Review

Clinical Lipidology

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The interaction between metabolism, cancer and cardiovascular disease, connected by 27-hydroxycholesterol

Oxysterols are metabolites of cholesterol that are produced in liver and other peripheral tissues as a means to eliminate cholesterol to bile acid. Recent studies have revealed that the most abundant circulating oxysterol 27-hydroxycholesterol (27HC) is the first identified endogenous selective estrogen receptor modulator. 27HC levels correlate well with that of cholesterol, and also rise progressively with age. 27HC affects estrogen receptor function by the antagonism of estrogen action and also by the direct modulation of the receptor function, and similar to estrogen/estrogen receptors, 27HC has many actions in various tissues. This review article introduces the recent progress in the understanding of the role of 27HC in breast cancer and cardiovascular dysfunction.

Keywords: 27-hydroxycholesterol • atherosclerosis • breast cancer • cholesterol metabolite • CYP27A1 • CYP7B1 • estrogen receptor • oxysterol • SERM

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27HC, its generation & metabolism

Oxysterols are metabolites of cholesterol that are produced in liver and other peripheral tissues. Although oxysterols were originally considered as substrates for bile acid synthesis and also as a means to eliminate cholesterol, especially from peripheral tissues to the liver, there is accumulating evidence that oxysterols have unique function in various tissues [1–3]. Some oxysterols are present in foods, however, most oxysterol with the exception of 7β-hydroxycholesterol (7βHC) is only slightly absorbed in the intestinal tract and rapidly metabolized in the liver ([4,5] Umetani, Unpublished Data). The most abundant circulating oxysterol is 27-hydroxycholesterol (27HC). 27HC is a hydroxylated product in the C27 position on the lateral chain of the cholesterol structure, and serum concentrations of 27HC correlate well with that of cholesterol [1]. 27HC levels also rise progressively with age [6]. There are large differences in the ratio of esterified to unesterified 27HC among tissues, ranging from more than 90% in serum to less than 40% in kidney, with around 30% unesterified in the aorta [7–9].

The enzyme that generates 27HC, sterol 27-hydroxylase (CYP27A1), is a mitochondrial P450 enzyme and is primarily expressed in the liver, but it is also expressed in peripheral tissues, but to a lesser extent [10]. CYP27A1 catalyzes oxidation of cholesterol at C27 position to form 27HC and cholestenoic acid with NADPH, adrenodoxin and adrenodoxin reductase as co-factors [11–13]. CYP27A1 gene expression is upregulated by growth hormone and insulin-like growth hormone-1 [14], and downregulated by steroid hormones and inflammatory cytokines [14–16]. While 27HC affects cellular cholesterol homeostasis as a potent suppressor of cholesterol synthesis by regulating SREBP action *in vitro* [17,18], its effect on SREBP action *in vivo* is not clear [19]. 27HC is metabolized by another P450 enzyme, oxysterol 7α-hydroxylase (CYP7B1). CYP7B1 is expressed in the brain, particularly in the hippocampus, but is also expressed in liver and other peripheral organs [20,21]. Although the physiological concentration of these substrates should be considered, CYP7B1

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has a relatively broad substrate specificity for steroids and sterols such as dehydroepiandrosterone (DHEA), 5α-androstane-3β, 17β-diol (3Adiol), 25-hydroxycholesterol, pregnenorone, 17β-estradiol (E_2) and 27HC, as a 7α-hydroxylase [21–23]. Clinically, the importance of CYP7B1 has been reported in prostate cancer together with ER-β and 3Adiol because of its enzymatic function against androgen and estrogen, and CYP7B1 inhibitors can be used as chemoprevention and in the treatment of prostate cancer [23–25]. CYP7B1 shows male-predominant expression in the liver [8,26]. E_2 induces transcription of CYP7B1 in culture cells through PI3K-Akt pathway [27].

