidized 7-dehydrocholesterol to 24- and 25-hydroxy-7-dehydrocholesterol (Fig. 8a). Desmosterol was oxidized to 24*S*,25-epoxycholesterol and 27-hydroxydesmosterol. These oxysterols may be of relevance to SLOS.

Although the rate of 24-hydroxylation of desmosterol is only ~0.1 min^{-1} [34] this is similar to that measured with cholesterol as substrate. Even with cholesterol as substrate, the catalytic efficiency (k_{cat}/K_m) is only ~140 M^{-1} s^{-1} [34]. Despite the low efficiency, the enzyme has an important role, in that *Cyp46a1*$^{-/-}$ mice have learning defects [35] and the enzyme is a potential issue in Alzheimer's disease [36].

In support of our findings on in vitro activities of (human) P450, it has been reported that brain levels of 24*S*,25-epoxycholesterol in *Cyp46a1*$^{-/-}$ mice are only 20% of wild-type (mice) [37]. This epoxide is an inhibitor of 3β-hydroxysterol-Δ^{24}-reductase (DHR24, Fig. 1) and leads to the accumulation of desmosterol in cultured cells [38].

It should also be pointed out that P450 46A1 is inhibited by several drugs, can oxidize several drugs, and is also stimulated by certain drugs, e.g., efavarinz [13, 39, 40] (Fig. 8b). The rates of oxidation of several of these drugs are higher than the rates of the sterols mentioned above, although the contributions to the overall metabolism of any of these drugs in vivo are unknown (this could be established with the *Cyp46a1*$^{-/-}$ mice). However, the drug efavarinz has also been reported to stimulate the oxidation of cholesterol in vivo (mice) [41].

Contributions of P450s 7A1, 11A1, 27A1, and 46A1 to Cholesterol Homeostasis

In the course of a collaborative study with Prof. Damjana Rozman on modeling cholesterol homeostasis in mice, we considered the possibility that the four (human) P450s that oxidize cholesterol might also act on cholesterol precursors (Fig. 1) and thereby influence the homeostatic balance [4]. Previous studies had already revealed new reactions of two of these P450s (7A1 and 46A1) (vide supra). (Some of the cholesterol precursors were not commercially available, and therefore results are limited). The results are summarized in Tables 2 and 3.

In some cases rates could be determined but not in all, due to the lack of available standards. When oxidation occurred on the C-17 side chain, the site of hydroxylation could be established by addition of trimethylsilane functions and fragmentation by gas chromatography-mass spectrometry. However, this strategy is not possible for other sites of hydroxylation (i.e., ring methylenes). In some cases, NMR spectra were used to define structures, but in other cases the amount of highly purified

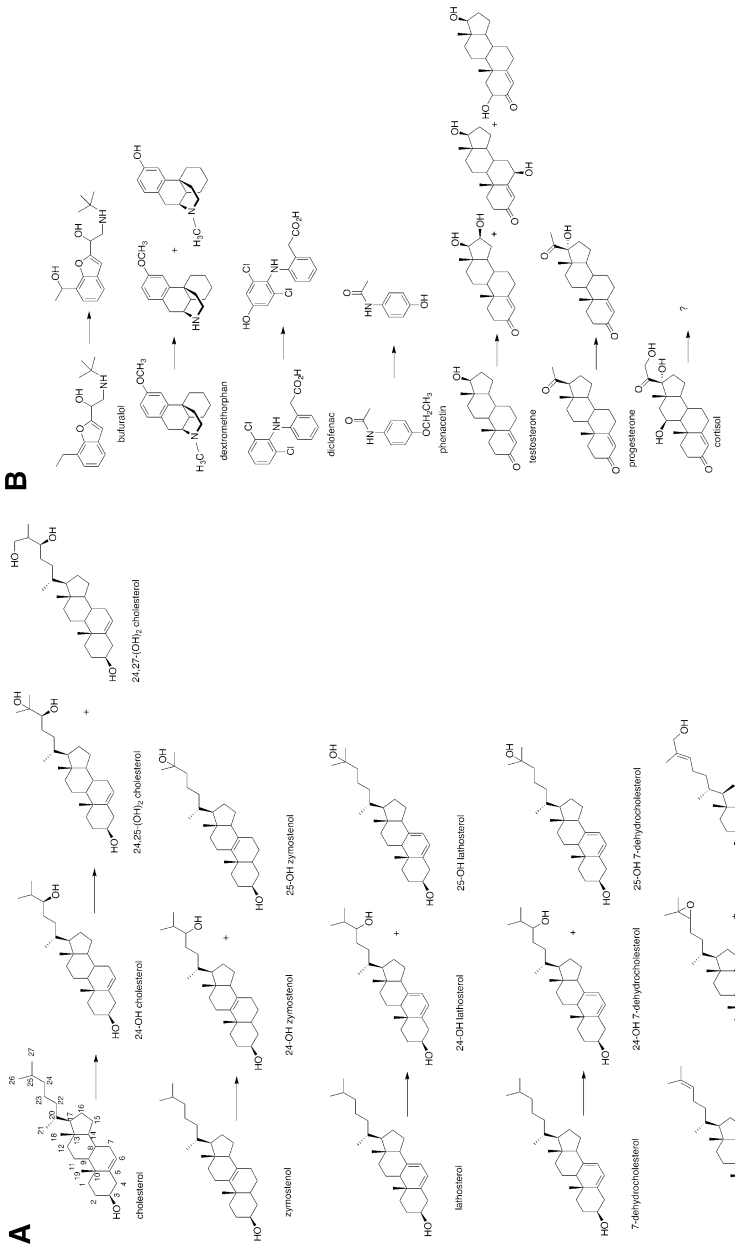

Fig. 8 Oxidations catalyzed by P450 46A1 [4, 12, 13, 34]. (**a**) Sterols. (**b**) Other steroids and drugs

