ELSEVIER

Cellular biochemistry of oxysterols derived from the diet or oxidation in vivo

Diane W. Morel and Chen Yi Lin*

*Department of Biochemistry, Allegheny University of Health Sciences, McP Hahnemann Medical School, Philadelphia, PA USA; and *Department of Endocrinology, Rush Presbyterian-St. Luke's Medical Center, Chicago, IL USA*

Oxidized derivatives of cholesterol, known as oxysterols, are present in the diet as contaminants of cholesterol-containing foods. They can enter the circulation through the diet or they are generated through peroxidation of lipoproteins or enzymatic oxidation of cholesterol. Like cholesterol, oxysterols are transported in serum with lipoproteins but, unlike cholesterol, they may also be transported by serum albumin. This additional means of transport may allow for more efficient removal of cellular sterols or transfer between tissues. It has been suggested that the physiologic regulation of cholesterol metabolism may be through generation of oxysterols. In addition, oxysterols potentially play a role in aspects of various diseases such as atherosclerosis and cancer, either as contributory or protective agents, most likely through their action as potent modulators of cholesterol metabolism and/or their direct effects on membrane structure and function. Thus, these compounds may represent normal physiologic processes as well as pathological ones and strategies to enhance or diminish oxysterol levels may prove useful in the future. (J. Nutr. Biochem. 7:495–506, 1996.)

Keywords: oxysterols; cholesterol metabolism; cell membrane; atherosclerosis; cancer

Introduction

Oxysterols are a group of sterols similar in structure to cholesterol (cholest-5-en-β-ol), but that contain an additional oxygen function such as a hydroxyl group, a ketone group, or an epoxide group on the sterol nucleus or on the side chain of the molecule. The structure of some common oxysterols is compared with that of cholesterol in *Figure 1.* These compounds may enter the blood circulation as contaminants of cholesterol-containing foods, or generated as a result of lipoprotein oxidation or intracellular catabolism. A wide variety of cellular effects have been attributed to oxysterols including effects on cellular membrane enzymes, cellular cholesterol, and phospholipid metabolism.

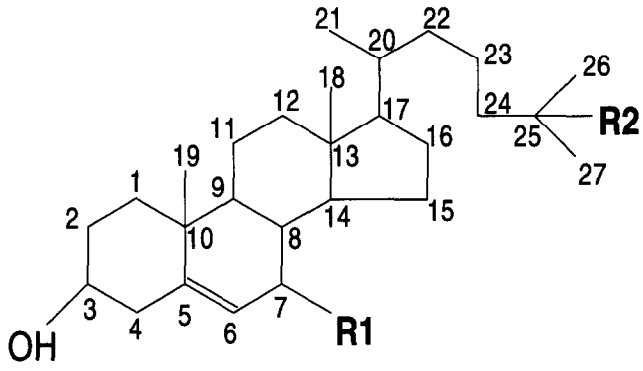
Oxysterols have been detected in plasma and aortic tissues of humans and in experimental animals. Using isotope-dilution mass spectrometry, Breur et al.[1] detected 7α-OHC, cholesterol-5β, 6β-epoxide, cholesterol-5α, 6α-epoxide, 7-oxocholesterol, cholestane-3β, 5α, 6β-triol, 250HC, and 260HC in human plasma, with 7α-OHC being the most abundant (115 ng/mL) oxysterol. Using HPLC, Harik-Khan et al.[2] estimated that the plasma concentration of 260HC in healthy humans is within the range of 100 to 300 ng/mL (approximately 0.3 to 0.9 μM). Smith and colleagues [3–6] reported that 7α- and 7β-OHC, 250HC, and 260HC were also found in the esterified form in human aorta and liver, at concentrations in the range of μg/g of dried tissue. Elevated oxysterol concentrations were also seen in aortic tissues of rabbits after the animals were made hypercholesterolemic by feeding a high cholesterol diet.[7]

That oxysterols in the body may arise from dietary sources is supported by several observations. Significant quantities of oxysterols have been detected in cholesterol-containing food; the major oxysterol species found in traditional Western foods are 7β-hydroxycholesterol (7β-OHC), cholesterol-α-epoxide, cholestan-3β, 5α, 6β-triol, 7-ketocholesterol, and 25-hydroxycholesterol (250HC).

Address reprint requests to Diane W. Morel, Ph.D. at Department of Biochemistry, Allegheny University of Health Sciences, 2900 Queen Lane, Philadelphia, PA 19129 USA
Received April 3, 1996; accepted June 21, 1996.

655 Avenue of the Americas, New York, NY 10010

0955-2863/96/$15.00
PII S0955-2863(96)00101-5



	RI	R2
Cholesterol	H	H
25-Hydroxycholesterol	H	OH
7-Hydroxycholesterol	OH	H
7-Ketocholesterol	=O	H

Figure 1 Schematic of sterol structure showing differences between cholesterol and several common oxysterols. Esters of cholesterol and oxysterols have fatty esters attached by ester bond at the 3B-position.

These oxysterols have been detected in relatively high concentrations, approximately 10 to 150 μg/g dry weight, in commercially dried egg yolk,[8–10] heated tallow,[11] cream,[12] and fresh butter and cheese;[13] furthermore, concentrations of oxysterols in cholesterol-containing foods reportedly increase after certain food processes, such as deep frying.[12,13] In a clarified butter product called ghee, about 12% of the total sterols are oxysterols.[14]

There is also evidence that dietary oxysterols can be readily absorbed. Emmanuel et al.[15] have shown that human subjects, given an oral dose of mixed oxysterols, including 7β-OHC, 7-ketocholesterol, cholestane-α-, and β-epoxides showed rapidly increasing postprandial plasma concentrations of these oxysterols within 2 to 3 h; these levels returned to baseline within 3 to 5 h. In another experiment by Hodis et al.,[7] feeding New Zealand White rabbits with a high cholesterol (1%) diet was also shown to increase plasma oxysterol concentrations substantially as well as to increase the oxysterol content of aortic tissues.

