Sulfation of 25-hydroxycholesterol regulates lipid metabolism, inflammatory responses, and cell proliferation

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INTRACELLULAR LIPID ACCUMULATION, inflammatory responses, and apoptosis are the major pathogenic events of many metabolic disorders, including atherosclerosis and nonalcoholic fatty liver diseases (NAFLD) (25, 42). Increasing evidence shows that nuclear receptors play critical roles in the regulation of lipid metabolism and inflammatory responses (11, 79). Nuclear receptors are ligand-activated transcription factors that affect processes as diverse as reproduction, development, inflammation, and general metabolism through regulating target gene expression (20). Their natural ligands, such as orphan nuclear receptors, remain unknown in many cases. Many nuclear receptors function as sensors of cellular cholesterol and lipid levels and elicit gene expression changes that maintain lipid homeostasis and protect cells from lipid overload (77). Examples include receptors for fatty acids (peroxisome proliferation-activated receptors, PPARs) (51, 91), oxysterols (liver X receptors, LXRs), bile acids (farnesoid X receptor, FXR), and retinoic acids (retinoic acid receptors, RXRs) (56).

There have been many efforts in recent years to understand the coordination of nuclear-receptor activities. Oxysterol sulfation as a regulatory pathway has grown out of recent studies in the past seven years, including discovery of a novel oxysterol sulfate, identification of a key enzyme hydroxysterol sulfotransferase 2B1b (SULT2B1b) involved in oxysterol sulfate synthesis, and investigation into the role of oxysterol sulfates in regulation of lipid metabolism, inflammatory responses, and cell proliferation. Ten years ago, our laboratories began investigating the role of intracellular cholesterol transport proteins in the regulation of bile acid synthesis and cholesterol degradation (68, 75). We found that bile acid synthesis via the acidic, “alternative”, pathway was limited by mitochondrial cholesterol uptake. This barrier could be overcome by increasing expression of the intracellular cholesterol transporter StarD1 (68, 73, 75). This suggests a physiological role for StarD1. Increases in StarD1 expression also led to upregulation of biliary cholesterol secretion and downregulation of cholesterol, fatty acid, and triglyceride biosynthesis (63–65, 86). A search for these regulatory effects’ mechanisms led to the discovery of a novel sulfated oxysterol with potent regulatory properties and, subsequently, a novel regulatory pathway. Functional studies have shown that this regulatory pathway plays a critical role in lipid metabolism, inflamma-
Oxysterol Sulfation as Regulatory Pathway

There are two main pathways involved in cholesterol degradation to primary bile acids in hepatocytes (44, 81). The classic, “neutral” pathway is believed to be the major pathway, at least in humans and rats (67, 89). The first and rate-limiting step of this pathway is the highly regulated microsomal cholesterol 7a-hydroxylase (CYP7A1) (24). The alternative, “acidic” pathway is initiated by mitochondrial sterol 27-hydroxylase (CYP27A1). Bile acids activate nuclear receptors and initiate an overall physiological response that regulates various biosynthetic and metabolic pathways in the liver (44). The acidic pathway generates oxysterol intermediates, such as 25- or 27-hydroxycholesterol (25HC and 27HC), which have been reported to be able to regulate cholesterol metabolism (30, 37). These oxysterols serve as biologically active molecules for regulation of many gene expressions involved in cholesterol biosynthesis and transport (3, 13, 27). Increases in CYP27A1 activity in peripheral tissues downregulate cholesterol synthesis through the sterol regulatory element-binding protein (SREBP) pathway as well as enhance the efflux and elimination of cholesterol via liver X receptor (LXR) (35). Overexpression of CYP27A1 in cultured hepatocytes resulted in minimal increases (~50%) in the rates of bile acid biosynthesis compared with the sevenfold increase seen following overexpression of CYP7A1 (40). Interestingly, increases in mitochondrial cholesterol delivery protein StarD1 expression dramatically increases cholesterol catabolism to bile acids both in cultured primary hepatocytes and in vivo (68, 75). Increases in StarD1 expression in vivo not only increased the rate of bile acid synthesis to the same level as overexpression of CYP7A1 but also produced a similar composition of bile acids in vivo (75). This finding made it possible to study the function of the alternative pathway by manipulating the expression of StarD1 protein. To understand why hepatocytes need two pathways for bile acid biosynthesis, it was hypothesized that the classic pathway is for bile acid synthesis, and the alternative pathway serves as a regulatory pathway: when intracellular cholesterol levels increase, StarD1 would deliver cholesterol to the mitochondria where CYP27A1 is located; the generated regulatory oxysterols would leave the mitochondria and activate or inactivate nuclear receptors, which would enter nuclei for transcriptional regulation. To test this hypothesis, StarD1 was overexpressed in hepatocytes, and nuclear [14C]cholesterol derivatives were characterized. Following the overexpression, water-soluble [14C]oxysterol products in nuclei were isolated and purified by chemical extraction and HPLC. Analysis of the purified [14C]-labeled products with enzymatic digestion, HPLC, and MS-MS identified the water-soluble oxysterol as 5-cholest-3β, 25-diol 3-sulfate (25HC3S). Further experiments detected this cholesterol metabolite in the nuclei of normal human liver tissues, indicating that this novel oxysterol sulfate is a physiological product and that oxysterol sulfation is a physiological reaction (74). Although other oxysterol sulfates have also been found in vivo, 25HC3S has been shown to be the major sulfated oxysterol (data have not been published). Since 25HC is initially synthesized in mitochondria, sulfated in cytosol, and translocated to nuclei, this oxysterol is believed to be a regulatory molecule as a sensor of cholesterol levels.