Table 2 Summary of enzymatic reactions with cholesterol-oxidizing P450s and various sterols [4, 22, 34] (see Figs. 1, 6, 8, and 9)

P450	Sterols	Products
P450 7A1	Zymostenol	(Not determined)
	Lathosterol	7-ketocholestanol cholesterol-7α,8α-epoxide
		Cholestanol-7α,8α-epoxide
	7-dehydrocholesterol	7-ketocholesterol
	Desmosterol	7α-hydroxydesmosterol
	Cholesterol	7α-hydroxycholesterol
P450 11A1	Zymostenol	(Not determined)
	Lathosterol	(Not determined)
	7-dehydrocholesterol	7-dehydropregnenolone
	Desmosterol	Pregnenolone
	Cholesterol	Pregnenolone
P450 27A1	Zymostenol	25-hydroxyzymostenol
		27-hydroxyzymostenol
	Lathosterol	25-hydroxylathosterol
		27-hydroxylathosterol
	7-dehydrocholesterol	25-hydroxy-7-dehydrocholesterol
		27-hydroxy-7-dehydrocholesterol
	Desmosterol	27-hydroxydesmosterol
	Cholesterol	27-hydroxycholesterol
P450 46A1	Zymostenol	24-hydroxyzymostenol
		25-hydroxyzymostenol
	Lathosterol	24-hydroxylathosterol
		25-hydroxylathosterol
	7-dehydrocholesterol	24-hydroxy-7-dehydrocholesterol
		25-hydroxy-7-dehydrocholesterol
	Desmosterol	24*S*,25-epoxycholesterol
		27-hydroxydesmosterol
	Cholesterol	24*S*-hydroxycholesterol

material was not sufficient to obtain spectra. Thus, several sterol oxidation products still remain uncharacterized (Tables 2 and 3).

Of the P450s that normally oxidize cholesterol (7A1, 11A1, 27A1, 46A1—not considering 3A4, vide infra), P450 11A1 had seemed to be the most selective in its requirements, and only cholesterol, desmosterol, and 7-dehydrocholesterol were found to be substrates (Tables 2 and 3). The other three P450s oxidized a variety of sterols. However, this inventory would present a misleading picture of P450 11A1, in that it can oxidize ergosterol and lumisterol 3 (Fig. 9), a number of vitamin D-related secosteroids (Fig. 10), and even a rather different-looking drug candidate (Fig. 11). A number of drugs are also reported to inhibit and stimulate P450 11A1 [46].

Table 3 Metabolism of sterol intermediates from the Bloch and Kandutsch–Russell pathways (Fig. 1) by P450 enzymes that are known to metabolize cholesterol [4]

Bloch pathway	P450 7A1	P450 11A1	P450 27A1	P450 46A1
Lanosterol			15%	
FF-MAS	1%		5%	5%
T-MAS	5%		50%	1%
Zymosterol	65%		75%	4%
7-Dehydrodesmosterol	25%		83%	9%
Desmosterol	+	+[a]	+	+[b]
Kandutsch–Russell pathway	P450 7A1	P450 11A1	P450 27A1	P450 46A1
Dihydrolanosterol			16%	
Zymostenol	45%		+	+
Lathosterol	+[c]		+	+
7-Dehydrocholesterol	+[d]	+	+	+[e]

For percent conversion, each P450 was present (in a reconstituted system with either NADPH-P450 reductase (7A1, 27A1, 46A1) or NADPH-adrenodoxin reductase and andrenodoxin) at 1 μM and the substrate concentration was 10–25 μM; the reaction time was 15 min. Shown is the % conversion of substrates to products that were not identified. The identified products (indicated with a "+" sign) are presented in Table 2. See Figs. 1, 8, and 9

[a]The k_{cat} for formation of pregnenolone was 4.6 ± 0.2 min^{-1}, K_m 1.2 ± 0.4 μM [4]

[b]The k_{cat} for formation of 24*S*,25-epoxycholesterol was 0.033 ± 0.001 min^{-1}, K_m 2.2 ± 0.3 μM [34]. The k_{cat} for formation of 27-hydroxydesmosterol was ~0.044 min^{-1} [34]

[c]The k_{cat} for formation of 7-ketocholestenol was 3.7 ± 0.2 min^{-1}, K_m 1.8 ± 0.3 μM; k_{cat} for formation of 7α,8α-epoxycholestenol was 7.1 ± 0.4 min^{-1}, K_m 2.1 ± 0.3 μM [22]

[d]The k_{cat} for formation of 7-ketocholesterol was 2.2 ± 0.1 min^{-1}, K_m 1.1 ± 0.1 μM [22]

[e]The k_{cat} for formation of 24-hydroxy-7-dehydrocholesterol was 0.024 ± 0.001 min^{-1}, K_m 0.24 ± 0.01 μM and the k_{cat} for formation of 25-hydroxy-7-dehydrocholesterol was ~0.11 min^{-1} [34]

P450 27A1 is also not very selective in that it oxidizes both sterols (Fig. 3, Tables 2 and 3) and some vitamin D secosteroids (Fig. 10). It is of interest that a major enzyme product, 27-hydroxycholesterol, has been studied in the context of a link between obesity, hypercholestemia, and breast cancer [47]. In our own studies on the related P450 27C1, a vitamin A_1 desaturase, we also found traces of this activity toward vitamin A_1 with P450 27A1 [48].

Consideration of some of the P450 reactions (Tables 2 and 3) allowed for refinement of a metabolic model that was consistent with the observed levels of cholesterol in mice [4].