In addition to dietary sources, plasma oxysterols may arise through lipoprotein peroxidation in vivo. Several studies have provided evidence supporting this possibility. First, according to Hughes et al.,[16] 7-ketocholesterol and 7β-OHC were detected in low-density lipoproteins (LDL) after the lipoprotein was oxidized in vitro in the presence of cuprous ions. Chisolm et al.[17] identified 7β-hydroperoxycholes-5-en-3β-ol as the primary cytotoxin of oxidized LDL and demonstrated its presence in atherosclerotic lesions. Second, studies from Hodis' laboratory showed that administration of probucol, a known antioxidant, to cholesterol-fed rabbits significantly reduced the concentrations of oxysterols (7-hydroxycholesterol, 5,6-epoxycholesterol, and cholestanetriol) in their plasma and aortic tissues.[18]

The third source of oxysterols generated in vivo is thought to be intracellular enzymatic reactions.[19–21] Studies by Johnson et al.[22] revealed that feeding rats a cholesterol-rich diet (5% cholesterol) substantially increased the quantity of 250HC in their livers. By feeding the animals deuterium oxide and comparing the incorporation of deuterium into cholesterol and 250HC, the investigators concluded that the elevated 250HC was produced endogenously from exogenous cholesterol, but not from contaminants of cholesterol-autoxidation.

Parenchymal hepatocytes have been thought to be the primary, if not the sole, cell type possessing a functional enzyme system that could convert cholesterol into oxidized derivatives, namely the cytochrome P-450 system. Within the cytochrome P-450 family, 27-hydroxylase is an important enzyme responsible for the hydroxylation of the cholesterol side chain.[23] Studies from several laboratories have suggested that 27-hydroxylase can be detected in a variety of cells including fibroblasts, macrophages, brain, and kidney cells.[19,24,25] Recently, studies from Bjorkhem's laboratory[24] showed that cholesterol-enrichment in cultured human alveolar macrophages increased the conversion of cholesterol to 27-hydroxycholesterol (270HC) and its oxidized derivative, 3β-hydroxy-5-cholestanoic acid, and promoted their excretion to the serum-containing medium. These data suggest that enzymatically generated oxysterols may account for a significant percentage of oxysterols found in serum or other tissues. In addition, the conversion of cholesterol to 270HC in fibroblast mitochondria has been proposed to mediate the suppression of cholesterol biosynthesis.[26]

Taken together, the data available clearly indicate that oxysterols are naturally occurring derivatives of cholesterol that may enter the circulation in a number of ways. In this review, the metabolism of oxysterols, their effects on the metabolism of other lipids, and possible roles in various disease processes will be discussed.

Oxysterol metabolism

Oxysterols and bile acid synthesis

Most sterols, including cholesterol, are eliminated from the body through bile secretion. Synthesis of bile acids occurs in the liver through 7α-hydroxylation of cholesterol; the oxysterol, 7α-OHC, so formed can then be used to synthesize both cholic acid, the most common bile acid, and chenodeoxycholic acid. It is generally accepted that the catabolism of oxysterols is similar to that of cholesterol. Using radiolabelled 260HC as a model, Javitt et al.[19] have demonstrated that both cholic acid and muricholic acid were the metabolites of oxysterols in rats with a bile fistula. In animals that do not synthesize muricholic acids, such as hamsters,[27,18] rabbits, and humans,[30,31] the proportions of chenodeoxycholic and cholic acids derived from 260HC can account for the proportions of these bile acids normally found in bile.

Although the products are similar, it is not clear if oxysterols and cholesterol share the same pathways for bile acid synthesis. According to Ogishima et al.,[32] highly purified 7α-hydroxylase from rat liver has no activity toward 26OHC-derived 3β-hydroxy-5-cholenoic acid, the monohydroxyl bile acid precursor of chenodeoxycholic acid in humans. Based on this, Javitt et al.[19] have hypothesized a family of discrete P-450 7α-hydroxylases that catalyze different (oxy) sterols independently and are subject to separate regulation.

Oxysterols and steroidogenesis

As with cholesterol, which serves as a precursor for many of the steroid hormones, there is some evidence that oxysterols, especially those with a hydroxyl group on the side chain, can serve as substrates for steroid hormone synthesis in steroidogenic cells. For example, in cultured syncytiotrophoblasts, addition of 25OHC to a serum-free medium led to an increase in cellular progesterone content,[33,34] suggesting its use in synthesizing progesterone. Using isolated placental mitochondria as a source of enzyme, Tuckey et al.[35] showed that hydroxycholesterols were comparable to cholesterol as substrates for side chain cleavage. In other experiments, the binding affinity of hydroxycholesterols to the side chain cleavage enzyme, cytochrome P450scc, was even higher than that of cholesterol.[36]

Transport of oxysterols

Because of the similarity in structure between oxysterols and cholesterol, it has also long been assumed that transport in serum would be similar, i.e., wholly with various lipoprotein fractions. In the early 1980s, work by Peng et al.[37] in squirrel monkeys and by Javitt et al.[31] showed that oxysterols associated primarily with LDL and high-density (HDL) lipoproteins. In contrast, in vitro studies at the same time suggested that added oxysterols associated only partially with lipoproteins, with the remainder in the higher density fraction of serum.[38] Recent studies have demonstrated that unesterified oxysterols associate readily with serum albumin.[39] As shown in *Figure 2*, there is an equilibrium in the association of 25OHC between albumin (in LPDS) and the lipoproteins; as the concentration of lipoprotein in a mixture increases, there is a decrease in albumin-associated 25OHC and an increase in lipoprotein-associated 25OHC. The marked decrease in the "specific activity" of 25OHC association with albumin (*Figure 1B*) suggests that the association with albumin is low affinity but high capacity. It is thus likely that the serum "oxysterol-carrier protein" hypothesized by Bjorkhem et al.[18] is albumin and may facilitate the removal of excess cholesterol-derived oxysterols from cell membranes.

