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Role of Oxysterols in the Regulation of Cholesterol Homeostasis: A Critical Evaluation†

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Introduction

There exists a wealth of knowledge on cholesterol synthesis and metabolism. Research in this area has been awarded a number of Nobel prizes during the last five decades. Maintenance of cholesterol homeostasis is very important from a clinical point of view, and disturbances in this homeostasis may have serious consequences. Research during the last decades has focused on the key enzymes involved in cholesterol synthesis and metabolism. Although there exists a lot of general knowledge regarding the mechanisms involved in this regulation, there are still several unclear points. The importance of oxysterols is one of the uncertainties. Many oxysterols have a high potential to downregulate the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase (for a general review, see ref 3). Since oxysterols are formed in biological systems by both enzymatic and nonenzymatic mechanisms, it has been suggested that they may be important physiological regulators of cholesterol homeostasis.[4,5]

In the present Account the "oxysterol hypothesis" will be critically examined and results of some recent experiments will be reviewed.

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Ingemar Björkhem was born in 1941 in Lund, Sweden. He started to study medicine in 1961 at the Karolinska Institute and got his basic training in biochemistry at the department of chemistry at this institute. He received M.D. and Ph.D. degrees in 1969. In 1986 he was appointed professor of "Biochemical Research on Atherosclerosis" at the Karolinska Institute, a position that he still holds. He is also head of the Clinical Research Center at the Huddinge Hospital. His research interests have broadened from basic studies on the mechanism of formation of bile acids from cholesterol to research on regulation of key enzymes involved in cholesterol synthesis and degradation and atherogenic mechanisms.

Formation of Monooxygenated Oxysterols

Monooxygenated derivatives of cholesterol may be formed directly from cholesterol by autoxidation or by the action of a specific monooxygenase. Another possibility is that formation is secondary to enzymatic or nonenzymatic lipid peroxidation. The various structures that will be referred to in this text are depicted in Figure 1.

Autoxidation of Cholesterol. This subject has been thoroughly reviewed and will not be discussed here.[3,6] It can be concluded that the major autoxidation products are the 7-oxygenated compounds **2**−**6** and the 5,6-oxygenated compounds **7**−**9**. To a lesser extent, 20- and 25-hydroxycholesterol are also formed.

Enzymatic Formation of Oxysterols. Enzymatic formation of oxysterols can be divided into two classes: (a) direct enzymatic action on cholesterol or another related sterol and (b) enzymatic activity leading to the formation of radicals which in turn attack cholesterol. All reactions of the first type seem at present to be cytochrome P-450 dependent.

One of the most important enzymes of type a is the hepatic cholesterol 7α-hydroxylase, which catalyzes 7α-hydroxylation of cholesterol. This is the initial and rate-determining reaction in mammalian bile acid biosynthesis (for a review, see ref 7). This enzyme is

† Nomenclature and abbreviations: A *sterol* is a steroid containing a hydroxyl group at C-3 and most of the skeleton of cholestane. Additional carbons may be present in the side chain.[1] An *oxysterol* is a sterol containing one or more additional oxygen functionalities. Closely related compounds may also be called "oxysterols". Note: The name 27-hydroxycholesterol has been used for the compound (25*R*)-cholest-5-ene-3β,26-diol according to recent terminology.[2] The mitochondrial hydroxylase forming 27-hydroxycholesterol is accordingly named sterol 27-hydroxylase (previously called sterol 26-hydroxylase). Abbreviations: HMG-CoA, hydroxymethylglutaryl coenzyme A; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; LDLR, LDL receptor; ER, the endoplasmic reticulum; EPR, electron paramagnetic resonance; CHO, Chinese hamster ovary.

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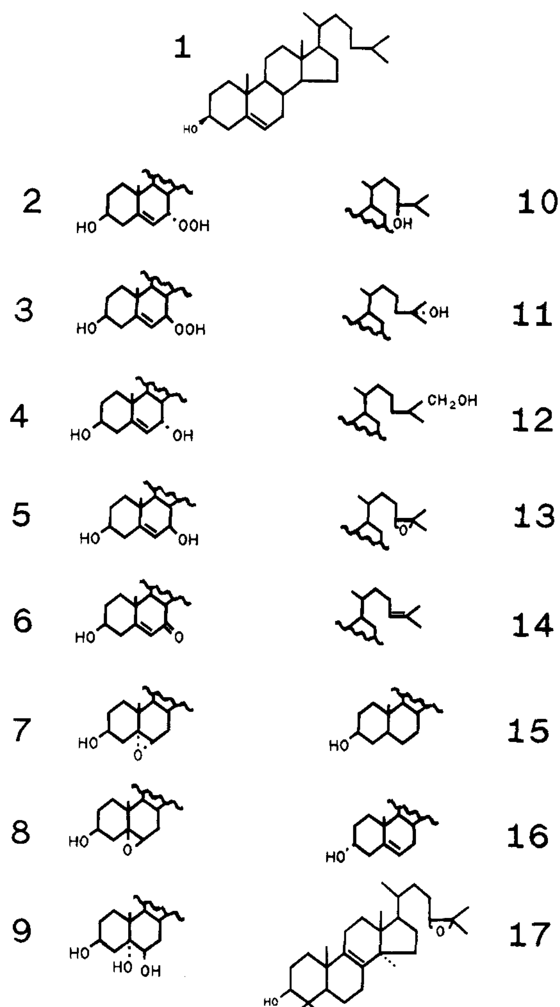