There is valuable information available about the physiological roles of CYP27A1 and CYP7B1 from mutational study in human and knockout/transgenic mice. In the bile acid production, CYP27A1 acts both in the classical and alternative pathways of the conversion from cholesterol to bile acids, whereas CYP7B1 is only involved in the alternative pathway. In addition, CYP27A1 is also involved in the reverse cholesterol transport and vitamin D3 biosynthesis [28]. Therefore, it needs to be noted that in addition to not making 27HC, loss of CYP27A1 results in a number of problems associated with cholesterol and bile acid metabolism, thereby the phenotypes caused by the loss of CYP7B1 and by the loss of CYP27A1 may be due to different mechanisms of action. In addition, there are differences in the phenotypes caused by the suppression/deficiency of CYP27A1 or CYP7B1 between human and mice [29,30]. In humans, functional deficiency of CYP27A1 causes a rare disorder, CTX (cerebrotendinous xanthomatosis), connected with sterol deposition in tissue macrophages and increased risk of neuronal dysfunction and premature atherosclerosis due to diminished reverse cholesterol transport, regardless of normal circulating cholesterol levels [12,13]. In mice, loss of CYP27A1 results in a number of problems associated with cholesterol and bile acid metabolism, the combination of which contributes to the cardiovascular function. However, mice with CYP27A1 deficiency are still able to produce bile acids, and decreased bile acid synthesis caused by CYP27A1 deficiency is due to the involvement of CYP27A1 in the classical pathway, and not due to the decreased 27HC levels. Indeed, CYP27A1 overexpressed mice do not show a significant difference in cholesterol homeostasis, regardless of increased serum 27HC levels [31]. CYP7B1 mutation in humans causes spastic paraplegia 5A (SPG5A), an autosomal recessive neurologic disorder, which is due to the defect in cholesterol and neurosteroid metabolism. The phenotypes of mice with CYP7B1 deficiency are described below.

27HC, the first identified endogenous selective estrogen receptor modulator

Using cell-based assays and *in vitro* assays, we discovered that 27HC is a competitive ER antagonist in the vasculature [9]. 27HC binds directly to ER-α (K_i= 1.32 μM) and ER-β (K_i=0.42 μM). The Km of 27HC for its catabolic enzyme CYP7B1 is 24 μM [32], which is much higher than the Kd of 27HC for the ERs. Thus, unesterified 27HC achieves levels above the Kd value for the ER activity.

The generation of nitric oxide (NO) by inducible type and endothelial type of nitric oxide synthases (iNOS and eNOS, respectively) promotes endothelial cell growth and migration, and prevents leukocyte adhesion, thrombosis and vascular smooth muscle cell proliferation. Reduced vascular synthesis of NO causes several disorders, including hypercholesterolemia and diabetes mellitus [33–35]. E_2 regulates vascular functions such as: vasodilation and re-endothelialization after vascular injury through its modulation of iNOS and eNOS. With the known functions of ER in the regulation of NOS in EC, the pathophysiologic implications of the findings on 27HC were determined *in vivo.* Increasing 27HC levels in mice by diet-induced hypercholesterolemia or pharmacologic administration decreased estrogen-dependent expression of vascular eNOS and iNOS, repressed carotid artery re-endothelialization and increased aortic tension [9]. Parallel findings were shown in mice with elevated 27HC due to deletion of CYP7B1. The binding of 27HC to nuclear receptors is highly ER specific, and 27HC did not affect other nuclear receptor activity tested [9]. Indeed, although some reports show that 27HC is a weak agonist for liver X receptor (LXR) [36] *in vitro* or cell culture models, 27HC does not show strong direct activity as an LXR agonist in certain cell lines [37] and *in vivo* [9,38]. In addition to the antiestrogenic effects of 27HC in vascular endothelial cells in the presence of estrogen, we also identified proestrogenic actions of 27HC in hepatoma and colon cancer cells, indicating that the effect of 27HC is tissue specific [9]. Thus, 27HC is the first identified endogenous selective estrogen receptor modulator, or SERM, and it has important biological actions *in vivo*.