Assays using mitochondria isolated from wild-type and Cyp27a1 gene knockout mice indicated that 25HC is synthesized by CYP27A1. Incubation of either cholesterol or 25HC with mitochondrial and cytosolic fractions resulted in the synthesis of 25HC3S (53). Real-time RT-PCR and Western blot analysis showed the presence of SULT2B1b in hepatocytes. The expression is highly regulated by insulin (53). 25HC3S, but not 25HC, decreased SULT2B1b mRNA and protein levels, which indicated feedback regulation. Specific siRNA decreased SULT2B1b mRNA, protein, and activity levels and decreased the biosynthesis of 25HC3S. These findings demonstrated that mitochondrial CYP27A1 synthesizes 25HC, which is subsequently 3-sulfated to form 25HC3S by SULT2B1b (53).

Oxysterol Sulfation by SULT2B1b Reduces Intracellular Lipid Levels

SULT2B1b is the key enzyme for the sulfation of 25HC to 25HC3S (36, 53). Recent studies have shown that decreases in intracellular lipid levels by increasing SULT2B1b expression are caused by 25HC sulfation and 25HC3S formation in human aortic endothelial cells and diet-induced NAFLD mouse model (7, 8). Increases in SULT2B1b expression decrease intracellular lipid levels and SREBP-1 expression in the presence of 25HC but has no effect in the absence of 25HC or in the presence of synthetic LXR agonist T0901317. Knockdown studies, which used siRNA-SULT2B1b in HEPG2 cells, have shown that in the presence of 25HC total triglycerides and cholesterol levels increase, along with expression of SREBP-1, fatty acid synthase (FAS), and acetyl-CoA carboxylase-1 (ACC1). These data indicate that decreases in intracellular lipid levels are caused by 25HC sulfation rather than inactivation of oxysterols. The mechanism for decreasing intracellular lipid levels following increasing SULT2B1b expression in the presence of 25HC has been confirmed by determining protein and mRNA expression of the genes involved in lipid metabolism both in vitro and in vivo (7, 8).

Increases in SULT2B1b expression substantially decreased atherosclerotic lesions in an LDLR−/− mouse model. After being fed a high-fat diet for 10 wk, the mice were infected twice with recombinant adenovirus for 6 days. After the feeding, macrophages swollen by the accumulation of cytoplasmic lipid (foam cells) were present in the intimal layer of the aorta. Infiltration of lymphocytes was also found in the atherosclerotic lesions. However, in the SULT2B1b-expressed mice, no foam cells were observed; lymphocytes had also decreased (8). Sulfated oxysterol, 25HC3S, as well as overexpression of SULT2B1b suppress lipid biosynthesis, whereas unsulfated oxysterol 25HC increases lipid synthesis both in vitro and in vivo. This suggests that oxysterol sulfation by SULT2B1b functions as a regulatory pathway for lipid metabolism (18, 71).