Figure 1. Structure of cholesterol and various oxysterols: **1**, cholesterol; **2**, 7α-hydroperoxycholesterol; **3**, 7β-hydroperoxycholesterol; **4**, 7α-hydroxycholesterol; **5**, 7β-hydroxycholesterol; **6**, 7-oxocholesterol; **7**, cholesterol 5α,6α-epoxide; **8**, cholesterol 5β,6β-epoxide; **9**, cholestane-3β,5α,6β-triol; **10**, 24-hydroxycholesterol; **11**, 25-hydroxycholesterol; **12**, 27-hydroxycholesterol; **13**, 24,25-epoxycholesterol; **14**, desmosterol; **15**, cholestanol; **16**, epicholesterol; **17**, 24,25-epoxydihydrolanosterol.

located only in the liver. Thus, product **4** may have a dual origin, arising from autoxidation as well as from enzymatic monooxygenation. However, autoxidation always yields a mixture of **4** and **5**. Also 20-hydroxycholesterol can be formed both by autoxidation[3] and by enzymatic means.[8] 20(*S*)- and 22(*R*)-hydroxycholesterol are intermediates in the synthesis of steroid hormones (for a review, see ref 3, p 318).

Other enzymatic side chain hydroxylations of cholesterol include the formation of 24-hydroxycholesterol, for example, in rat liver mitochondria[9] and rat[10] and bovine[11] brain microsomes. The enzyme or enzymes responsible for the 24-hydroxylation have not been identified, although pig liver mitochondrial 27-hydroxylase has some 24-hydroxylase activity.[12] The mitochondrial 27-hydroxylase has a broad substrate specificity and is found in many different organs in the body (for a review, see ref 13). Cholesterol is one of the substrates of this enzyme, which plays an important role in bile acid biosynthesis. 25-Hydroxylation of cholesterol is commonly encountered in liver mitochondrial preparations together with the dominating 27-hydroxylation.[14] It is not yet known whether a specific 25-hydroxylase exists or the reaction is catalyzed by the 27-hydroxylase, possibly after a posttranslational modification of the enzyme.[13] 24-, 25-, and 27-hydroxycholesterols **10**−**12** accumulate in mouse liver during cholesterol feeding,[15] most probably as a consequence of enzymatic reactions.

It has been shown that incubation of the cholesterol precursor mevalonate with rat liver homogenate under conditions where cyclization of squalene 2,3-epoxide to lanosterol is blocked results in accumulation of squalene 2,3:22,23-diepoxide.[16] When the latter is incubated with rat liver microsomes, 24(*S*),25-epoxycholesterol (**13**) is formed.[17] It was later shown that this compound is also formed in rat liver homogenates from mevalonate when squalene epoxide cyclization is not blocked,[18] representing an alternate pathway for the formation of oxysterols. 24(*S*),25-Epoxycholesterol has been found in human liver in surprisingly high amounts.[19] This so-called squalene dioxide pathway was recently reviewed in *Accounts*.[20]

Cholesterol 5α,6α-epoxide (**7**) is mostly associated with autoxidation, but has also been shown to be enzymatically formed in different systems.[21−23] It is also formed by the action of agents such as hydroxyl radical or hydrogen peroxide on cholesterol.[3]

Formation of Oxysterols as a Consequence of Enzymatic and Nonenzymatic Lipid Peroxidation. Lipid peroxidation is a special form of autoxidation, where the primary target molecules are polyunsaturated fatty acids and their derivatives. These compounds are more susceptible to peroxidation than cholesterol due to the presence of double-allylic hydrogens which are easily extracted as a result of stabilization of the radical formed. Cholesterol, a

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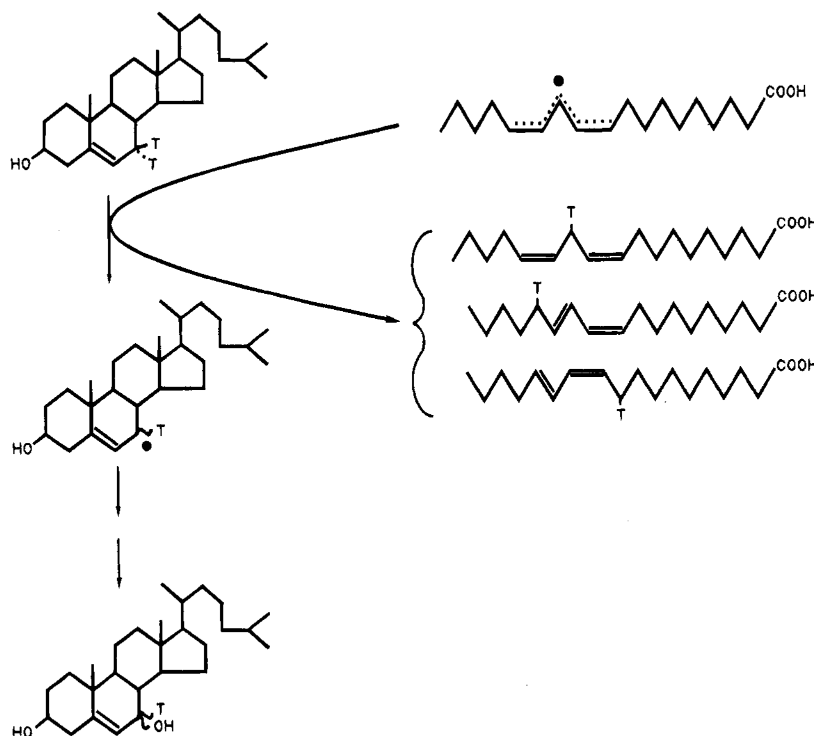