The extent of association with albumin or with lipoproteins is related to the extent of oxysterol esterification.[40] When esterification is blocked (4°C or by inhibitors), the distribution of 25OHC tends toward albumin, whereas when serum is incubated under conditions that permit esterification, the distribution shifts towards lipoproteins. Another player regulating the distribution of oxysterol in serum is cholesterol ester transfer protein (CETP), which allows

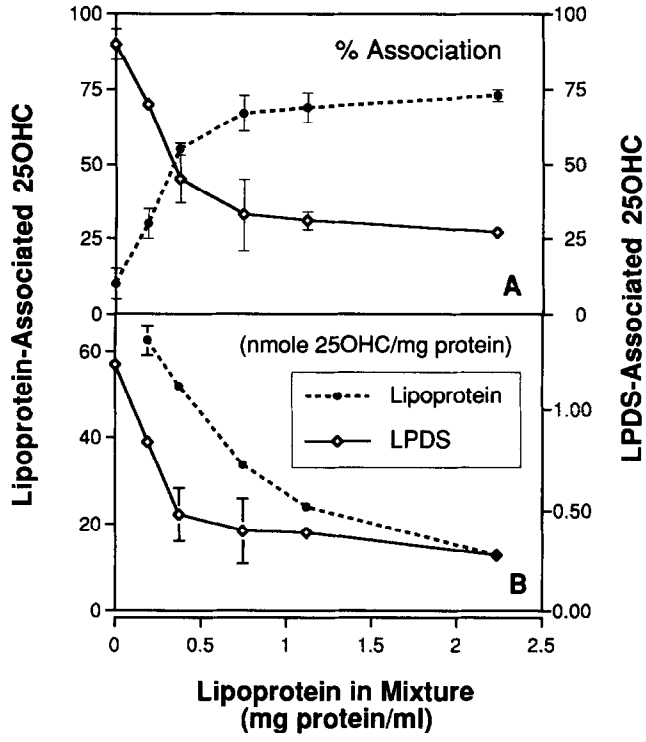


Figure 2 Partitioning of 25OHC between lipoproteins and LPDS. Human LPDS (57 mg protein and 60 nmol 25OHC/mL) was isolated from [3H]25OHC-prelabelled serum, and mixed with an increasing volume of 25OHC-free total lipoproteins (5.0 mg protein/mL). After incubation at 4°C for 18 hr, the lipoproteins and LPDS were reisolated by density ultracentrifugation; radiolabel was quantitated by liquid scintillation counting and used to determine distribution. Data are mean ± range for duplicate samples.

transfer of HDL-associated 25OHC esters to LDL, presumably in exchange for triglyceride.[40] Thus, in the presence of an antibody to CETP, 25OHC esters accumulate in HDL.[40] The major factors that modulate the distribution in serum of oxysterols such as 25OHC are illustrated in the cartoon shown in *Figure 3*.

Esterification of oxysterols

There is ample evidence that oxysterols are readily susceptible to esterification reactions. Fatty acyl esters are the predominant form of oxysterols found in extrahepatic tissues such as the aorta[23,37] and these esters are also found in plasma associated with various lipoproteins.[41,42] Using purified lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for esterification of cholesterol in serum, or the non-lipoprotein fraction of serum as a source for the enzyme, studies in vitro have shown that oxysterols are readily esterified and that the esterification can be inhibited by agents known to inhibit the esterification of cholesterol.[40,43] Similarly, tissue culture studies have shown that oxysterols can be esterified by acyl CoA:cholesterol acyltransferase, the intracellular enzyme responsible for esterification of cholesterol.[44] Although oxysterols have an additional oxygen function that might be available for esterification of a fatty acid, monoester formation, most likely at

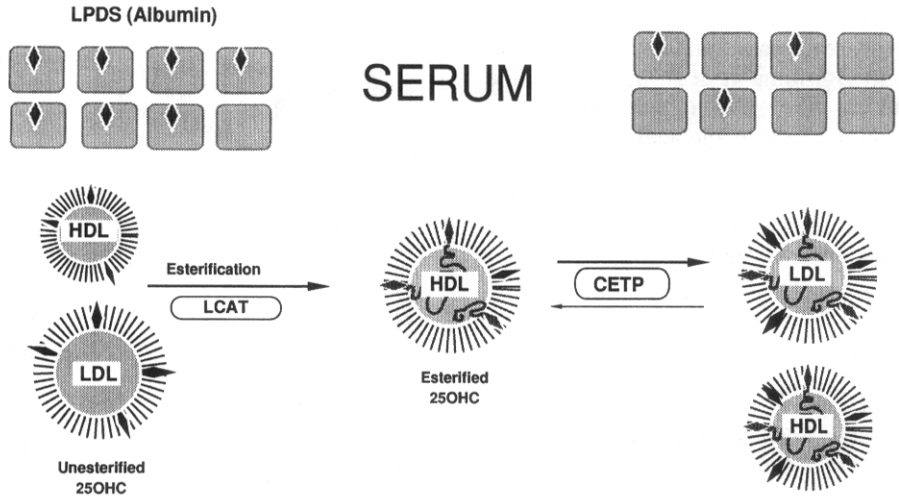


Figure 3 Schematic illustrating the distribution of the oxysterol 25-hydroxycholesterol in serum and the role of esterification via lecithin:cholesterol acyltransferase (LCAT) and ester transfer via cholesteryl ester transfer protein (CETP) in redistribution of oxysterol in serum.

the 3β-position of the sterol nucleus (*Figure 1*), appears to predominate (40,44).

Oxysterol actions

Maintenance of cholesterol homeostasis in cells is essential; it is maintained through regulation of cholesterol biosynthesis,[45] uptake of lipoprotein-associated cholesterol[46] and efflux of membrane cholesterol to extracellular acceptors.[47,48] Accumulating evidence indicates that many oxysterols display potent regulatory effects on cellular cholesterol metabolism.[49] They are capable of modulating the biosynthesis,[50,51] esterification,[52] uptake, and efflux of cholesterol[53] at relatively low concentrations.