Impact of 27HC on breast cancer

Breast cancer is the second most common malignancy after skin cancer in women, with approximately 1,000,000 new cases diagnosed worldwide each year. The risk of ER-positive breast cancer increases particularly in postmenopausal women despite of the decline of circulating estrogen levels. Endocrine-based therapies against ER-positive breast cancer with synthetic SERMs or aromatase inhibitors are often ineffective or resistance

develops [39], suggesting that there are yet unknown, important ER-mediated mechanisms [40]. There is increasing evidence that obesity is closely related with several types of cancer development and progression [41].

Our two recent papers by Wu *et al.* [42] and Nelson *et al.* [43], together with our previous report [44] shed light on the link between 27HC and the progression of breast tumor, in which the impact of estrogen/ER in cancer development and progression has been widely studied. In MCF-7 cells, 27HC increases cell number and has a potent impact on ER-mediated processes involved in breast cancer cell growth [44]. We further investigated the role of 27HC in breast cancer progression *in vivo*. First, we compared mRNA expression of *cyp27a1* and *cyp7b1* in ER-positive tumors versus normal breast tissue samples in the Cancer Genome Atlas (TCGA 2012). *cyp27a1* expression was similar in normal breast and tumors, in contrast, *cyp7b1* expression was decreased in ER-positive tumors compared with normal breast tissue. In addition, there were greater 27HC levels in tumor samples compared with controls. Furthermore, survival of cancer patients was markedly poorer for patients with low versus high tumor *cyp7b1* expression. In mouse models, 27HC promoted the tumor growth and metastasis by independent mechanisms (Figure 1) [43]. Interestingly, 27HC treatment also increased macrophage infiltration and angiogenesis in the tumor, and locally produced 27HC by CYP27A1 in macrophages may determine the likelihood of a higher tumor grade. In addition, elevated levels of 27HC or *cyp27a1* gene expression, or decreased levels of *cyp7b1* expression correlated with tumor metastasis. Thus, 27HC, which is an endogenous SERM that does not require aromatization, has a potent impact on ER-mediated processes in breast cancer cells *in vitro* and *in vivo*.

This discovery will be of great interest, because similar mechanisms are potentially operative in a number of other steroid hormone-responsive cancers, and also 27HC and its regulatory enzymes in cancer may explain treatment failures with aromatase inhibition. Therefore, assessments of 27HC or the enzyme abundance in tumors may aid in personalizing hormone-based therapy. However, the limitation of these studies should be noted. For example, these studies were performed in postmenopausal women, who have reduced estrogen levels. In addition, estrogen is also produced from stromal cells of adipose tissue [45], and increased fat mass in women with obesity can enhance the aromatization of adrenal androgens and consequently increase estrogens [46]. From cell culture assays, 27HC has proestrogenic effects in breast cancer cells in the absence of estrogen, in contrast, it shows suppressive effects on the ER activity in the presence of estrogen [9]. Sometimes, the effects of 27HC in cell culture and *in vivo* are different, therefore, how 27HC acts against breast tumors in premenopausal women should be investigated. In this regard, it is interesting that 27HC promotes tamoxifen-resistant tumors in the similar fashion as E_2 [43], suggesting that 27HC promotes breast tumors that have acquired resistance to chemical therapy such as tamoxifen and other SERMs.

Impact of 27HC on atherosclerosis & metabolism

Atherosclerosis is a complex disease, and inflammation is a major component that is involved in the pathological process [47,48]. Oxidized lipids modulate