Figure 2. Abstraction of hydrogen from cholesterol by a linoleic acid radical in a lipoxygenase system. 7-[^{3}H]Cholesterol was incubated with linoleic acid and soybean lipoxygenase as described.[24] Some ^{3}H label could be reisolated in linoleic acid or an isomer thereof after the incubation.

major component in cell membranes together with the polyunsaturated membrane lipids, becomes a second target of the radical chain reaction leading to the formation of compounds **2**–**9**.

The mechanism of formation of 7-oxygenated cholesterol in a soybean lipoxygenase system was recently studied.[24] Incubation of cholesterol containing two atoms of deuterium in the 7-position with soybean lipoxygenase and linoleic acid in the presence of oxygen gave a mixture of all three 7-oxygenated products **4**–**6**. Conversion into 7-oxygenated products was associated with a very high intermolecular isotope effect; this suggests that the rate-limiting step in the overall conversion is likely to be the abstraction of hydrogen at C-7 in a radical reaction. Evidence that linoleic acid is to some extent directly involved was obtained with cholesterol labeled with tritium in the 7-position. Incubation of such a cholesterol resulted in a significant incorporation of tritium in the reisolated linoleic acid fraction. It was further shown that both the extraction of hydrogen from cholesterol and the subsequent addition of oxygen are nonstereospecific. It was thus concluded that the most important mechanism for oxidation of cholesterol at C-7 in the system involves participation of radicals and that a carbon-centered linoleic acid radical can extract hydrogen directly from cholesterol (Figure 2).

It is well established that liver microsomes in the presence of NADPH and in the absence of chelator are capable of oxidizing cholesterol and other 3β-hydroxy 5-unsaturated steroids yielding the common autoxidation products **4**–**9**.[25] In this case, the cholesterol oxidation is secondary to the enzymatic NADPH-dependent lipid peroxidations. The enzyme-catalyzed reaction is required, however, merely to reduce Fe^{III} to Fe^{II}, which in turn catalyzes ordinary autoxidation.[26] It has also been shown by EPR studies using spin traps that radicals are involved in these conversions.[27] The major radicals detected were lipid peroxyl radicals and superoxide, whereas only small amounts of hydroxyl radicals were detected. Lipid hydroperoxides are probably involved in the formation of cholesterol epoxides **7** and **8** by microsomal lipid peroxidation.[28]

Presence of Oxysterols in Different Biological Systems. Methodological Aspects

In most biological systems, oxysterols are present only in trace amounts; the ratio between oxysterols and cholesterol is often in the range 1/10 000 to 1/1000. A notable exception is atheromas, where the ratio may be more than 1/100 (unpublished).

Research aimed at examining the physiological importance of oxysterols has previously been hampered due to the lack of analytical procedures with sufficient sensitivity and accuracy. Since cholesterol exists at very high levels together with the oxysterols in all tissues, there is always a resulting artifactual formation of oxysterols during isolation and workup procedures unless very specific precautions are taken. We have developed specific and accurate methods based on isotope dilution–mass spectrometry for the most important oxysterols.[29,30] Individual deuterated standards are employed for the different compounds. When these methods are used for determination of the

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low levels of oxysterols present in the circulation, the most important limitation is related to the difficulties encountered in avoiding autoxidation.

The extent to which it is possible to avoid autoxidation is a matter of controversy. The levels of oxysterols reported to be present in the circulation have tended to decrease with increased advancements in technology. According to Kudo et al.,[31] most of the oxysterols previously detected in plasma are artifacts formed during isolation and workup. More recently, we have clearly shown that, in rat, 7α-hydroxycholesterol (**4**), 7β-hydroxycholesterol (**5**), 7-oxocholesterol (**6**), and 24-hydroxycholesterol (**10**), 25-hydroxycholesterol (**11**), and 27-hydroxycholesterol (**12**) are formed from cholesterol in vivo and are present both in liver and in the circulation.[32] This was achieved by exposure of rats to an $^{18}O_2$-containing atmosphere in a closed system. Under such conditions, ^{18}O can only be incorporated in monooxygenated oxysterols in vivo and any artifactual formation would result in an unlabeled product. Formation of oxysterols by mechanisms not involving molecular oxygen would also result in unlabeled products. We observed little or no incorporation of ^{18}O in the 5,6-oxygenated steroids. As a result, the possibility can not be excluded that most of the latter compounds are formed artifactually during workup of the sample.