Oxysterols and cholesterol biosynthesis

HMG-CoA reductase, the enzyme catalyzing the rate-limiting step in cholesterol biosynthesis, is among the most highly regulated enzymes in nature. As illustrated in the cartoon shown in *Figure 4,* many oxysterols are potent inhibitors of HMG-CoA reductase, effective at concentrations less than 100 nM. In the early 1970s, Kandutsch and Chen showed that oxidized derivatives of cholesterol produced by introducing a ketone or hydroxyl group are several orders of magnitude more effective than pure cholesterol in specifically suppressing HMG CoA reductase activity.[54,55] At concentrations as low as 50 to 70 nM, oxysterols such as 25OHC decreased reductase activity and sterol synthetic rate by 50% in cultured mouse fibroblast-like L-cells.[50,55] In avian myeloblasts, 25OHC at 250 nM produced an 80% decrease in the reductase activity after 60 min.[56] In contrast, cholesterol in the same concentration range exhibited no effect on sterol synthesis and HMG-CoA reductase activity.[54,55]

The molecular mechanisms by which oxysterols and high concentrations of cholesterol inhibit sterol synthesis

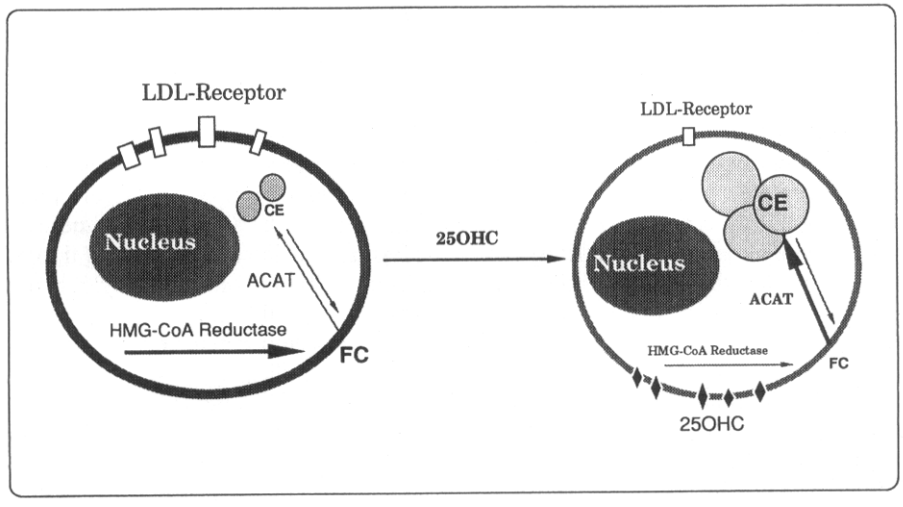


Figure 4 Schematic representing the influence of an oxysterol such as 25-hydroxycholesterol (25OHC) on various aspects of cellular cholesterol metabolism including esterification via acyl:cholesterol acyltransferase (ACAT), synthesis via HMG CoA reductase and LDL receptor expression.

have been investigated extensively during the last two decades. Earlier studies by Bell et al.[57] revealed that the addition of 250HC or 7-ketocholesterol to cultured human hepatoma HTC cells at a concentration of 2.3 μg/ml reduced the half life of HMG-CoA reductase from 3 to 4 hr to 24 to 36 min. Studies by Tanaka et al.[56] showed that 250HC caused a 4 fold increase in the rate of the reductase degradation within 90 min. Interestingly, this oxysterol-enhanced HMG-CoA reductase turnover could be largely suppressed when protein synthesis inhibitors, either cycloheximide or actinomycin D, were added.[58,59] These data suggest that oxysterols suppress HMG-CoA reductase by enhancing protein degradation.

Another important finding concerning the mechanisms of (oxy)sterol-mediated reductase degradation emerged from studies by Gil et al.[60] Using DNA engineering techniques, the investigators expressed a truncated HMG-CoA reductase protein that lacked the membrane-anchoring domain in a mutant CHO cell (UT-2 cells). This truncated protein retained normal enzymatic activity but was no longer anchored in the endoplasmic reticulum; its rate of degradation was substantially decreased (by 80%) and was no longer accelerated by oxysterol treatment. Thus, the loss of (oxy)sterol-enhanced degradation in this truncated protein suggested that the basis for both normal and oxysterol-enhanced degradation was attachment of the reductase protein to the ER membrane. Based on these findings, it was speculated that the presence of oxysterols in the ER membrane may (1) alter the physical interaction between the membrane and the reductase protein, triggering fast turnover or (2) activate other membrane-bound enzyme(s) that, in turn, accelerate the degradation of the reductase protein.

Other studies have suggested that oxysterols are capable of inhibiting the synthesis of HMG-CoA reductase at transcriptional and post-transcriptional levels. Pretreatment of chinese hamster ovary cells with 250HC blocked the incorporation of radiolabelled methionine into immunoprecipitable HMG-CoA reductase polypeptide;[61] in mutant cells with enhanced HMG-CoA reductase expression, such treatment virtually blocked any incorporation.[62] In agreement with these observations, Trzaskos et al.[63] reported that addition of 250HC to cultured CHO cells resulted in rapid and parallel decreases in the levels of reductase mRNA and the rate of protein synthesis. These 250HC-mediated changes in the transcription and translation of the reductase also required protein synthesis.