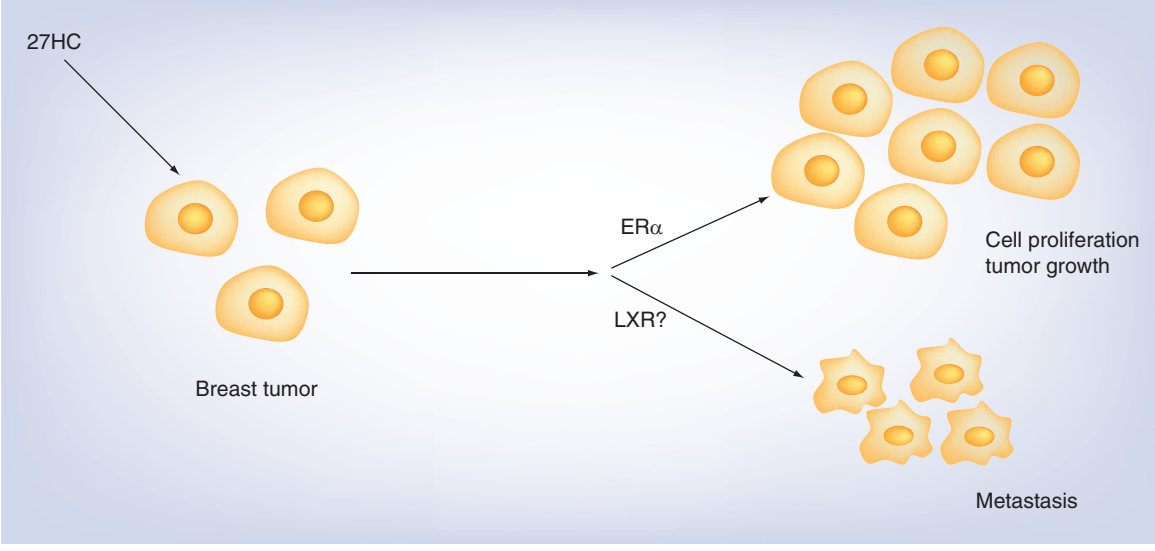

Figure 1. 27-hydroxycholesterol promotes breast tumor progression and metastasis. 27HC promotes cell proliferation and tumor growth of breast cancer cells via ER-α. It also increases tumor metastasis through actions independent from ER function, probably via LXR.
27HC: 27-Hydroxycholesterol; ER: estrogen receptor; LXR: Liver X receptor.

inflammation-related gene expression, which induces proatherogenic responses and plaque instability [49]. Oxysterols, such as 7βHC and 7keto-cholesterol but not cholesterol with the same levels, have cytotoxic effects and cause superoxide anion production [50]. In contrast, 27HC is also important for cholesterol elimination from macrophages, and this works as an atheroprotective effect [13]. Furthermore, in mice CYP27A1 shows gene dose-dependent effect which is likely due to affected reverse cholesterol transport, and atherosclerosis is accelerated in *cyp27a1*$^{+/-}$, but reduced in *cyp27a1*$^{-/-}$ mice [51]. As described above, plasma 27HC concentration is strongly correlated with the concentration of cholesterol. Compared with plasma 27HC levels, 27HC levels in atherosclerotic lesions are much higher and approach millimolar concentrations [1]. The amount of 27HC in atherosclerotic lesions increases with the severity of the lesion and with the abundance of macrophages [10,52]. There is one report showing no correlation between plasma 27HC levels and CHD risk in the WHI study [53], however, the plasma levels of 27HC in that paper are very low compared with previously published data from human serum [1]. In addition to the abundance of cholesterol, CYP27A1 is also upregulated in atherosclerotic lesions [10,54], and its expression increases during monocyte to macrophage differentiation [55]. High cholesterol/fat diet increases plasma, hepatic and adipose levels of 27HC in mice [56]. 27HC is also locally produced as one of the major *de novo* cholesterol products in adipocytes and suppresses adipocyte differentiation, suggesting the prevention of the formation of new fat cells upon overfeeding with dietary cholesterol [57].

The role of estrogen in atherosclerosis and cardiovascular function is also complex. At the early stages of atherosclerotic lesion development, estrogen prevents atheroma formation, and it also protects vasculature from ischemia [58]. In contrast, estrogen has deleterious effects such as promoting inflammation, unstabilizing plaque and increasing coagulation and thrombosis, especially at the late stages when the fatty streak is established [59]. Work in multiple animal models and observational clinical studies suggests that estrogen has the potential to provide potent cardiovascular protection. However, following more recent clinical trials, it is now apparent that there are mechanisms that modify the vascular actions of estrogen, particularly in the setting of hypercholesterolemia. This raises the critically important and novel possibility that there are endogenous counter-regulatory mechanisms.