Effects of Oxysterols on Enzymes Involved in Cholesterol Homeostasis. The "Oxysterol Hypothesis"

HMG-CoA Reductase (HMGR). In cells, oxysterols regulate HMGR transcriptionally and posttranscriptionally via degradation of the HMGR protein.[33] The activity of HMGR in various cell systems is suppressed by many oxysterols. The commonly found autoxidation products **4**–**9** have been shown to inhibit HMGR activity in mouse and human fibroblasts, human leukocytes, and Chinese hamster ovary cells (reviewed by Smith[34]). Most interest has, however, focused on the side-chain-hydroxylated products **10**–**12** and related compounds such as epoxides **13** and **17**.

The oxysterol hypothesis was originally formulated by Kandutsch and Chen on the basis of the observation that, in cultured cells, purified cholesterol is unable to suppress HMGR activity or sterol synthesis. Mutant CHO cells of several classes have been shown to resist regulation of HMGR by both LDL and 25-hydroxycholesterol.[35-37] 25-Hydroxycholesterol has been shown to mimic the action of LDL not only with regard to HMGR but also for other enzymes of importance in cholesterol biosynthesis.[35] It has therefore been suggested that oxysterols are physiological mediators of the suppression of HMGR by LDL.[5] Using cytochrome P-450 inhibitors, it has been suggested that cytochrome P-450 catalyzed oxidation of cholesterol is necessary for LDL-induced downregulation of HMGR.[38] It has also been suggested that oxysterols are supplied by LDL, originating from autoxidation of cholesterol or from dietary sources. However, as discussed above, human plasma contains only low levels of oxysterols[29,31] and, with the possible exception of 24- and 27-hydroxycholesterol, circulating oxysterols are most probably not of regulatory importance.

The LDL Receptor (LDLR) and HMG-CoA Synthase (HMGS). As a result of work carried out by Brown, Goldstein, and co-workers, the regulation of LDLR, one of the most important proteins in cholesterol homeostasis, is perhaps the most well characterized. The expression of LDLR is transcriptionally downregulated by oxysterols and LDL in intact cells.[33] Also, the regulation of LDLR by LDL can be blocked by cytochrome P-450 inhibitors,[39] indicating a potential intracellular formation of regulatory oxysterols.

HMGS is also transcriptionally downregulated by oxysterols and LDL. The regulation of LDLR and HMGS appears to be parallel, with the same transcription factors acting on the genes encoding these proteins.[40-42] The possible involvement of oxysterols in the regulation of these transcription factors, termed SREBP-1 and SREBP-2 (sterol regulatory element binding protein), has not been established. Some of the effects of oxysterols on HMGS are similar to those observed on HMGR, though the former enzyme has not been studied as thoroughly. For example, 25-hydroxycholesterol (**11**) and LDL inhibit HMGS activity in CHO cells, and in 25-hydroxycholesterol resistant cells both HMGS and HMGR activities are elevated.[35] Like HMGR, HMGS is regulated transcriptionally by sterols. However, in contrast to HMGR, the protein is not degraded by sterols.[36,43] The synthase is cytosolic whereas the reductase is membrane-bound.

Recently, Roger Davis et al.[44] proposed a regulatory mechanism linking LDLR, cholesterol 7α-hydroxylase, and oxysterols. On the basis of experiments using CHO cells transfected with cholesterol 7α-hydroxylase and selected for resistance toward 25-hydroxycholesterol, it was suggested that cholesterol 7α-hydroxylase could inactivate 25-hydroxycholesterol and as a result upregulate the expression of LDLR. However, no firm conclusions can be drawn from these experiments as control cells were not resistant toward 25-hydroxycholesterol and the assumed product, 5-cholestene-3β,7α,25-triol, was never identified. We later demonstrated that 25-hydroxycholesterol is not a substrate for cholesterol 7α-hydroxylase and that a specific

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oxysterol 7α-hydroxylase is involved in the metabolism of 25-hydroxycholesterol.[45]

Acyl-CoA:Cholesterol Acyltransferase (ACAT). Much less is known about the regulation of ACAT than of HMGR, HMGS, and LDLR. ACAT is a hydrophobic integral membrane protein which has never been purified to homogeneity in an active form. From early studies, it has been reported that 25-hydroxycholesterol is a potent stimulator of cholesterol esterification in intact cells.[46] Also LDL stimulates cholesterol esterification. The stimulation of ACAT by 25-hydroxycholesterol or LDL has been shown to be insensitive to the protein synthesis inhibitor cycloheximide,[47] indicating that ACAT can be regulated posttranslationally by sterols. There are indications that activation of ACAT by oxysterols involves protein phosphorylation.[48]

More recently, human ACAT has been functionally expressed in ACAT-deficient CHO cell mutants.[49] At present, it is not known if the protein encoded by the cDNA used for the expression constitutes the entire enzyme. Interestingly, the expressed protein was recently shown to be activated by cholesterol as well as by 25-hydroxycholesterol in a cell-free system.[50] 25-Hydroxycholesterol but not cholesterol was also shown to activate this protein in a certain line of intact insect cells.[50] Why cholesterol fails to activate ACAT in intact cells when added to the cell medium can at present only be a matter of speculation.