Sulfated Oxysterol, 25HC3S, Suppresses Lipid Biosynthesis Via LXR/Srebps Signaling

SREBP-1c controls triglyceride biosynthesis, while SREBP-2 regulates cholesterol biosynthesis. The expression of SREBP-1c is regulated by LXRs (9, 31). Although many oxysterols have been
reported to be LXR ligands and to regulate LXR response gene expression (4, 45, 46, 50, 58, 69, 82), the regulatory mechanism of the gene expression by oxysterols had not been elucidated until a report that overexpression of SULT2B1b inactivated the LXR’s response to multiple oxysterol ligands, indicating that oxysterol sulfation by SULT2B1b is involved in an LXR signaling pathway (22). Recent results have demonstrated that oxysterol sulfation is not a protective mechanism to prevent oxysterol accumulation (2), and other data indicate that the sulfation is another regulatory pathway for maintenance of intracellular lipid homeostasis (57, 76, 92). High intracellular cholesterol levels generate regulatory oxysterols that can be further sulfated to be another regulatory oxysterol.

LXRs play an important role in the regulation of lipid metabolism (19, 21). The clinical application of LXR agonists in the treatment of lipid accumulation is controversial. Oxysterols, as the agonists, upregulate the expression of cholesterol reverse transporters, including ABCA1 and ABCG1, via activation of LXR/RXR heterodimers. These transporters have been shown to mediate the efflux of cellular cholesterol and phospholipids, which has potential for prevention of lipid accumulation in such conditions as atherosclerosis (12, 85). Administration of synthetic LXR ligands, however, elevated plasma triglyceride levels and induced the lipogenic pathway through the SREBP-1c signaling pathway, whose promoter is an LXR direct target in hepatocytes in vitro and in vivo (21, 28, 38, 43, 78). Pharmacological activation of LXR leads to production of large, triglyceride-rich VLDL lipoprotein particles (38). 25HC, as an LXR ligand and a direct precursor of 25HC3S, increased SREBP-1, FAS, ABCA1, and ABCG1 mRNA levels, increased intracellular lipid levels, induced apoptosis, and decreased cell viability. In contrast, administration of 25HC3S decreased intracellular lipids. Coincidentally, 25HC3S inhibited LXR activation and SREBP expression, which subsequently decreased ACC1, FAS, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) mRNA levels (57, 76). These results were supported by administration of LXR ligands in vivo in mice (21, 28, 78), indicating that increase in SREBP-1c expression by the LXRα activation is the major course for its hyperlipidemia and lipogenesis. It should be noted that the sulfation product 25HC3S decreases SREBP-1c expression and activation but does not increase ABCA1 and ABCG1; rather, it decreases SREBP-1 expression and activation, which results in decreased intracellular lipid levels (57, 92). It is also noteworthy that synthetic sulfated forms of some oxysterols also display antagonistic activity against LXR (26, 84). Importantly, in vivo studies show that only two peritoneal administrations of 25HC3S decreased serum triglycerides, without any liver toxicity, and decreased SREBP-1c expression at the mRNA level in liver tissues (93).

Levels of endogenous cellular 25HC3S are low (unpublished data). It was hypothesized that 25HC3S is initially synthesized in mitochondria and then in cytoplasm and acts on nuclei. The levels of 25HC3S could be significantly increased when cholesterol delivery to the mitochondria increases. The effects of the pair of oxysterols, 25HC and 25HC3S, seem to be coordinately regulated, which appears to play an important role in lipid metabolism. Their different regulatory effects indicate the importance of their physiological role, as shown in Fig. 1.