Oxysterols and cholesterol esterification

Many oxysterols are potent regulators of intracellular cholesterol esterification. As depicted in *Figure 4,* oxysterols in general are potent activators of cholesterol esterification. In the early 1970s, Brown and Goldstein[64] reported that incubation of cultured human fibroblasts with oxysterols markedly increased cellular cholesteryl ester content as a consequence of markedly increased activity of the membrane-bound acetyl CoA:cholesterol acyltransferase; 250HC concentrations as low as 5 μg/ml caused an 8 fold increase in the specific activity of the enzyme. Similar effects have been observed in rat hepatocytes,[52] rabbit intestinal cells,[65] bovine adrenal steroidogenic cells,[65] rabbit aortic smooth muscle cells,[66] as well as several transformed macrophage and intestinal cell types.[67] In rat hepatocytes, the incorporation of fatty acid into cholesteryl esters, but not triglyceride and phospholipid, was enhanced. Because cholesterol achieved comparable enhancement of ACAT only at much higher concentrations,[52] it has been speculated that the true activator of ACAT in mammalian cell may be an oxysterol rather than cholesterol itself.[52,68] According to Drevon et al.,[52] 250HC-treatment produced a 2 fold increase in ACAT activity in both intact rat hepatocytes and in the microsomes isolated from these cells, suggesting that the 250HC-mediated ACAT activation does not require the integrity of cells, and therefore is most likely not under transcriptional or translational control. This was supported by the studies of Field[66] and Kusuhara et al.[69] showing that addition of protein synthesis inhibitors did not reverse the stimulatory effect of 250HC on ACAT activity, suggesting that oxysterols modulate ACAT activity post-translationally.

Although not well understood, several possible mechanisms might be responsible for activation of ACAT. First, it is possible that binding of 250HC at ACAT causes a ligand-induced structural change in the ACAT protein that converts the enzyme from an inactive to an active form.[70] It has been speculated that the ligand-induced configurational changes may occur through interactions among multiple subunits in the ACAT holoenzyme because the molecular mass of the functional ACAT enzyme is approximately 220 kDa,[52] which is about four times the values estimated by SDS-PAGE.[71] Supporting this model is the concentration dependence of oxysterol-induced ACAT activation and the sigmoidal shape of the ACAT activity versus 250HC concentration curve, suggesting that the enzyme is under allosteric regulation.[71] The second possibility is that there may be an oxysterol-induced specific covalent modification of the ACAT protein, which, in turn, activates the enzyme. There has been evidence showing that inhibition of cellular phosphorylation and dephosphorylation reactions by kinase and phosphatase inhibitors could eliminate 250HC-induced ACAT activation,[67,72,73] suggesting a role for phosphorylation in activation or deactivation. Recently, Corton et al.[74] reported that microsomal ACAT activity from the rat liver was unaffected by incubation with the purified catalytic subunits of protein phosphatase 1, 2A, or 2C, the primary protein (serine/threonine) phosphatases in cytosol of mammalian cells, arguing that dephosphorylation does not seem to participate in the activation of ACAT activity. The third possibility is that oxysterols activate ACAT catalysis, not by acting on the ACAT protein, but rather, by facilitating translocation/delivery of cholesterol to the ACAT catalytic site(s). Data accumulated over the past few years suggests a clustered distribution of cholesterol or specific domains within the cell membranes,[75] which could be disrupted by the ability of oxysterols to modify packing of cholesterol and phospholipids in membranes.[76,77] This idea is supported by the studies of Tabas et al.,[78] in which incubation of macrophages with 250HC led to a shift of cellular cholesterol from a cholesterol oxidase-accessible pool, presumably plasma membrane cholesterol, to an inaccessible pool.

Oxysterols and fatty acid metabolism

The modulation of cellular fatty acid metabolism by oxysterols is another example of the multiple functions of oxysterols. In 1990, Seillan[79] reported that various oxysterols produced different effects on fatty acid distribution in cultured bovine aortic smooth muscle cells. Both 250HC and 22α-OHC treatment enhanced the incorporation of radiolabelled fatty acids into triacylglycerides, with a concomitant reduction in the labelling of phospholipids. Structurally different oxysterols, 20-OHC and cholestanetriol had the opposite effect. In contrast, the work of Drevon et al.[52] suggested that 250HC enhanced incorporation of fatty acid specifically into cholesteryl esters and not triglyceride or phospholipid in cultured fibroblasts.

In addition to their effects on cellular fatty acid distribution, oxysterols have been shown to modify the cellular metabolism of arachidonic acid and its oxygenated metabolites, the eicosanoids. In 1988, La Housa[80] reported that oxysterols potentiated serum-induced arachidonic acid release and prostaglandin biosynthesis in NRK 49F cells, a rat kidney derived fibroblast cell line. Among the oxysterols tested, 7α- and 7β-OHC were more potent than 250HC. The rapid increase in arachidonate release was not accompanied by increased cyclooxygenase activity or cellular free calcium levels. It has been speculated[81] that oxysterols may activate phospholipase A2 or protein kinase C or, alternatively, insert into the plasma membrane and alter enzyme activity.

Oxysterols and LDL receptor gene expression

Most non-hepatic cells meet their requirements for cholesterol by uptake of LDL cholesterol via a specific transmembrane protein, the LDL receptor.[46] Expression of the LDL receptor is controlled by the cell's need for cholesterol, with increased gene transcription when cellular cholesterol is needed and decreased mRNA synthesis when intracellular cholesterol accumulates. As illustrated in *Figure 4,* many oxysterols are potent inhibitors of LDL receptor gene expression. In 1975, Brown and Goldstein[82] reported that prior incubation of cultured human fibroblasts with cholesterol, 250HC, or LDL progressively reduced the ability of cells to bind radiolabelled LDL at the high affinity site; kinetic studies indicated that this reduced binding was due to a decrease in the number of LDL receptors. Studies by Ellsworth et al.[83] demonstrated that incubation of Hep G2 cells with 25 μM 250HC for 24 hr decreased the mRNA level for LDL receptors by 40 to 50%; because its half life was unaltered, the decrease in LDL receptor was due to reduced transcription of the LDL gene.