Role of Oxysterol-Binding Protein in the Regulation of Cholesterol Homeostasis

The observation that several oxygenated cholesterol derivatives regulate HMGR led to a search for a protein that could mediate these effects. A candidate was found with affinities for different oxysterols that closely correlated the potency of the oxysterol to suppress HMGR in isolated mouse fibroblast cell cultures.[51,52] In an effort to elucidate the metabolic role of this protein it was purified from hamster liver cytosol[53] and the corresponding cDNA cloned both from rabbit[54] and human.[55] By overexpression of the protein in CHO cells coupled to an immunofluorescence assay, Ridgway et al. were able to examine the distribution of the protein.[56] Under normal conditions, the protein was mainly located in the cytoplasm with a small amount present in vesicles near the nucleus. Addition of 25-hydroxycholesterol led to a marked change in the distribution of the protein with the majority of it bound to the Golgi apparatus. Interestingly, the protein mRNA was expressed in all animal cell lines and hamster tissues examined,[56] suggesting that some vital function may be associated with it.

Taylor[57] studied the ability of oxysterols to promote the degradation of HMGal, a fusion protein consisting of the membrane-spanning domain of HMGR and β-galactosidase constructed in Simoni's laboratory.[58] The degradation of this protein is supposed to mimic the controlled degradation of HMGR, one of the mechanisms by which this enzyme is regulated. It was shown that good correlation exists between the binding affinity of different oxysterols to the oxysterol-binding protein and degradation of HMGal and HMGR activity.[57] However, no *direct* evidence has been presented to support a role for this protein in the regulation of cholesterol homeostasis.

Attempts To Identify the Oxysterol(s) Believed To Be Involved in the Regulation of HMG-CoA Reductase

According to current concepts, regulatory oxysterol or oxysterols are formed intracellularly rather than internalized from LDL. There are some indications that the formation of these steroids may be dependent upon cytochrome P-450. Gupta et al.[38] have shown that in a specific line of cultured cells the cytochrome P-450 inhibitor ketoconazole totally abolishes the suppressive effect of LDL on HMGR. Thus, it was concluded that the putative regulatory oxysterols are formed via a cytochrome P-450 dependent pathway.[38,59,60] It has also been demonstrated that ketoconazole abolishes the suppressive effect of LDL on LDLR expression, while 25-hydroxycholesterol is suppressive even in the presence of ketoconazole.[39]

No agreement has been reached regarding the identity of the regulatory oxysterol(s), and several candidates have been proposed. All of these compounds have an oxygen function in the side chain in common, and all are likely to be dependent upon cytochrome P-450 for their formation. The mitochondrial 27-hydroxylating cytochrome P-450 is active on cholesterol and is expressed in most tissues.[13] It has also been shown that the levels of mRNA for this enzyme parallel the cholesterol biosynthesis capacity in different tissues.[61] In accordance with this, 27-hydroxycholesterol (**12**) has been suggested to be a regulator of HMGR by several groups.[62-64]

Recently, we attempted to evaluate the possible role of the sterol 27-hydroxylase in the downregulation of HMGR activity by dietary cholesterol in mouse liver.[65] Mouse liver mitochondria are able to catalyze 27-hydroxylation of cholesterol. When cholesterol labeled

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with deuterium in the 26- and 27-positions was used as substrate, a kinetic isotope effect was observed for the 27-hydroxylation. Use of this deuterium-labeled cholesterol thus allowed a specific suppression of 27-hydroxylation. Feeding mice with unlabeled pure cholesterol inhibited the hepatic HMGR. When the mice were fed with the same amount of deuterium-labeled cholesterol, the degree of suppression was the same as when unlabeled cholesterol was used. If mitochondrial 27-hydroxylation was important, reduced suppression of HMGR when the mice were fed deuterated cholesterol would have been expected due to the isotope effect.

Additional evidence against the role of 27-hydroxycholesterol as an important regulator of cholesterol homeostasis comes from studies on patients with the rare disease cerebrotendinous xanthomatosis (CTX) (for a review, see ref 66). We have shown that such patients lack sterol 27-hydroxylase activity, and mutations in the gene encoding the enzyme have been identified in some patients.[67–69] These patients have slightly increased cholesterol biosynthesis, possibly due to the reduced formation of chenodeoxycholic acid associated with this disease.[66] Chenodeoxycholic acid absorbed from the gastrointestinal system is a potent suppressor of HMGR.[66] The plasma levels of cholesterol in CTX patients are normal. If the 27-hydroxylation of cholesterol was obligatory for the regulation of HMGR, the consequences of the enzyme deficiency for cholesterol homeostasis would probably be far more dramatic. In addition, isolated fibroblasts from CTX patients have been shown to have a normal basal HMGR activity. Furthermore, the downregulation of HMGR upon addition of LDL to the culture medium was similar in fibroblasts from CTX patients and controls.[70]

25-Hydroxycholesterol (**11**) is one of the most potent naturally occurring oxysterol suppressors of HMGR known.[34] This compound is also formed enzymatically, possibly by 27-hydroxylase due to a broad substrate specificity of this enzyme.[13] 25-Hydroxycholesterol is, however, present in very low concentrations in biological tissues. Saucier et al. have shown that several oxysterols accumulate in mouse liver after cholesterol feeding and have suggested that these oxysterols, in particular 24(*S*)-hydroxycholesterol (**10**), are responsible for the downregulation of HMGR activity.