25HC3S Suppresses Inflammatory Responses Via a PPARγ/IκBα Signaling Pathway

The nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) regulates a large group of gene expressions in nuclei, including cytokines/chemokines and immunoreceptors, and promotes proinflammatory responses. NF-κB exists in an inactive form in the cytoplasm when bound to the inhibitory protein IκBα, which is the key step in regulation of inflammatory responses. The cytosolic levels of IκBα regulate inflammatory responses via controlling NF-κB nuclear translocation (17, 34, 61). The IκBα promoter region contains the PPAR response element (PPRE) and has been shown to be a PPARα/γ target gene (14). Recent studies have shown that losartan inhibits LPS-induced inflammatory signaling through a PPARα-dependent mechanism (5).

Nuclear receptors and PPARs, as well as LXRs, play a critical role in the regulation of inflammatory responses. PPARγ and LXR regulate inflammatory responses in different ways and respond to distinct signaling pathways (11). Activation of PPARγ suppresses inflammatory responses. PPARγ are ligand-inducible transcription factors and regulate gene expression by binding with RXRs as heterodimeric partners with PPRE (66). This complex then binds to cognate sequences in promoter regions of target genes that are involved in lipid metabolism and inflammatory responses (54). Comparative cDNA microarray studies have shown that LXR and PPARγ can repress several proinflammatory gene expressions but distinct (29, 47, 62, 87).

The role of oxysterols in inflammatory responses is controversial. Recent studies have shown that 25HC3S acts as an agonist of PPARγ, and 25HC acts as an LXR agonist in THP-1-derived macrophages (94). Both oxysterols regulate inflammatory responses but via different pathways. Several laboratories have shown that oxysterols, including 25HC, can activate LXRs and, subsequently, repress proinflammatory gene expression following LPS and cytokine stimulation (62, 100).
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25HC3S inhibits inflammatory responses through increasing inflammatory responses (94) as shown in Fig. 2. Thus, the two oxysterol signaling pathways stimulate inflammatory responses by activation of the IκB kinase IKK. 25HC3S decreases NF-κB protein levels and increases PPARγ in nuclei; 2) suppresses the expression of the TNFα-induced NF-κB-dependent reporter gene, which is PPARγ dependent; 3) induces IκBα expression; and 4) inhibits expression of a number of NF-κB target genes, including IL-1β and TNFα. TNF binds to the Toll-like receptor, whereas LPS binds to the TLR4 receptor. Both signaling pathways stimulate inflammatory responses by activation of the IκB kinase IKK. 25HC3S suppresses inflammatory responses through increasing nuclear PPARγ and increasing IκBα expression and then its cytosolic protein levels, subsequently decreasing NF-κB translocation. Meanwhile, the increased PPARγ represses TNFα expression, which directly inhibits IκBα ubiquitination and degradation. In conclusion, 25HC3S inhibits inflammatory responses through increasing IκBα expression and decreasing IκBα degradation via the PPARγ/IκB/NF-κB signaling pathway. Thus, the two oxysterols, 25HC and 25HC3S, play an important role in regulation of inflammatory responses (94) as shown in Fig. 2.

**Oxysterol Sulfation Induces Hepatocyte Proliferation**

Hepatocytes are unique parenchymal cells, for they are able to proliferate under specific conditions such as injury or excision (32, 59). This property is a foundation for the remarkable capacity of the liver to regenerate following acute injuries that lose hepatic mass. Hepatocyte proliferation is also a critical protective response in chronic liver diseases. When hepatocytes are destroyed by lipid accumulation, inflammation, and other injuries, they will be replaced to maintain adequate liver function. The regulatory mechanisms of hepatocyte proliferation have not been completely elucidated but are believed to include many growth factors and cytokines arising from both extrahepatic sites and the liver (32, 59). The ability of quiescent hepatocytes to proliferate in response to physiological stimuli is a key point to promote liver regeneration (48). The process of proliferation is regulated by cyclin expression and cyclin-mediated activation of cyclin-dependent kinases (Cdks). Cyclin A and cyclin E are important for S-phase entry and progression. The formation of the cyclin E:Cdk2 and cyclin A:Cdk2 complexes phosphorylates the retinoblastoma (RB) protein, which subsequently releases bound E2F transcription factor and stimulates expression of proliferation-specific target genes (95). Cyclin B is associated with Cdk1, which promotes cells from the G2 into mitosis (57).