The initial focus in understanding how oxysterols regulate LDL gene expression has been on the DNA sequences adjacent to the LDL receptor encoding gene which regulate its transcriptional expression (*cis*-elements) and to identify the nuclear proteins which interact with the *cis*-elements to modify gene expression (*trans*-elements). The cloning of the human LDL receptor gene and subsequent expression in CHO cells revealed that a 42-bp promoter sequence in the 5′-flanking region of the receptor gene controls the expression of the receptor-coding region in a sterol-responsive manner.[84] Within this 42-bp sequence, two contiguous 16-bp direct repeat sequences (or repeats), designated as repeat-2 and repeat-3, were identified. When introduced separately into the promoter region of a chimeric viral thymidine kinase reporter gene, repeat-3 constitutively promoted transcription of the reporter gene regardless of whether (oxy)sterols were present, whereas repeat-2 strongly suppressed repeat-3 when sterols (10 μg/ml cholesterol plus 0.5 μg/ml 250HC) were present.[85] By point mutation, Smith et al.[86] revealed that a 10-bp sequence within repeat-2 was essentially responsible for the sterol-mediated suppression of the LDL-receptor gene expression. This 10-bp sequence, designated as sterol regulatory element-1 (SRE-1), was also found in the 5′-flanking regions of the HMG-CoA reductase[87,88] and synthase genes.[84–86] These findings are consistent with the observations of Molowa et al.[89] that treatment of Hep G2 cells with mevalonate, 250HC, LDL cholesterol or Lovastatin altered the transcriptional expressions of the genes for HMG-CoA reductase, synthase, and LDL receptors in a coordinated fashion. Similar results have been seen in a line of 250HC-resistant CHO cells in which the mRNA levels of all three proteins increased dramatically, and were not suppressed by 250HC treatment.[90,91]

The SRE-1 in the promoter region of all three genes contains a common consensus sequence resembling 5′-CACC(C/G)CAC-3′. This consensus sequence, however, plays different regulatory roles on different genes. For instance, this consensus sequence serves as an enhancer to increase the transcription of HMG-CoA synthase and LDL receptor genes in the absence of (oxy)sterols, and is inactivated when (oxy)sterols are present.[86,87] On the other hand, the SRE-1 consensus sequence serves as a suppressor of the transcription of the HMG-CoA reductase gene in the presence of (oxy)sterols.[88] Apparently, this difference in transcriptional activity is not due to the structural differences between various *cis*-elements, but rather, reflects the importance of nuclear-binding proteins (*trans*-elements) in controlling transcription of these genes.

In 1988, Dawson et al.[85] reported that in the promoter region of the LDL receptor, the repeat-3, but not the repeat-2 sequence, contains a specific binding sites for Sp1, a known nuclear binding protein. Mutation at the repeat-3 abolished the binding of Sp1 to the sequence and the promoter activity simultaneously. However, subsequent experiments revealed that in the absence of SRE-1 (in the repeat-2), the binding of Sp1 to repeat-3 was not sufficient to achieve high levels of transcription and the regulation was not sterol-responsive.[85,92]

Among several possible proteins examined as sterol-responsive *trans*-elements, the oxysterol-binding protein (OBP) has received much attention. OBP was first identified because of its high binding affinity to radiolabelled oxysterols in mouse fibroblast-like L cells by Kandutsch and Thompson[93] in the late 1970s, and then purified to homogeneity by Dawson et al.[94] from hamster liver cytosol in 1989. On SDS-PAGE, OBP contained a doublet of peptides with molecular weights of 101 kDa and 96 kDa. Both the 100 kDa and 96 kDa proteins were shown to be encoded by a single OBP gene cloned from a rabbit cDNA library, and specifically bound to an anti-OBP antibody derived from an OBP tryptic peptide, suggesting that they are closely related

and one may be a modified or proteolyzed form of the other. Earlier studies by Taylor et al.[95] revealed a strong, positive correlation between the binding of oxysterols to OBP and their potency in suppressing HMG-CoA reductase activity. Based on these findings, as well as the evidence that OBP binds to 250HC specifically and with high affinity,[96] it has been proposed that OBP may be one of the sterol-responsive *trans*-elements contributing to the regulation of LDL receptor gene expression. Unexpectedly, however, subsequent studies by Ridgway et al.[97] revealed that upon addition of 250HC, OBP translocated from the cytosol to the Golgi apparatus rather than to the cell nucleus. This finding suggests that OBP most likely functions as an intracellular oxysterol transport protein that delivers oxysterols between intracellular organelles rather than as a sterol-responsive *trans*-element.

Further studies[92,98] have identified a 38 kDa protein, designated as sterol regulatory element binding protein-1 (SREBP-1), from nuclear extracts of human HeLa cells, which specifically recognizes the SRE-1 sequence in the LDL receptor gene promoter region. The binding of this protein correlated perfectly with the sterol-regulated transcription activity, indicating that SREBP-1 is indeed a sterol-responsive *cis*-element. SREBP-1 is synthesized as a 125 kDa precursor that is anchored in the nuclear envelope and that endoplasmic reticulum is cleaved to generate a soluble N-terminal fragment of 68 kDa. According to Wang et al.[99] the addition of (oxy)sterols (10 μg/mL cholesterol plus 1 μg/mL 250HC) reduced the transcription of the LDL receptor gene by inhibiting the cleavage of the 125 kDa SREBP-1 precursor, as well as increasing the degradation of the 68 kDa functional SREBP-1 protein. This proposal is supported by the observation that addition of an inhibitor of neutral cysteine protease abolished the catabolism of the 68 kDa protein, and restored the transcription of the sterol-regulated genes.

Oxysterols and cellular membrane effects

Another aspect of cellular cholesterol homeostasis is removal of cellular cholesterol and transport to the liver for further catabolism, the so-called process of reverse cholesterol transport. Recent studies by Kilsdonk et al.[53] indicated that oxysterols are also capable of suppressing the efflux of cell membrane cholesterol to extracellular acceptors such as HDL_3, synthetic HDL-like particles, and small unilammellar vesicles. The effect of 250HC on cholesterol efflux was dose-dependent, with significant effects seen at concentrations as low as 50 ng/mL, despite the continual removal of 250HC by the same acceptors and even albumin. It was also independent of a shift in the cellular ester to free cholesterol ratio because inhibition of ACAT had no effect. Changes in membrane characteristics or domains in membranes may explain the effect at least in part, because partial inhibition was observed with isolated membranes.