Using the same approach as above, we have investigated the possibility that mitochondrial 24-hydroxylation of cholesterol is important for cholesterol-induced downregulation of HMGR in mouse liver.[65] When cholesterol labeled with deuterium in the 23,-23,24,24,25-positions was used, there was a significant isotope effect in the 24-hydroxylation of this substrate in mouse liver mitochondria. However, the deuterated cholesterol possessed the same capacity as the unlabeled cholesterol to suppress the hepatic HMGR activity in mice when supplied in the diet. This result is compatible with the assumption that mitochondrial 24-hydroxylation of cholesterol is not obligatory for dietary cholesterol induced downregulation of HMGR.

A compound that has attracted much interest as a potential physiological suppressor of HMGR is 24(*S*),-25-epoxycholesterol (**13**). According to a report by Spencer et al., this compound is present in surprisingly high amounts in human liver.[19] Recently however, using a specific assay based on isotope dilution–mass spectrometry, we demonstrated that mouse liver contained less than 25 ng of 24,25-epoxycholesterol/(g of liver).[71] Its possible regulatory role remains to be established.

The putative precursor of 24(*S*),25-epoxycholesterol, 24(*S*),25-epoxydihydrolanosterol (**17**), has also been isolated from human liver.[72] Gupta et al.[38,59] suggested that the dependence on cytochrome P-450 for the downregulation of HMGR by LDL could be explained by a P-450 catalyzed formation of endogenous polar steroids from 24(*S*),25-epoxydihydrolanosterol. It was later found that 24(*S*),25-epoxydihydrolanosterol may be a translational downregulator of the synthesis of HMGR in certain strains of cultured cells[73] under conditions where conversion to 24,25-epoxycholesterol is blocked. This was found to be the case even in cells starved for mevalonate. It was also shown that transcriptional regulation of HMGR by 24-(*S*),25-epoxydihydrolanosterol requires conversion of this steroid into 24(*S*),25-epoxycholesterol. It was hypothesized that lanosterol epoxide is the mevalonate-derived factor required for downregulation of HMGR in mevalonate-deprived cells.[73]

Attempts To Define General Structural Requirements for Downregulation of HMG-CoA Reductase

Another approach to determine the possible regulatory role of oxysterols is to identify the general structural requirements of a steroid which would enable it to suppress HMGR activity in vivo. Such an investigation may furnish indirect information as to the necessity of a certain oxidation for the downregulation of the enzyme. To this end, we have investigated the importance of the steroid side chain and the 3β-hydroxy Δ^5 structure in cholesterol.

As a result, it is suggested that 24-, 25-, and 27-hydroxylations are not of critical importance for downregulation of HMGR. There is also no *direct* evidence that 24,25-epoxides of cholesterol and/or lanosterol are important. In this context results obtained by Erickson and Nes are of interest.[74] The authors fed mice with various cholesterol analogues and studied their effect on hepatic cholesterol synthesis. Some of the analogues contained a steroid side chain markedly different from that of cholesterol or were even completely devoid of a steroid side chain. Nevertheless, the suppressive effect on the HMGR was similar for all of these analogues of cholesterol. As a result of

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certain limitations in the feeding experiments which will be described below, the results do not completely rule out the possibility that side-chain oxygenations are critical for the regulation of HMGR by cholesterol even if the combined results from our laboratory and from that of Erickson and Nes make it seem less likely.

Interestingly, we[75] and others[76] have demonstrated that dietary cholestanol, the 5,6-saturated analogue of cholesterol, is a poor inhibitor of HMGR. This suggests that the 5,6-double bond is necessary for the downregulation of the enzyme by cholesterol. If an intermediate conversion of cholesterol is necessary, it may therefore be dependent on the presence of the 5,6-double bond. Epoxidation of the 5,6-double bond or allylic oxidation in the 7-position gives rise to oxysterols that are known to be efficient inhibitors of cholesterol synthesis in various cell systems.[34]

In a recent study, the possibility that modification of cholesterol in the region around the 5,6-double bond is required for the suppression of HMGR in the liver of mice fed dietary cholesterol was investigated.[77] Specifically, the possibility that epoxidation or allylic oxidations are involved in the regulation was examined. To this end cholesterol analogues containing structures which interfered with oxidations and isomerizations were used. Introduction of a highly electronegative fluorine at the 6-position could be expected to have a stabilizing effect on the 5,6-double bond and prevent not only epoxidation but also other chemical modifications that are dependent upon the 5,6-double bond. 6-Fluorocholesterol was, however, found to be an effective suppressor of HMGR in mice. Similarily, introduction of a methyl group at the 6-position should also affect steric conditions whereas the chemical properties should be similar to those of cholesterol. However, 6-methylcholesterol was also found to be an effective suppressor of the enzyme. Allylic oxidation of cholesterol at C-7 by nonenzymatic radical mechanisms is likely to be markedly reduced if the two hydrogens at the 7-position are substituted with deuterium.[24] 7,7-$^2H_2$-labeled cholesterol did, however, suppress the hepatic HMGR in mice as efficiently as did unlabeled cholesterol.