Other Oxidation Products of Cholesterol

Cholesterol is known to be hydroxylated at the 4β position by P450 3A4 [49, 50]. The reaction is slow ($k_{cat}/K_m \sim 7\ M^{-1}\ s^{-1}$) but very real, and several efforts have been made to utilize levels of 4β-hydroxycholesterol as a "biomarker" or "noninvasive" measure of P450 3A4 [51–53].

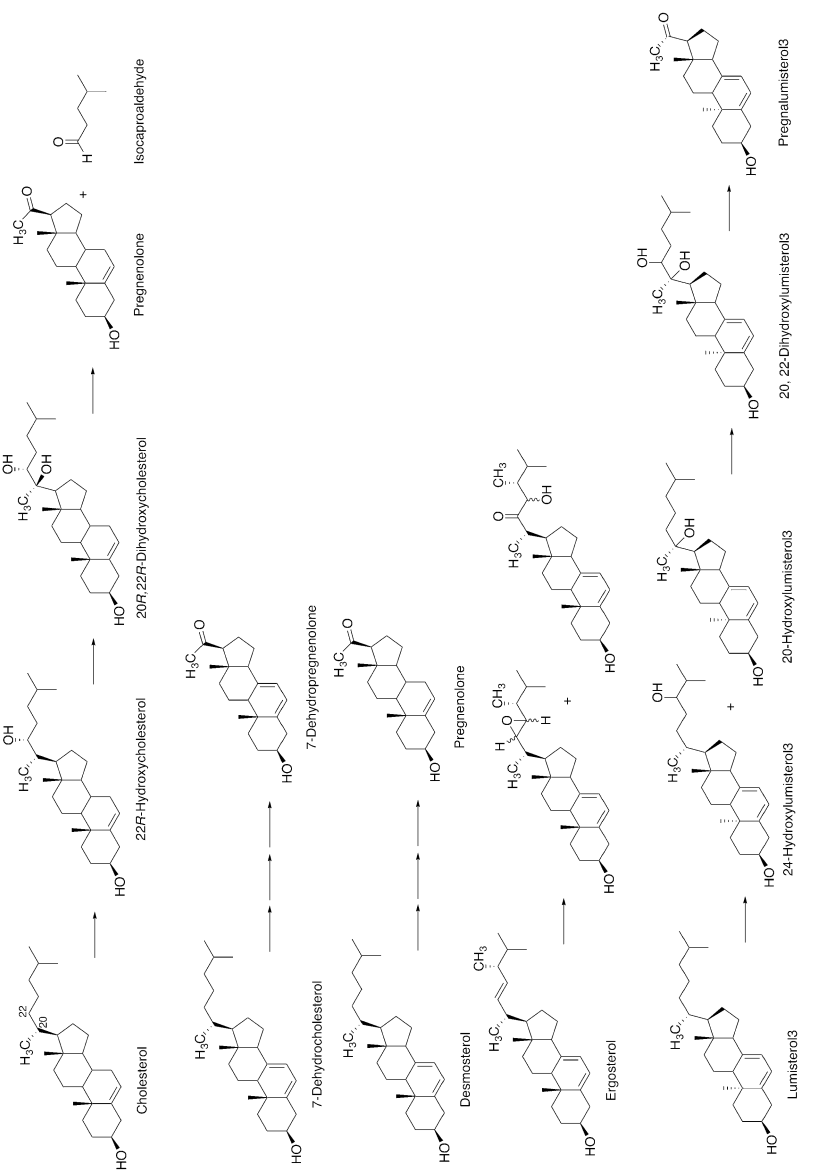

Fig. 9 Oxidation of sterols catalyzed by P450 11A1 [4, 42]

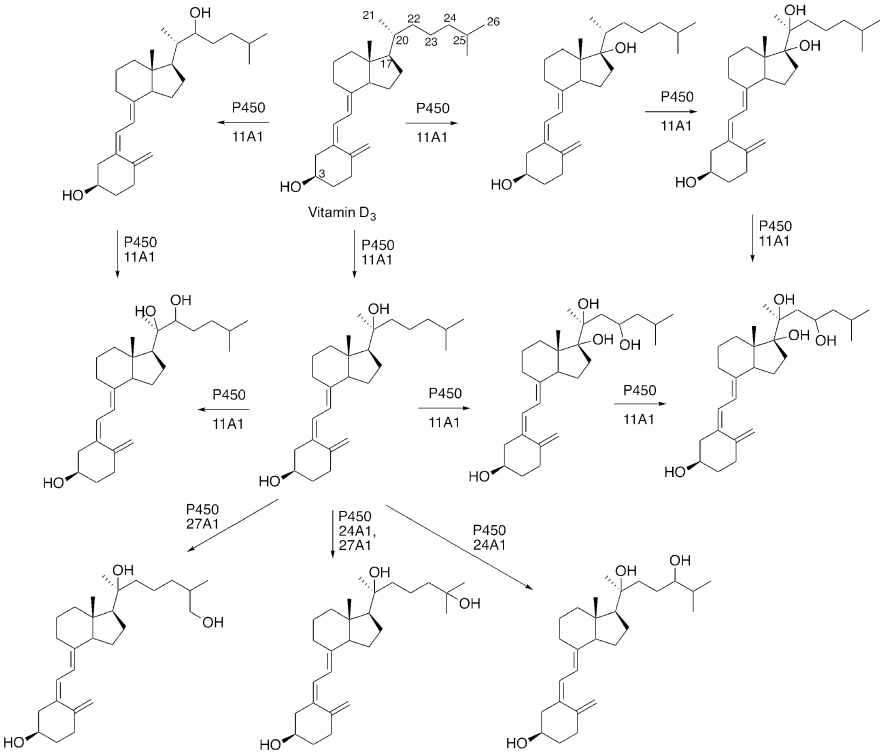

Fig. 10 Oxidation of Vitamin D secosteroids by P450 11A1 [43, 44]