Based on the results showing that 27HC as a SERM inhibits both transcriptional and nontranscriptional estrogen-dependent production of NO in vascular endothelial cells, we tested the impact of 27HC on atherosclerosis by crossing apoE-deficient mice, commonly used as an atherosclerosis model, with *cyp7b1*$^{-/-}$mice and studied the resulting littermates [60]. This animal model enabled us to compare the impact of 27HC with or without hypercholesterolemia. We found that elevations in 27HC via the deletion of *cyp7b1* caused exaggerated atherosclerosis without altering lipid status in the setting of normo- and hypercholesterolemia; in addition, estrogen-related atheroprotection is markedly attenuated. Furthermore, dose-related capacity of E_2 is observed to reverse the exaggerated atherosclerosis with elevated endogenous 27HC. The effects of 27HC on atherosclerosis are ER-α-dependent. Taken together, these results show that 27HC is an important contributing factor in the loss of estrogen protection from vascular disease, and has a potent impact on atherosclerotic lesion development regardless of the blood cholesterol levels (Figure 2). Elevated 27HC also caused increased inflammatory cytokine production in aorta. This suggests that 27HC also induces inflammatory responses in vasculature. Indeed, in monocytes/macrophages 27HC upregulates proinflammatory genes via ER-α, and also activates NFκB in EC via the activation of JNK and ERK. Subsequently, 27HC increased monocyte adhesion to EC in cell culture and *in vivo*. Since estrogen blunts NFκB activation in EC [61], it is indicated that 27HC promotes atherosclerosis via unique proinflammatory processes mediated by ER-α, the impact of elevated 27HC occurs through the direct modulation of ER function rather than through the antagonism of estrogen action, and it potently impairs the beneficial effects of ER on vascular function.

Although the results of hormone replacement therapy to date are surprisingly negative in clinical trials [62,63], this study will lead to a greater understanding of how estrogen replacement therapy is ineffective or even harmful in postmenopausal women after a certain period without estrogen replacement after menopause has passed. Strategies to lower 27HC may complement existing approaches targeting cholesterol to prevent vascular disease.

Future perspective

Considering that estrogen/ER play many important roles in various tissues in men and women, 27HC may have more actions than have been investigated so far. One area of great interest is the impact of 27HC in brain function, and there is growing evidence to show the importance [64]. Hypercholesterolemia is a risk factor for Alzheimer's disease, and cholesterol does not pass through the blood–brain barrier, in contrast 27HC does. This implies that 27HC has an important role in the cholesterol metabolism in the brain. Indeed, elevated