The results suggest that a transformation of cholesterol in the region C-3 to C-7 is not required for the downregulation of HMGR and demonstrate that the 5,6-double bond is essential. Since epicholesterol (5-cholesten-3α-ol) was significantly less efficient than cholesterol in suppressing the hepatic HMGR, it is thus possible that the configuration of the hydroxyl group at the 3-position is also essential. It may be mentioned that, in addition to 3β-hydroxy Δ^5 steroids, certain 3-oxo Δ^4 steroids such as 25-hydroxycholest-4-en-3-one[57] also possess a weak suppressive capacity. 15-Oxygenated steroids may be highly suppressive, even in the presence of a 5α-hydrogen in the steroid molecule.[78,79]

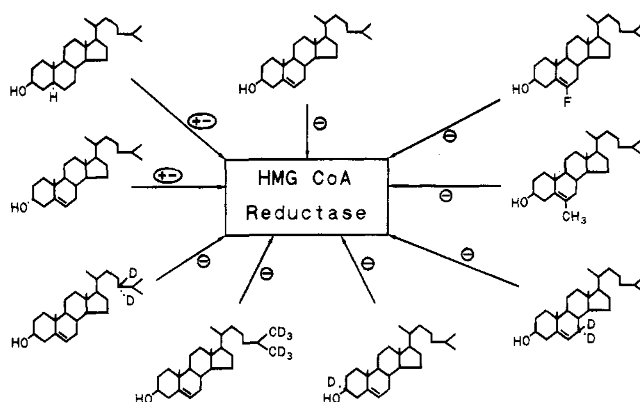

Figure 3. Summary of effect of cholesterol and different cholesterol analogues on HMG-CoA reductase activity in mouse liver. The mice were fed sterols for 24 h and sacrificed before the HMG-CoA reductase activity was assayed in liver preparations.[65,77]

There are certain limitations with experiments that involve the feeding of cholesterol analogues. An exchange of steroids, for example, through membranes during intrabody transport cannot be excluded. If this was the case, a possibility would exist that the steroid molecule reaching the putative regulatory site, regardless of whether an oxygenation has occurred or not, may not be the same molecule that was administered. Thus, more solid conclusions can probably be drawn from the experiments in which cholestanol, 6-methyl- and 6-fluorocholestanol, and epicholesterol were administered rather than from those in which cholesterol specifically deuterated in different positions was utilized. However, in general, conclusions drawn from the various experimental approaches are in agreement. Use of side-chain-fluorinated cholesterol analogues recently described by Schroepfer and colleagues[80] in feeding experiments may supply important additional information on the possible importance of the side chain in the regulation of HMGR by cholesterol.

Figure 3 summarizes the results obtained from the various experiments carried out in order to define the structural requirements of a steroid which would enable it to suppress HMGR in mice.

The sensitivity for structural variation in the steroid for the induction of regulatory events is compatible with sterol incorporation in membranes in the regulation of cholesterol homeostasis. The endoplasmic reticulum, in which both HMGR and ACAT are localized, has been reported to contain only low levels of cholesterol whereas the plasma membrane is considered cholesterol-rich (review[81]). There is some evidence to suggest that the cholesterol content of the plasma membrane is "sensed" by cholesterol-metabolizing enzymes in the ER through an as yet unidentified mechanism.[82] Certain compounds may act to mimic increasing (oxysterols) or decreasing (e.g., progesterone, lysophosphatidylcholine) cholesterol levels in the plasma membrane. Perhaps the most convincing evidence is the demonstration that lysophosphatidylcholine is able to inhibit cholesterol esterification in intact cells after 15 min of incubation, even though lysophosphatidylcholine has been re-

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ported to require hours in order to cross plasma membranes.[83] In addition, recent work by Rudney and colleagues has suggested that modulation of the ratio of cholesterol to sphingomyelin in plasma membranes in cells is a determining factor for HMGR activity.[84]

Are Oxysterols Physiological Regulators of HMG-CoA Reductase in Vivo? Evidence for and against the Hypothesis

The basic mechanism by which HMGR is regulated in vivo is still obscure, and the role of oxysterols, uncertain. The results presented above have not lent further support for the current model that oxysterols are important in this regulation.

Major differences exist between in vitro versus in vivo cellular systems used in most studies on the regulation of HMGR. In the former, cells are exposed to static levels of LDL or oxysterol, whereas in vivo, cells are exposed to a continuous flux of cholesterol through the system. According to our investigations and some previous reports, the steady-state levels of cholesterol and oxysterols within the cell in vivo are to a large extent unaffected by cholesterol influx. It seems likely that cholesterol biosynthesis is regulated by the flux of cholesterol rather than by the absolute concentration. This hypothesis is supported by the finding that feeding mice 2% cholesterol in the diet resulted in a 70% increase of oxysterol levels in liver.[65] The same dietary conditions resulted in suppression of HMGR activity by about 90%. Exposure to only 0.05% cholesterol in the diet resulted in a 50% reduction in activity. The small increase in oxysterol levels expected in the latter case is unlikely to be consistent with the hypothesis that HMGR is regulated by steady-state levels of oxysterols.