4β-Hydroxycholesterol can be further oxidized to other sterols (Fig. 12). P450 7A1 catalyzes 7α-hydroxylation, at a rate described as slower than for cholesterol but not quantified [49]. P450s 7B1 and 39A1 did not catalyze the reaction. It is unclear as to whether 4β,7α-dihydroxycholesterol is further oxidized to a 4β-hydroxy bile acid. P450 27A1 oxidizes 4β-hydroxycholesterol to 4β,27-dihydroxycholesterol and then to 3β,4β-dihydroxy-5′-cholestenoic acid (Fig. 12). P450 46A1 oxidizes 4β-hydroxycholesterol to 4β-24-dihydroxycholesterol (Fig. 12) [49].

If 4β-hydroxycholesterol is a product of P450 3A4, then cholesterol is a substrate of the enzyme. The level of total cholesterol in the liver and brain is very high, although much is not free (~5 mM in serum, calculated 560 μM in liver [50]). If cholesterol is a substrate of P450 3A4, then it follows that it should also be an inhibitor. Inhibition of P450 3A4-mediated drug oxidations could be shown with the purified enzyme and in human hepatocytes, although there were technical problems, largely associated with the insoluble nature of cholesterol [50]. The in vitro steady-state kinetics appeared to be noncompetitive, but an explanation for this has been provided [50]. Other oxidation products of cholesterol and 7-dehydrocholesterol are

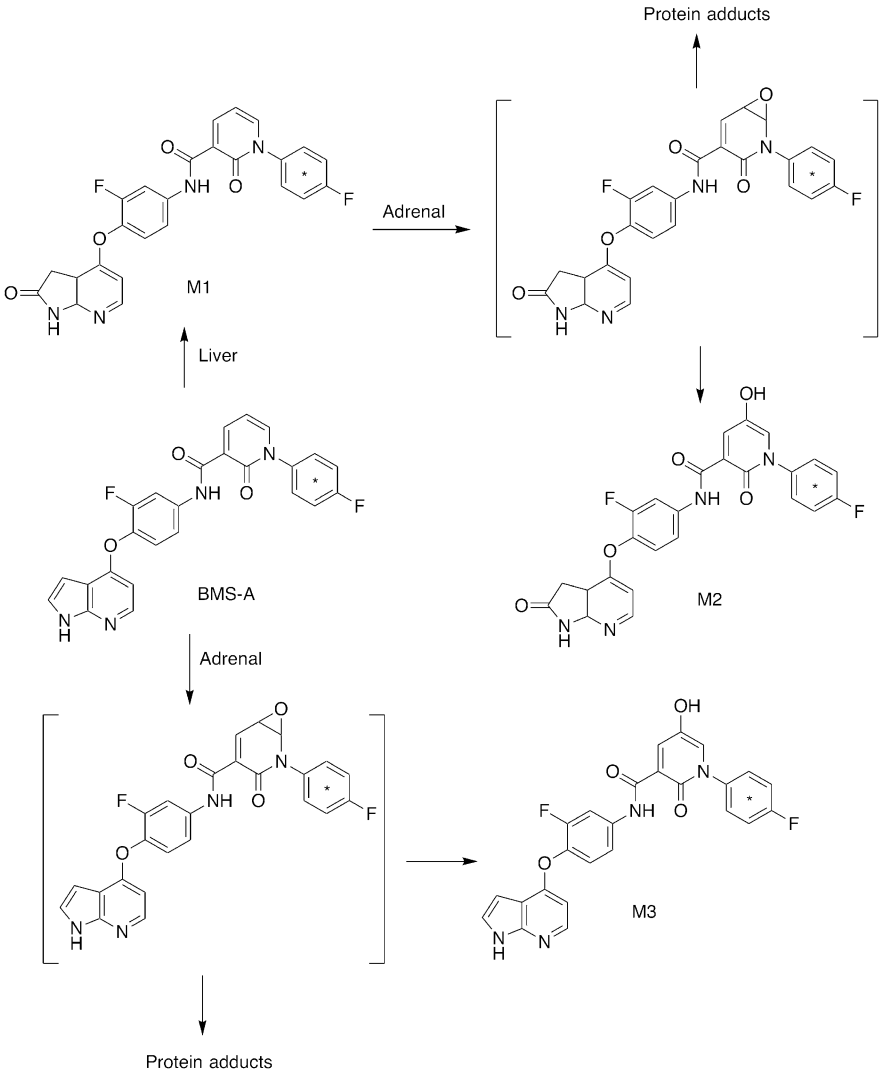

Fig. 11 Bioactivation of an experimental drug (BMS-A) by P450 11A1 [45]. The asterisk indicates the site of radioactive label in the study

known. Some of these are probably the result of lipid peroxidation and possibly other radical processes [21, 54, 55].