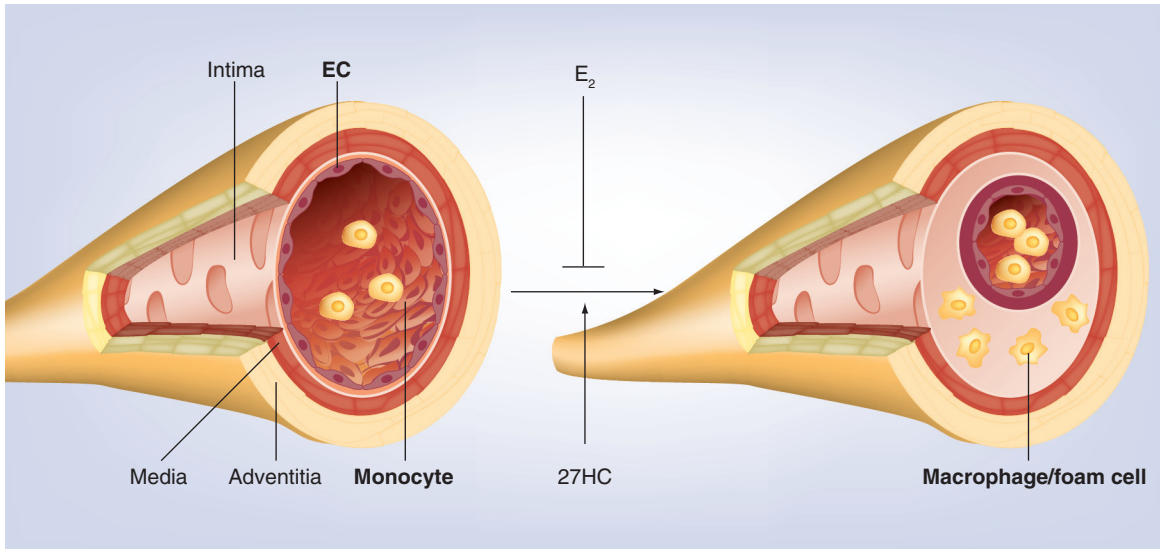

Figure 2. 27-hydroxycholesterol develops atherosclerosis in an estrogen receptor-dependent manner. Estrogen suppresses atherosclerotic lesion development, while 27HC stimulates inflammatory responses via estrogen receptor-α in macrophages and vascular endothelial cells. 27HC also promotes monocyte/macrophage infiltration into the intimal layer, leading to atherosclerosis development.
27HC: 27-Hydroxycholesterol; EC: Endothelial cells.

27HC is linked with Alzheimer's disease progression, although the accumulation in brains of patients is likely secondary to neurodegeneration caused by decreased CYP7B1 activity. The sequence variations in the *cyp7b1* gene have been reported in the patients of spastic paraplegia type 5 with possibly decreased CYP7B1 activity that causes high cholesterol and 27HC levels, especially in the brain [65–69]. Mutations in *cyp27a1* gene cause CTX, which results in the accumulation of cholesterol in brain and progression of neurological dysfunction. In addition, 24(*S*)-hydroxycholesterol, one of the major oxysterol in brain, is also metabolized by CYP27A1 [70]. Therefore, it is intriguing to investigate the roles of 27HC/CYP27A1/CYP7B1 in the brain in the relationship with ER function.

Another area of great interest is the impact of 27HC through GPR30, recently identified membrane estrogen receptor [71,72]. Although it is still controversial whether GPR30 has physiological function as an estrogen receptor [73], this receptor may have an important role in a certain context. It is still unknown whether 27HC is a ligand to this receptor, however, it is possible that 27HC functions via this receptor in certain tissues.

There are still unknown physiological functions for the role of 27HC even in metabolism, cancer and cardiovascular system. In addition, our discovery of 27HC as an endogenous SERM suggests the existence of other endogenous ligands for ER or other steroid receptors. Further work will be warranted to explore the role of 27HC as an important regulator of the crosstalk between metabolism, cancer and cardiovascular diseases.

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Executive summary

- 27-hydroxycholesterol (27HC) is a cholesterol metabolite that is produced and catabolyzed by P450 enzymes, sterol 27-hydroxylase (CYP27A1) and oxysterol 7α-hydroxylase (CYP7B1), respectively, and its concentration correlates well with that of cholesterol.
- 27HC is the first identified endogenous selective estrogen receptor modulator, and suppresses estrogen-induced genomic and nonnuclear action of estrogen receptor (ER) in vascular endothelial cells.
- In breast cancer patients, the tissue expression of *cyp7b1* reversely correlates with overall outcome, and 27HC promotes breast tumor cell proliferation and metastasis.
- 27HC promotes atherosclerosis via unique proinflammatory processes mediated by ER-α, the impact of elevated 27HC occurs through the direct modulation of ER function rather than through the antagonism of estrogen action, and potently impairs the beneficial effects of ER on vascular function.
- Further investigation will be warranted to explore the role of 27HC as an important regulator of the crosstalk between metabolism, cancer and cardiovascular diseases.

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