Whereas in a phospholipid bilayer, membrane fluidity depends on the length of fatty acyl chains and their degree of unsaturation, the presence of cholesterol in eukaryotic cell membranes adds another degree of regulation of membrane fluidity. Cholesterol inserts into bilayers with its long axis perpendicular to the plane of the membrane; it prevents the crystallization of fatty acyl chains by fitting between them but sterically blocks the excess motion of fatty acyl chains.[100] Like cholesterol, many oxysterols can insert into membranes and modulate their fluidity. In erythrocytes, the insertion of oxysterols into the membrane resulted in a shrinking of the cells (echinosis) and an increased resistance of the erythrocytes to osmotic lysis.[101] These changes indicated that insertion of oxysterol into the red cell membrane resulted in an expansion of the surface area of the membrane and, therefore, an increase of the hemolytic volume of the cell.[102] Using Fourier transform infrared and Raman spectroscopy, Rooney et al.[103] have shown that insertion of an oxysterol into human erythrocyte membranes at 10% of total membrane sterol immobilized lipid acyl chains to a degree equivalent to enriching total membrane cholesterol by 50%. The addition of oxysterols to dioleoylphosphatidylcholine (DOPC) to form a mixed monolayer reduced the heat content required to achieve the gel to liquid crystalline phase transition as monitored by differential scanning calorimetry. In addition, whereas 7-ketocholesterol and 7-hydroxycholesterol displayed condensing effects on DOPC packing and reduced the permeability of DOPC liposomes to glucose, the addition of 250HC behaved like a "spacer" molecule and increased the permeability of liposomes to glucose.[76]

Oxysterols also exert profound effects on membrane cholesterol metabolism. The addition of oxysterols to the cholesterol-containing lipid bilayer potentiated the cholesterol-facilitated phospholipid gel-to-liquid crystalline phase transition in a synergistic fashion,[76] suggesting that oxysterols may disturb the interaction between cholesterol and membrane phospholipids, rendering cholesterol more effective.

Because of their direct effects on membrane structure, it is not surprising that many oxysterols potently modulate the enzymatic activity of many membrane-associated proteins. It has been shown that oxysterol treatment suppressed the rate of endocytosis in both L-cells and mitogen (Con A)-stimulated T-lymphocytes,[104] decreased the activities of plasma membrane 5′-nucleotidase and Na^+/K^+ ATPase activities in rabbit aortic smooth muscle cells[105] and inhibited membrane Ca^{++}/Mg^{++} ATPase activity in bovine aortic smooth muscle cells.[106] The oxysterol (250HC)-induced suppression of endocytosis was caused by the depletion of membrane cholesterol, because it could be reversed by cholesterol supplementation.[107] In contrast, inhibition of membrane 5′-nucleotidase and Na^+/K^+ ATPase activities by oxysterol (cholestane-3β, 5α, 6β-triol) could be demonstrated in both intact cells as well as the isolated plasma membranes, suggesting that oxysterol exert this regulatory effect locally within the membranes.[105] According to Rooney et al.[103] insertion of 7α-OHC into human erythrocyte membrane (10% of total membrane sterol) resulted in an increased membrane protein helical structure, comparable to that observed for erythrocyte membranes enriched with pure cholesterol by 50%. Similar effects were also seen when membrane Ca^{++}/Mg^{++} ATPase activity was inhibited in the presence of 250HC. The suppression of membrane Ca^{++}/Mg^{++} ATPase activity, together with an increased membrane permeability to ionic particles upon oxysterol treatment, led to a marked elevation in the concentration of

intracellular calcium. Because intracellular calcium plays such an important role in signal transduction, it is conceivable that the oxysterol-induced cell responses are complex and multidimensional.

Thus, oxysterols exhibit profound regulatory effects on both the structure and function of biological membranes. They achieve these effects by (1) inserting into the phospholipid bilayer, thereby modulating the interaction among membrane lipids as well as the secondary structure of membrane embedding proteins, and (2) depleting membrane cholesterol.

Possible role(s) of oxysterols in disease processes

Atherosclerosis

A strong and positive correlation between serum cholesterol levels and the incidence of atherosclerosis has been appreciated for several decades, and chronic hypercholesterolemia is thought to be the first and most important risk factor for atherosclerosis. However, because most oxidized derivatives of cholesterol are more atherogenic than pure cholesterol in vivo,[9,43] more potent in producing lipid loading of cells in vitro,[108,109] and present in LDL oxidized in vitro[10] and present in elevated concentrations in hypercholesterolemic serum,[9,22] it is possible that oxysterols may play an even more important role than cholesterol in the pathogenesis of atherosclerosis.

Several lines of evidence suggest that oxysterols contribute to the development of atherosclerosis. That oxysterols are much more potent than cholesterol in causing vascular endothelial damage and inducing plaque formation was shown by Imai et al.[110] in studies where New Zealand White rabbits fed newly purified cholesterol (1 g/kg × 7 weeks) developed significantly less atherosclerotic lesions than those fed with unpurified cholesterol; the impurities were identified thin layer and gas chromatography as 250HC, 7α- and 7β-OHC, and small amounts of cholestane-3β, 5α, 6β-triol.[111] Intravenous injection of a mixture of 250HC and cholestanetriol (2.5 mg/kg body weight) produced similar vascular endothelial cell damage but with more acute morphological characteristics such as severe endothelial cell degeneration, intimal edema, as well as infiltration of neutrophils and macrophage-monocytes.[112] In other studies, White Carneau pigeons fed a diet containing 0.05% cholesterol plus a trace amount of cholestane-3β, 5α, 6β-triol for 3 months developed much more severe atherosclerosis than those fed pure cholesterol alone.[7] Furthermore, inhibition of lipoprotein peroxidation in vivo by administration of antioxidants such as probucol to cholesterol-fed rabbits reduced the levels of plasma oxysterols and plasma oxidized LDL concomitantly with reducing the extent of atherosclerosis.[12]