A variety of oxidized products (≥15) can be generated from treatment of cholesterol and 7-dehydrocholesterol with radicals [21, 55], and some of these compounds are found in cells [56]. However, a set of 7-dehydrocholesterol-derived oxysterols found in *Dhcr7*-deficient Neuro2a cells and SLOS human fibroblasts are attributed to metabolic processes, not nonenzymatic radical processes (Fig. 13) [54]. Some of

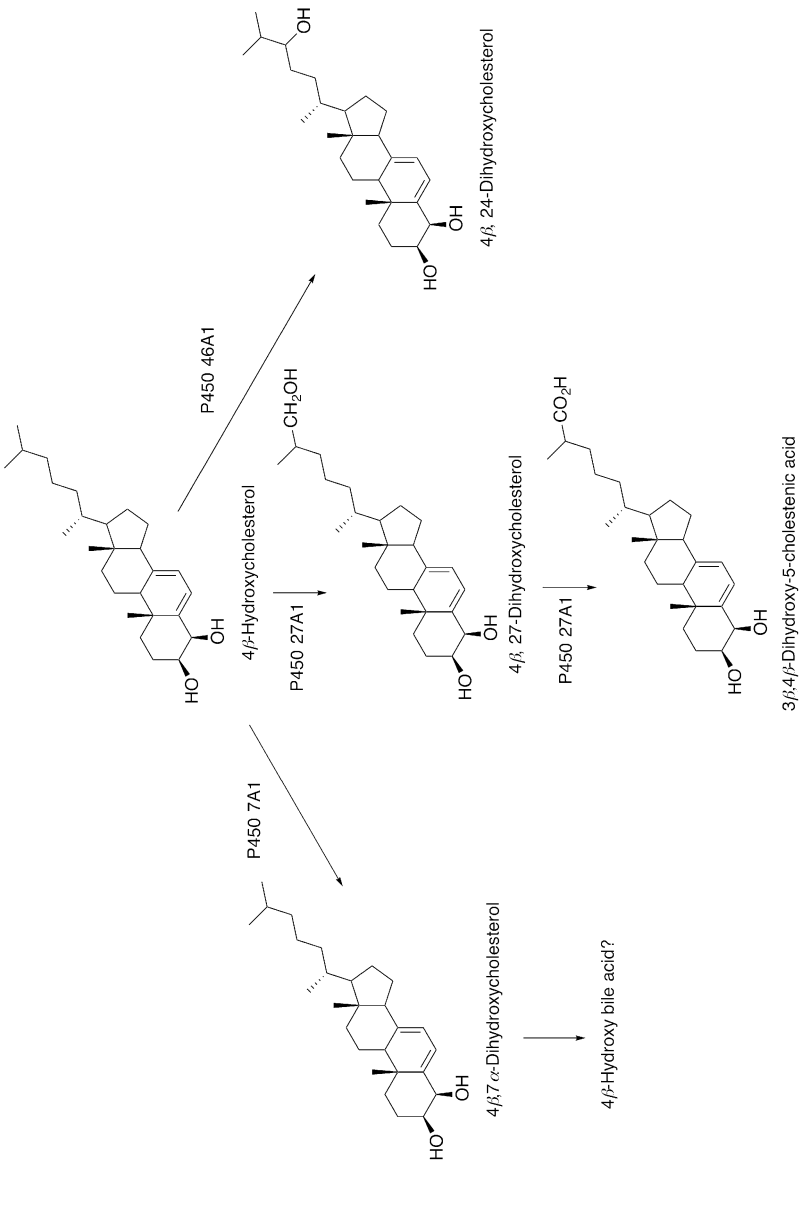

Fig. 12 Oxidations of 4β-hydroxycholesterol [49]

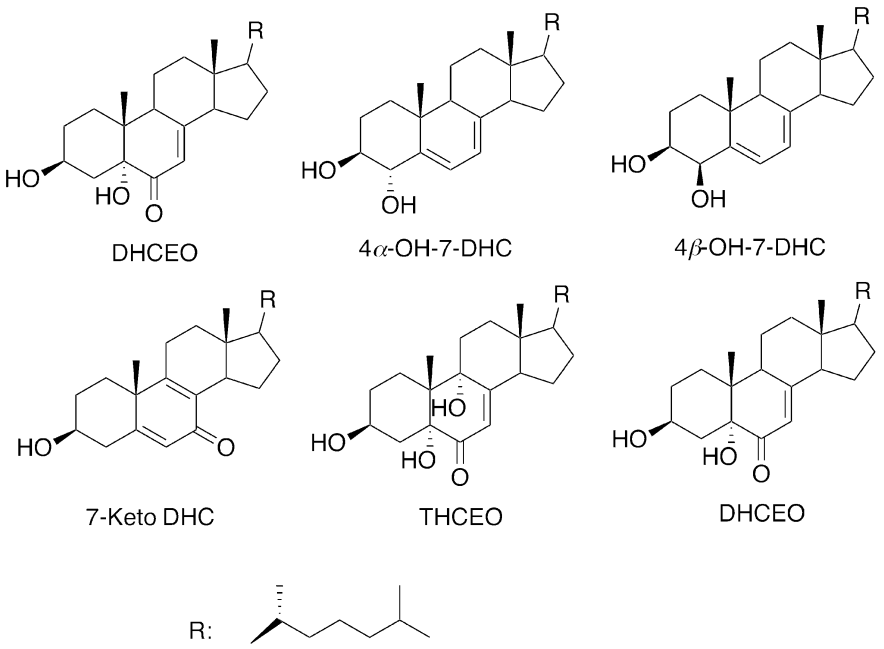

Fig. 13 Oxysterols derived from 7-dehydrocholesterol and identified in *Dhcr7*-deficient cells and/or SLOS human fibroblasts [54]. DHCEO 3β,5α-dihydroxycholest-7-en-6-one, 4α-OH-7DHC 4α-hydroxy-7-dehydrocholesterol, 4β-OH-7-DHC 4β-hydroxy-7-dehydrocholesterol, 7-keto DHC 7-ketocholestra-5,8-dien-3β-ol, THCEO 3β,5α,9α-trihydroxyholest-7-en-6-one, DHCEO 3β,5α-dihydroxycholes-7-en-6-one

these may be derived from the radical products [21], in some cases by reduction reactions [21]. While the origin of these six compounds (Fig. 13) is yet unknown, it is very likely that some of these arise by P450 oxidations, e.g., 4β-hydroxylation of 7-dehydrocholesterol (Fig. 12). 7-Dehydrocholesterol interconverts with 8-dehydrocholesterol through the action of Δ^8–Δ^7 isomerase (Fig. 5), and 7-hydroxylation of this could occur (followed by dehydrogenation). However, the involvement of P450s in the formation of these compounds remains only speculatory. One of the technical problems with doing incubations with microsomes is that the membranes contain not only high concentrations of cholesterol but also many of the minor sterols, requiring the use of radioactive or heavy-isotope substrates [22]. Nevertheless, many of these oxysterols have biological activities and are certainly of interest, e.g., in the hedgehog signaling pathway [57].