Several reports have implied that SULT2B1b is involved in the control of cell growth and survival: 1) expression increases in liver regeneration after partial hepatectomy (55); 2) increases in T cell proliferation activated by influenza A virus-infected mice (23); and 3) its ability to protect cells from 7-ketocholesterol-induced apoptosis by sulfating this oxysterol (36). Interestingly, increases in SULT2B1b activity in mouse liver tissues increase PCNA-positive cells and promotes hepatic proliferation (97). Cytosolic SULT2B1b protein levels are coincreased with FoxM1b, CDK2, and cyclin in cultured primary rat hepatocytes (53, 97). These increases in expression of proliferative genes play an important role in hepatic response to injury (41, 90). Knockdown of SULT2B1b expression by siRNA downregulates these gene expressions involved in cell cycle regulation (97), indicating that the increase in SULT2B1b expression is associated with the proliferation of hepatocytes. More interestingly, the sulfated oxysterol 25HC3S increases the mRNA levels of cyclins (cyclin A, cyclin B, cyclin E), E2F3 transcription factor, and Cdc25 in mouse liver. Furthermore, 25HC3S decreases gene expression to suppress the initiation of apoptosis (96, 97), such as Apaf1, Tnf, and Tnfrsf10b while increasing the mRNA levels of genes related to antiapoptosis, including Bag1 and Bnip3 (15, 70, 83). Furthermore, the PCNA-positive cells are significantly increased in mouse liver tissues following administration of endogenous and exogenous 25HC3S (96, 97). It is concluded that oxysterol sulfation promotes hepatic proliferation.

The LXR signaling pathway is downregulated in liver tissues treated with 25HC3S or overexpression of SULT2B1b in mice (8, 57). Our study has shown that induction of PCNA expression by 25HC3S can be blocked by synthetic LXR agonist T0901317 (96). It is also noticed that the ability of LXR activation by T0901317 is stronger than the LXR inhibition by 25HC3S (7, 57). Supportingly, it has been reported
that the inhibition of proliferation by LXR agonists in LNCaP cells is mediated by decreasing expression of cyclin A and Cdc25a (49). Thus, it is concluded that the effect of 25HC3S on proliferation is through inactivation of LXR signaling (96, 97). Oxysterol (substrate) reduction may cooperate with the generated oxysterol sulfate (product) in switching off the LXR system for the induction of proliferation (96, 97). Oxysterols including 25HC have been reported to induce apoptosis and growth arrest (88), whereas SULT2B1b shows more effect on proliferation when 25HC is present than when it is absent, supporting the conversion of 25HC to 25HC3S by SULT2B1b. These results further confirm the physiological significance of oxysterol sulfation in promoting liver regeneration (97).

A Novel Regulatory Pathway in Lipid Homeostasis, Inflammatory Responses, and Cell Proliferation

Intracellular lipid accumulation, inflammatory responses, and subsequent apoptosis are the major pathogenic events of many metabolic disorders, including atherosclerosis and NAFLDs. Based on the results reviewed in this manuscript, one possible regulatory pathway has been proposed here for the role of oxysterol sulfation in the maintenance of lipid homeostasis, inflammatory responses, and cell proliferation via regulation of the activity of nuclear receptors LXRs and PPARs. Components in this proposed pathway are as follows.

1. When intracellular cholesterol levels are increased, the mitochondrial cholesterol delivery protein StarD1 delivers cholesterol into mitochondria, where regulatory oxysterols such as 25HC and 27HC are synthesized by CYP27A1 (52, 53). These oxysterols can, in turn, activate LXR and, subsequently, regulate expression of its target genes, which are involved in cholesterol metabolism, efflux, and uptake (1, 33, 37, 46). On the other hand, the oxysterols also increase the expression of genes involved in fatty acid and triglyceride biosynthesis. Oxysterols can be sulfated by SULT2B1b to oxysterol sulfates when cholesterol delivery to mitochondria is increased (6, 74). Oxysterol sulfates can be desulfated by sterol sulfatase (STS) for maintenance of intracellular homeostasis (6, 74).