The toxic effects of oxysterols have also been demonstrated in vitro. Incubation of cultured rabbit aortic smooth muscle cells with 2 to 10 μM 250HC and cholestane-3β, 5α, 6α-triol inhibited cell growth and suppressed the de novo synthesis of cellular cholesterol.[108,112] In cultured human endothelial cells, 7β-OHC treatment (50 μM) enhanced uptake of Trypan blue dye and release of the cytosolic enzyme lactate dehydrogenase. Several recent studies have demonstrated a causal relationship between the occurrence of oxysterols in oxidized LDL and its cytotoxicity.[10,11]

It is also clear that significant quantities of various oxysterols and their esterified counterparts are found in the aortic tissues of hypercholesterolemic and atherosclerotic subjects.[23–26] Using HPLC and GC-mass spectroscopy, 250HC, 260HC, 7-OHC and their palmitate, and oleate mono- and diesters have been identified in human aortae. Recently, Carpenter et al.[37] reported that the content of 260HC and 7β-OHC were significantly higher in atherosclerotic aortae than in normal aortae; in atherosclerotic aortae, the amount of 260HC was proportional to that of cholesterol deposited in the tissues, revealing a positive correlation between the quantity of oxysterols in the lesions and the degree of atherosclerosis.

Although the aforementioned studies suggest that oxysterols are atherogenic molecules, it has also been speculated however, that the conversion of cholesterol to oxysterols may actually facilitate the excretion of excess cellular cholesterol in a more polar form.[18] In human alveolar macrophages, cholesterol enrichment activated 27-hydroxylase, catalyzing conversion of cholesterol to more polar 270HC, which was rapidly and almost completely removed from the cell in the presence of serum. The finding that 27-hydroxylase activity is found, not only in hepatocytes, but also in a number of cell types, including macrophages, fibroblasts, and endothelial cells,[14,18] suggests that the transformation of cholesterol to polar oxysterols is not uncommon. It remains to be determined whether oxysterols found in vivo are pro-atherosclerosis factors or represent ongoing lesion regression.

Cancer

Oxysterols have been implicated as a double edge sword in the area of cancer, perhaps playing roles in carcinogenesis and in cancer suppression. The earliest experimental evidence for the possibility that cholesterol oxidation products are carcinogenic emerged from studies in which ovariectomized mice injected subcutaneously with purified preparations of progesterone developed less cancer than those injected with an impure progesterone; analysis of the crude progesterone revealed that each mouse had received 20 μg of cholesterol oxidation products.[113] In further experiments, injection of various oxysterol derivatives into mice or rats led to sarcoma formation at an incidence of 10 to 60% in different experiments, significantly higher than incidence in vehicle-treated animals. Using a mouse model of skin cancer, Black et al.[114] demonstrated a positive correlation between the formation of cholesterol-α-epoxide and the incidence of skin cancer. In epidemiological studies, an increase in gastrointestinal cholestane-3β, 5α, 6β-triol was linked to patients with adenomatous polyps of the colon (a precancerous lesion) or those with colon cancer.[115] Progressively increasing concentrations of cholesterol and cholesterol β-epoxides have been observed in women with normal, hyperplasial, and hyperplasial with atypical mammary epithelium, respectively.[116] The incidence of all types of benign tumors was 8.5 times more common in women who had detectable β-epoxide in their breast fluid as compared with those with no detectable amounts.[117] In vitro studies have

shown that cholesterol α-epoxides had significant, although relatively weak, mutagenic effects in vitro.[188]

In contrast to these observations are those suggesting that oxysterols may be effective in killing cancerous cells. In 1977, Cheng et al.[119] isolated 7β-OHC from a putative Chinese anticancer drug Bombyx cum Botryte, and found it effective in killing human hepatoma cells. In subsequent studies, 7β-OHC displayed highly preferential toxicity to cancerous and proliferating cells. Whereas confluent astrocytes cultures were unaffected by concentrations of 7β-OHC up to 30 mM, spontaneously transformed cell lines were killed by concentrations as low as 20 μM.[120] Interestingly, this cancer-killing effect was augmented after the oxysterol was further metabolized by the transformed cells to yield esters with naturally occurring fatty acids at the C3 position. In an effort to develop a novel anticancer drug, Rong et al.[121] synthesized a water-soluble derivative of 7β-OHC, namely, the sodium salt of the bis-hemisuccinate 7β-OHC, and found that it also displayed marked antitumor activity in vivo in mice bearing Krebs II carcinoma. These studies contrast with others that have suggested that oxysterols exhibit anti-proliferative effects on cancer cells through binding to anti-estrogen binding sites[122] or through their inhibitory effects on de novo cholesterol biosynthesis.[123] Clearly, further work is required to understand these processes.

Summary

It is clear that oxysterols comprise a class of potent compounds with widespread effects on the metabolism of cholesterol and other lipids as well as on the structure and function of cellular membranes. The metabolism of these compounds is in many ways analogous to that of cholesterol, but with some significant differences including association with serum albumin, rapid efflux from cells, and much more potent effects on cellular cholesterol homeostasis than cholesterol itself. A summary of these actions is shown in *Table 1*. There are also paradoxical indications that oxysterols may contribute to the pathogenesis of atherosclerosis or may reflect the ongoing battle against atherosclerosis. Likewise, they may have effects that could contribute to the development of certain cancers or may be effective reagents in the treatment of cancer. Further studies will hopefully delineate the role(s) of oxysterols in disease processes and the relative importance of dietary, peroxidation or enzymatically derived oxysterols.

Table 1 Summary of oxysterol actions

	Effect	Mode of action
Cholesterol metabolism	↓ cholesterol synthesis	HMG CoA reductase
	↑ cholesterol esterification	ACAT
	↓ LDL uptake	LDL receptor expression
	↓ cholesterol efflux	plasma membrane (?)
Cell function	toxicity	cholesterol depletion (?) membrane destabilization (?)
	↓endocytosis	cholesterol depletion
	↓plasma enzyme activity	↑fluidity of membrane
	↑ prostaglandin synthesis	enzyme activation (?)

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