Many of the oxysterols are still uncharacterized, and relatively few well-defined biological functions have been elucidated. 25-Hydroxycholesterol has been reported to activate the integrated stress response to program transcription and translation in macrophages [58]. Interestingly the enzyme that does the 25-hydroxylation is a di-iron oxygenase, not a P450 [59].

New Products of Other Steroids Generated by P450s

The side-chain cleavage reaction on cholesterol (or desmosterol) by P450 11A1 (Figs. 4 and 9) results in the key product pregnenolone, which goes on to produce a variety of hormones, mineralocorticoids, glucocorticoids, etc. Several new oxidation products have been identified in some of these reactions, although any biological activities of these are yet unidentified.

P450 19A1 is normally considered to have three (androgenic) major substrates: testosterone, androstenedione, and 16α-hydroxytestosterone [60]. These are oxidized to 17β-estradiol, estrone, and estriol, respectively. 5α-Dihydrotestosterone is a potent androgen, derived from the reduction of testosterone by steroid 5α-reductase. (This enzyme will also reduce other 3-keto Δ^4 steroids.) A deficiency of the enzyme is associated with a lack of masculine features that develop during puberty [61], and for the same biological reasons the enzyme has been a target for drugs used to treat benign prostate hypertrophy (e.g., finasteride, dutasteride) [62, 63].

5α-Dihydrotestosterone is oxidized by at least two human P450s. Oxidation by P450 19A1 follows a course similar to the 3-step 19-demethylation course observed for the classic three androgenic substrates. However, after the last step, the product differs in that it is formally two electrons more reduced than an estrogen, and therefore, aromatic products are not obtained (Fig. 14) [64]. Further P450 19A1 oxidation of the (major) "Δ1,10" product yields a 2-hydroxy product, but apparently never an estrogen (Fig. 14).

Liver microsomes oxidize 5α-dihydrotestosterone [64]. Some of the same products produced by P450 19A1 were found (Fig. 15), and these were attributed to the small amount of P450 19A1 present in liver [64, 66, 67]. However, the major products of 5α-dihydrotestosterone were produced by P450 3A4 and were characterized as the 18- and 19-hydroxy products, easily recognizable by the loss of the distinct methyl singlets in their ^{1}H NMR spectra. These two hydroxylated products were also identified in human plasma and urine samples [64]. The discovery of the 18- and 19-hydroxy products is somewhat remarkable, in that (1) hydroxylation of a methyl group is energetically less favorable than a methylene and (2) the same enzyme oxidizes testosterone (only differing in the electronics and pucker of the A ring) primarily at the 6β position.

P450 19A1 oxidizes androgens to estrogens, as already mentioned. In our reconsideration of the catalytic mechanism of this enzyme, we established that the active oxygenating species is a perferryl oxygen ("Compound I," FeO^{3+}) not a ferric peroxide (FeO_2^-) [65]. Also in that work we identified a new reaction product, 19-carboxyandrostenedione (Fig. 14) [65], which had been seen only in hog follicles previously [68] (19-carboxytestosterone was also formed from testosterone). A mechanism is proposed in which the third step of the P450 19A1 reaction leads to either abstraction of a hydrogen atom from the 1β- or 19-position, yielding either an estrogen or 19-carboxy androgen, respectively [65]. Subsequent work in this laboratory has shown that human P450 19A1 and the three hog P450 19A enzymes (19A1, 19A2, 19A3) differ in their balance of the estrogen and 19-carboxy androgen