2. Oxysterol sulfates such as 25HC3S inactivate LXRs and suppress SREBP-1c/2 processing, subsequently inhibiting lipid biosynthesis, suppressing apoptosis, and inducing cell proliferation pathways; 25HC3S activates PPARs, which leads to translocation into the nucleus and transcriptional upregulation of the gene expression, which are both involved in suppressing inflammatory responses (57, 76, 92).
3. The effects of oxysterols such as 25HC on lipid metabolism, inflammatory responses, and cell proliferation are generally opposite to those of 25HC3S; thus, intracellular oxysterol sulfation, like protein and inositol phosphorylation in many cellular functions, may represent another regulatory mechanism for these events (Fig. 3).

Maintenance of intracellular lipid homeostasis is critical to prevent development of hyperlipidemia, fatty liver diseases, and atherosclerosis. The present review connects cholesterol metabolism to regulation of triglyceride biosynthesis, inflammatory responses, and cell proliferation. There are two sources for intracellular cholesterol: 1) exogenous diet, and 2) endogenous biosynthesized cholesterol using acetate as the substrate. Acetate can be used not only for synthesis of cholesterol but also for synthesis of other lipids including free fatty acids and triglycerides. Acetate can also be degraded for generation of energy (Fig. 4). It is well accepted that the most effective way to prevent hyperlipidemia is diet control and physical exercise. Meanwhile, the most prescribed therapeutics for the treatment of hyperlipidemia, i.e., statins, have been focused on blocking the synthesis of cholesterol (inhibition of HMGCR). However, when the use of acetate for synthesis of cholesterol is blocked, the additional acetate will likely be used for synthesizing free fatty acids, which are potentially more toxic than cholesterol to the cells.

The proposed regulatory pathway of bile acid biosynthesis is able to regulate intracellular lipid homeostasis and inflammatory responses, along with cell growth and differentiation through the generation of potent regulatory oxysterol intermediates. Many studies continue to support the checks and balances found between the two pathways. Uprogelation of CYP7A1 leads to increased metabolism and elimination of cholesterol as bile acids. Uprogelation of StarD1 increases delivery of cholesterol to mitochondria generating the regulatory oxysterols including 25HC and 25HC3S as discussed in this manuscript. The generated 25HC3S is capable of negative feedback regulation of SULT2B1b expression (53). In disorders of this pathway, lipids are accumulated within cells at pathological levels, which causes inflammatory responses and subsequent apoptosis. A further understanding of this intracellular lipid regulation could advance our knowledge of liver regeneration and other related metabolic syndrome such as NAFLD/NASH (nonalcoholic steatohepatitis).

Perspective

NAFLDs and atherosclerosis are systemic disorders. Millions of people in the world are suffering from these diseases, which are the number one killers of human beings. NAFLD and atherosclerosis develop slowly and gradually. When noticed, it is often too late, because there is no easy and specific assay to diagnose their early stages. Currently, there is no effective FDA-approved therapy for metabolic syndrome. LXR agonists such as 25HC have been proposed as pharmacological agents for metabolic syndrome (10, 60). The concept was based on the oxysterol property, activating LXR and subsequently increasing cholesterol transporter expression, thus protecting the cell from cholesterol overload through increasing cholesterol outflow. However, this hypothesis receives a negative feedback: the increase in de novo lipogenesis because the oxysterols also act on the SREBP-1c signaling pathway as LXR agonists (10, 39). Sulfation of 25HC to 25HC3S has a dual consequence: the decrease in 25HC as an LXR agonist, and the increase in 25HC3S, which inhibits the LXR/SREBP signaling pathway. Oxysterol sulfates such as 25HC3S seem to be beneficial in the prevention of NAFLD and atherosclerosis development, lipid accumulation, inflammatory responses, and cell apoptosis, thereby preventing hepatic steatosis, which is a prerequisite for NASH (18, 71).

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AUTHOR CONTRIBUTIONS

Author contributions: S.R. conception and design of research; S.R. and Y.N. performed experiments; S.R. and Y.N. analyzed data; S.R. interpreted results of experiments; S.R. prepared figures; S.R. and Y.N. drafted manuscript; S.R. edited and revised manuscript; S.R. and Y.N. approved final version of manuscript.

REFERENCES

Review

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