The observation that oxysterols when added to isolated cells have a high potential to downregulate HMGR activity does not necessarily indicate that this potential is utilized under in vivo conditions. The finding that isolated cells are more highly responsive to oxysterols than to free cholesterol may be due to solubility problems and limitations in the transfer through cell membranes. Under physiological conditions, cholesterol is delivered to the cells mainly via LDL, which has been shown to be an effective downregulator of HMGR activity also in cultured cells.[33]

Among the various findings supporting the hypothesis that oxysterols participate in the regulation of cholesterol homeostasis, the following two have been regarded as the most important:

1. The demonstration that the cytochrome P-450 inhibitor ketoconazole seems to interfere with cholesterol-induced downregulation of cholesterol synthesis.[38] This finding supports the contention that a cytochrome P-450 dependent oxidation is involved in the regulation.

2. The presence of at least one oxysterol-binding protein in the cells. This protein is likely to have some biological function.

With regard to the ketoconazole experiments, the specificity of this compound as a cytochrome P-450 inhibitor is very difficult to evaluate. The experimental design of some of these experiments is also relatively complicated. Thus, other explanations for the interference cannot be excluded.

With regard to the presence of oxysterol-binding proteins in the cell, they may have other as yet unidentified ligands. Another possibility is that they may be important in the export of oxysterols from cells. Some oxysterols are cytotoxic; thus there may be a need for binding and elimination of such compounds (recently reviewed in ref 85). We have recently demonstrated that cultured human alveolar macrophages possess a very high capacity to convert intracellular cholesterol into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid and secrete these compounds to the medium.[86] It seems likely that oxysterol-binding protein(s) are important for the secretion of 27-hydroxycholesterol. This conversion is catalyzed by the sterol 27-hydroxylase, and in a very recent study (unpublished) of this novel pathway we have shown that inhibition of this enzyme resulted in a marked intracellular accumulation of cholesterol. By determining the concentration of 27-oxygenated cholesterol in plasma from a peripheral artery, from vena hepatica, and from vena porta, it could also be shown that there is a continuous flux of 27-oxygenated metabolites of cholesterol from extrahepatic tissues to the liver. Once in the liver, these compounds are converted into bile acids.[87] These clearance studies are compatible with the contention that up to 4% of the total synthesis of bile acids in humans may originate from 27-oxygenated metabolites of cholesterol that have been formed extrahepatically. The magnitude of this flux is such that there must be a need for an efficient transport mechanism for 27-hydroxycholesterol in extrahepatic cells, especially since 27-hydroxycholesterol is known to be cytotoxic.[88] In this regard it is interesting to note that the cytosolic oxysterol-binding protein seems to concentrate in the Golgi apparatus when the cell is exposed to oxysterols.[56] This finding is in accordance with the contention that the oxysterol-binding protein has a secretory function in the cell.

Future Directions of Research

During the last few years important progress has been made in the elucidation of molecular mechanisms in the regulation of cholesterol homeostasis, in particular the mechanisms active at the gene level. At present, it seems to be more important to define the signaling pathways that trigger regulation of some key enzymes than to define the possible role of oxysterols. Once the most important regulatory mechanisms are known, it will then be possible to evaluate the role of oxysterols.

The recent discovery of the membrane-bound transcription factors SREBP-1 and SREBP-2 which act on sterol regulatory elements located upstream of genes encoding HMGS and LDLR sheds light on the importance of membrane characteristics for the regulation of these genes. One possibility is that when the sterol

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or oxysterol concentration in the membrane drops below a certain threshold value, events are triggered which ultimately lead to the release of sterol regulatory element binding proteins with a subsequent increase of cholesterol uptake and synthesis.

At present, the transcriptional regulation of HMGR is not as well characterized as the transcriptional regulation of HMGS and LDLR. The presence of a sterol regulatory element in the gene for HMGR and the general similarity in the regulation of these enzymes suggests that the mechanisms of transcriptional regulation of these enzymes by sterols are similar. Future experiments with reconstituted membranes may be needed to evaluate the importance of this type of transcriptional regulation and also the possible role of oxysterols for this mechanism.

Posttranslational mechanisms may be of more importance than transcriptional mechanisms for regulation of HMGR.[89-91] The membrane domain of HMGR is essential for the regulated degradation of enzyme.[92] At present, the most promising experimental approach is that of Simoni and co-workers, using the HMGal fusion protein consisting of the membrane domain of HMGR and β-galactosidase.[93] This experimental design may prove to be of value in the near future also for defining the possible role of oxysterols in posttranslational regulation of HMGR.

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Concluding Remarks

Oxysterols are formed in various biological systems and have a high potential to downregulate HMGR activity and reduce cholesterol synthesis in different cell systems. There is, however, little *direct* evidence that they are important under in vivo conditions. The results of our investigations suggest that hydroxylations in the steroid side chain as well as transformations at C-3 to C-7 are not critical for the cholesterol-induced downregulation of HMGR in mice. At least one oxysterol-binding protein is present in most cells. The function of this protein may be to export specific oxysterols from cells. Whether or not oxysterols are involved in the regulation of HMGR and HMGS by a mechanism involving steroid regulatory element binding proteins cannot at present be evaluated.

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