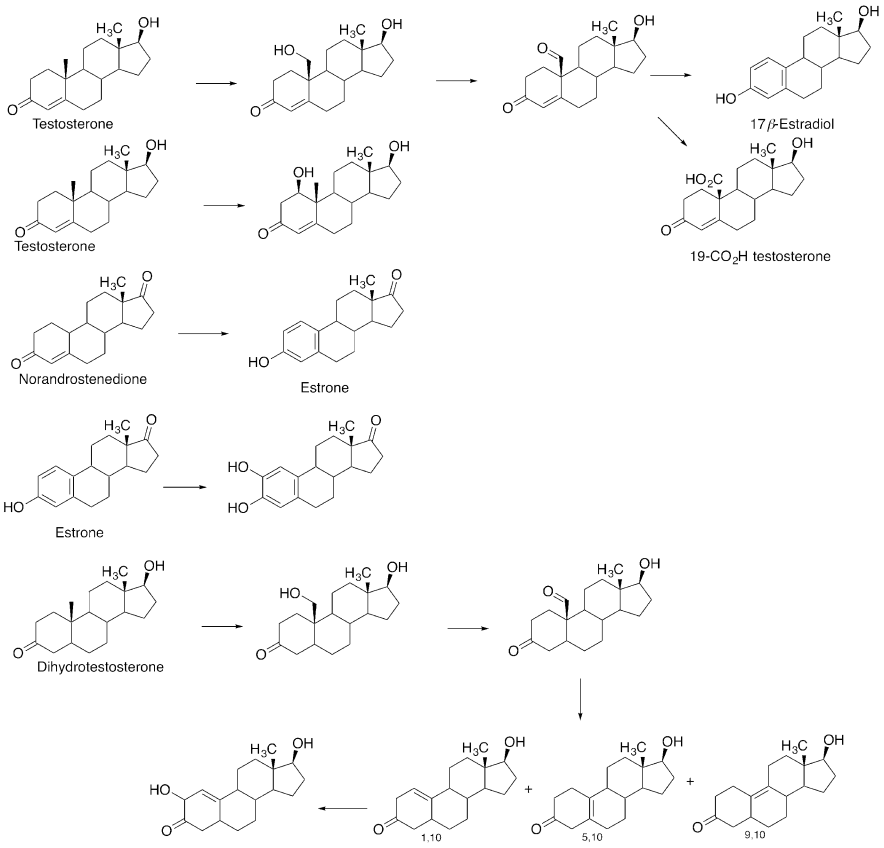

Fig. 14 Oxidation of steroids catalyzed by P450 19A1 [64, 65]

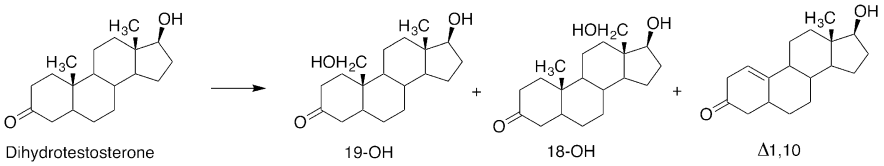

Fig. 15 Oxidations of 5α-dihydrotestosterone catalyzed by P450s 19A1 and 3A4 [64]

products. Some of the hog liver P450 19A enzymes also convert androgens to 1β-hydroxy products, which are apparently terminal [69]. Some amino acid residues controlling the balance of products have been identified by site-directed mutagenesis (Yoshimoto, Reddish, and Guengerich, unpublished results).

Overall, P450 19A1 catalyzes a number of reactions (Fig. 14). Some of these have been tested for possible biological activity, but the range of assays done to date is limited.

Another (human) enzyme that has revealed new steroid reaction products is P450 17A1 (Fig. 4). This is a complex enzyme and has been known to catalyze a 2-step reaction: progesterone is hydroxylated at the 17α position and then a lyase reaction cleaves between carbons 17 and 20 to yield androstenedione; pregnenolone undergoes the same course of events to generate dehydroepiandrosterone (Fig. 4). Our own work indicates that the two reaction steps are relatively distributive (as opposed to being processive), with only a fraction of either of the 17α-hydroxy steroids remaining on the enzyme and not equilibrating with the medium. An interesting point is that cytochrome b_5 stimulates the enzyme, reportedly the second step more than the first [70], although we have found that both steps are stimulated in our own studies.

P450 17A1 has now been shown to catalyze a plethora of oxidations (Fig. 16). The 16α- and 21-hydroxylations of progesterone and the conversion of pregnenolone to the 16,17-ene product were already recognized [71]. We also characterized several new products of the 17α-hydroxy steroids, including 16α,17α-dihydroxyprogesterone, 6β,16α,17α-trihydroxyprogesterone, 16-hydroxyandrostenedione, 16,17α-dihydroxypregnenolone, 16-hydroxydihydroepiandosterone, and a product of 16,17α-dihydroxypregnenolone with an additional oxygen in the B ring, suggested to be either a 7-hydroxy group or possibly a 5,6-epoxide (Fig. 16) (stereochemistry of 16-hydroxylation not established). Rates of formation of these new products were measured at high substrate concentrations but catalytic efficiency has not been determined [72]. The extent to which any of these products accumulate in tissues is yet unknown, as is any biological function, with the exception of 16-hydroxyandrostenedione, which is known to be converted by P450 19A1 to estriol, an abundant and characteristic estrogen during human pregnancy [73].

The formation of the oxygenated products of the 17α-hydroxy steroids (Fig. 16) is of interest not only in regard to any possible new biological functions of the products but also in regard to catalytic mechanism. One hypothesis proposed to explain the 17α,20-lyase reaction observed with P450 17A1 has been that the first step (17α-hydroxylation) involves a "classical" Compound I (FeO^{3+}) reaction but that a ferric peroxide (FeO_2^-) reaction is involved in the lyase reaction with the 17α-hydroxy substrates [74–76]. However, we clearly demonstrated that the 17α-hydroxy steroids are capable of undergoing simple oxygenations as well, probably catalyzed via P450 17A1 Compound I pathways. Further, the biomimetic oxygen surrogate iodosylbenzene, which cannot produce the ferric peroxide complex (FeO_2^-), supported all of the reactions, including the lyase reactions [72]. As we have pointed out [72], we cannot establish how much of the lyase reaction should be attributed to the Compound I versus a ferric peroxide mechanism under normal conditions (and other techniques have not addressed this question either). However, it is now established that a Compound I mechanism can be involved in the lyase reaction as well as the hydroxylations [72].

Furthermore, P450 17A1 has been shown to epoxidize the 16,17-position of $\Delta^{16,17}$-pregnenolone and $\Delta^{16,17}$-progesterone. Interestingly, $\Delta^{16,17}$-progesterone has been identified in human feces [77]. This product may potentially arise from the elimination of 16α-hydroxyprogesterone, one of the oxidation products of P450

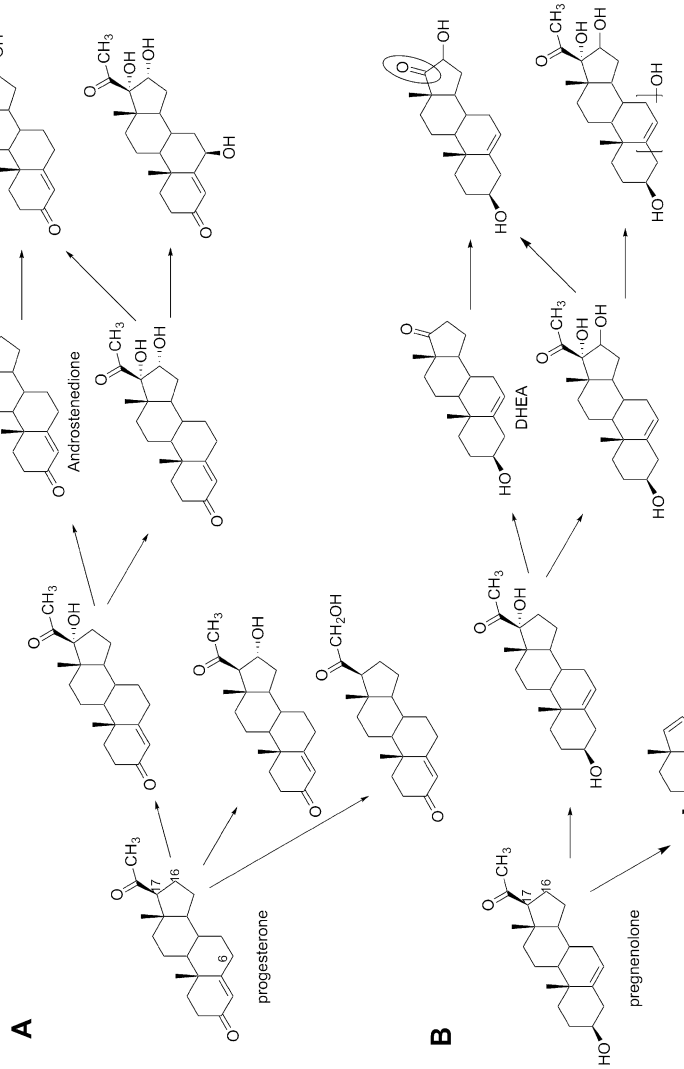

Fig. 16 Oxidations of steroids catalyzed by human P450 17A1 [71, 72]

17A1 from progesterone, which is potentially a substrate of a 16α-dehydratase enzyme found in gut microbiota [78]. Alternatively, a direct desaturation is possible. In addition to the epoxidation activity, P450 17A1 was found to significantly oxygenate the 21-position of both substrates to afford 21-hydroxy-$\Delta^{16,17}$-pregnenolone and progesterone. The 21-hydroxylation products were enhanced (over the epoxidation product) with the site-directed mutant P450 17A1 A105L. The Ala-105 residue, which corresponds to a leucine residue in other species, has been shown to be responsible for conferring enhanced 16α-hydroxylation activity in human P450 17A1. The reason for the switch in regioselectivity between the wild-type and site-directed mutant has been attributed to a hydrophobic interaction between the B′-helix and the F–G loop of the protein [71].

Conclusions and Future Directions

The classic pathways of sterol and steroid metabolism are relatively simple and have been very useful in considering normal homeostasis and disease states. The knowledge of additional reaction products has been possible largely because of the availability of recombinant enzymes for study and particularly new advances in analytical chemistry, particularly HPLC/UPLC, mass spectrometry, and NMR spectroscopy.

One of the conclusions is that the P450s involved in the metabolism of endogenous substrates are not as selective as they were once thought to be. All of these P450s can probably be targeted with drugs that bind in their active sites [2, 79], and some of these (e.g., 11A1, 46A1) will even catalyze oxidations of xenobiotic chemicals (Figs. 8 and 11) [12, 13, 45]. In many of the cases examined here, analogues of the classical substrates—including those occurring in metabolic pathways—are often substrates, usually with lower catalytic efficiency (Tables 2 and 3). In some cases [4], consideration of these minor pathways can help develop better models of metabolic flux.

There are two major areas for further investigation. One is more extensive analysis of the minor pathways such as those we have described here. In principle, this is very feasible and only involves pushing the existing technology further, i.e., using more enzyme and improving separations and the sensitivity of spectroscopic methods. A point to be made is that all of the studies cited from our own laboratory were done in the past 5 years (2011–2016) [4, 22, 26, 34, 64, 65, 72], and there is certainly an opportunity to do more. Many of the P450s under the “Steroids” column in Table 1 remain to be further interrogated. We have also mentioned that there are still many known oxysterols whose origin is unexplained, e.g., Fig. 13.

The other area for future research is more difficult, that is, the elucidation of function of the newly characterized oxidation products. One issue is the synthesis of these compounds in amounts substantial enough for testing, but that is certainly feasible through organic synthesis, although not trivial in some cases. The more difficult task comes in defining any biological functions. Exactly how to best do this is not clear. Using transgenic mice (e.g., knockout of a particular P450) is not

particularly useful in that many of the steroid-metabolizing P450 knockouts are lethal [2, 60] and eliminating a P450 eliminates both the major and minor metabolites. Realistically, a tiered strategy for addressing the question of function could be applied, involving experiments with (1) enzymatic or receptor activities of the main product (e.g., binding to a particular receptor, agonist activity), (2) cell culture, with either a readout related to hypothesized activity or, preferably, concentration-dependent transcriptomic profiling, and (3) animal studies. A caveat of the latter in vivo work is potential issues with routes of administration and metabolism, as well as species differences. Although these procedures require considerable resources and may be daunting, approaches such as these are the only path to progress. Another consideration, already in play with some of the oxysterols, is the relationship of concentrations of these with disease states in an animal model or in the clinic [26]. However, in such cases multiple compounds may change concentrations and even if a single one predominates, it may not be causal in its effects.

In summary, there is still more to be learned about new sterols and other steroids. Metabolism is still a developing field, even after a productive century of biochemistry.

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