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Free radical oxidation of cholesterol and its precursors: Implications in cholesterol biosynthesis disorders

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Abstract

Free radical oxidation of cholesterol and its precursors contribute significantly to the pathophysiology of a number of human diseases. This review intends to summarize recent developments and provide a perspective on the reactivities of sterols toward free radical oxidation, the free radical reaction mechanism, and the biological consequences of oxysterols derived from the highly oxidizable cholesterol precursor, 7-dehydrocholesterol. We propose that the rigid structures, additional substituents on the double bonds, and the well-aligned reactive C–H bonds in sterols make them more prone to free radical oxidation than their acyclic analogs found in unsaturated fatty acids. The mechanism of sterol peroxidation follows some well-established reaction pathways found in the free radical peroxidation of polyunsaturated fatty acids, but sterols also undergo some reactions that are unique to these compounds. Peroxidation of 7-dehydrocholesterol gives arguably the most diverse set of oxysterol products that have been observed to date. The metabolism of these oxysterols in cells and the biological consequences of their formation will be discussed in the context of the pathophysiology of the human disease Smith–Lemli–Opitz syndrome. Considering the high reactivity of sterols, we propose that a number of other cholesterol biosynthesis disorders may be associated with oxidative stress.

Keywords

peroxidation; autoxidation; oxysterol; 7-dehydrocholesterol; Smith–Lemli–Opitz syndrome

Introduction

Cholesterol is abundant in mammalian cells and tissues, and plays important roles in maintaining plasma membrane integrity [1,2], lipid-raft-mediated cell signaling [3,4], activation of the hedgehog pathway during embryonic development [5,6], and myelin

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formation [7]. Free radical oxidation of cholesterol has been implicated in a number of human diseases such as atherosclerosis [8], Alzheimer's disease [9], retinal degeneration [10], age-related macular degeneration [11], cataract [12,13], and Niemann–Pick C1 disease [14]. Recently, peroxidation of a cholesterol precursor, 7-dehydrocholesterol (7-DHC), was found to contribute to the pathophysiology of cholesterol biosynthesis disorder, Smith–Lemli–Opitz syndrome (SLOS) [15–24].

The mechanism of free radical oxidation of cholesterol has been extensively studied and many oxidation products, that is, oxysterols, have been identified [8,25–29]. Major efforts have also been devoted to study the biological activities of these oxysterols [30–33]. However, until recently little was known about the relative reactivity of cholesterol and other oxidizable lipids [15]. Significantly, the cholesterol precursors, 7-DHC and 8-DHC, were found to be among the most reactive lipid hydrogen atom donors to peroxy radicals, thus making them highly oxidizable [15,16]. 7-DHC accumulates in tissues and fluids (particularly high in the brain) of individuals affected with SLOS, an autosomal recessive disorder that is caused by mutations in the gene encoding 3 β -hydroxysterol-7-reductase (DHCR7; EC 1.3.1.21) (Scheme 1) [34–39]. The level of 8-DHC is also elevated in SLOS patients, comparable to that of 7-DHC [36,37,40], due to the functioning of 3 β -hydroxysterol-8,7-isomerase (Ebp; EC 5.3.3.5). Ebp catalyzes the equilibration between the 8- and the 7-double bond (e.g., zymostenol to lathosterol), and the equilibrium normally favors the 7-sterol as it can be subsequently converted to downstream products. However, when 7-DHC accumulates due to the defective 3 β -hydroxysterol-7-reductase (DHCR7), 8-DHC can be observed in significant amount as a result of the equilibrium. In fact, defects in each step of cholesterol biosynthesis causes a disorder, which results in accumulation of specific cholesterol precursors [34]. In the postsqualene cholesterol biosynthesis pathway, cyclization of squalene-2,3-epoxide gives the first sterol, lanosterol, which is followed by multistep transformations, leading to the ultimate product cholesterol (Scheme 1). Depending on whether the C24 double bond is reduced early or later by 3 β -hydroxysterol-24-reductase (DHCR24; EC 1.3.1.72), the pathway has been defined as the Kandutsch–Russell pathway or Bloch pathway, respectively [41,42]. Importantly, 7-DHC also serves as the biosynthesis precursor to vitamin D₃ in human skin, where ring-B is opened upon UV irradiation [43].

Oxidation of 7-DHC gives a complex mixture of oxysterols, which led us to re-examine the reactions involved in sterol free radical oxidation [16,17,19]. These oxysterols exert a variety of biological actions, such as reducing cell proliferation, inducing cell differentiation, modulating gene expression, forming adducts with proteins, etc. [18,21,44]. The mechanism of formation and the biological activities of cholesterol-derived oxysterols have been reviewed several times previously [30–33,45–49]. This review will focus on three different aspects: (1) rationalizing the reactivities of cholesterol and its precursors toward free radical oxidation; (2) reaction mechanisms involved in sterol oxidation and the effect of α -tocopherol on product distribution; and; (3) the biological consequences of the novel oxysterols derived from the cholesterol precursor, 7-DHC, and their role in SLOS.

Reactivities of cholesterol and its biosynthetic precursors toward free radical oxidation

The rate-determining step in the free radical chain oxidation of lipids is the propagation reaction of the peroxy radical, where generally two types of processes occur (Scheme 2) [48]: (a) a hydrogen atom is transferred from a donor to the chain-carrying peroxy radical (*hydrogen atom transfer*); (b) a peroxy radical adds to a double bond (*peroxy radical addition*). We will discuss these two types of reactions separately below.

Hydrogen atom transfer

Utilizing a peroxy radical clock, we have determined the hydrogen atom transfer rate constants (k_H) of polyunsaturated fatty acids (PUFAs) and sterols to linoleate peroxy radicals [15]. We found that cholesterol is a moderately oxidizable lipid with a k_H of $11 \text{ M}^{-1}\text{s}^{-1}$ at 37°C , about one-sixth of the rate constant for linoleate ($62 \text{ M}^{-1}\text{s}^{-1}$) [50], but 10 times that of the acyclic monounsaturated oleate ($0.88 \text{ M}^{-1}\text{s}^{-1}$) [50]. 7-DHC, the immediate cholesterol precursor with one additional double bond at C7, gives a rate constant of $2260 \text{ M}^{-1}\text{s}^{-1}$, the largest rate constant known for a lipid molecule. Mechanistic and product analysis suggest that H9 and H14 are the reactive hydrogen atoms [17], which make the k_H of 7-DHC $1130 \text{ M}^{-1}\text{s}^{-1}$ per hydrogen atom, a rate constant that is more than 35 times that of bis-allylic hydrogen atoms found in PUFAs (for linoleate, $k_H = 31 \text{ M}^{-1}\text{s}^{-1}/\text{H-atom}$). This was a surprising finding at the time because 7-DHC only has mono-allylic positions and in the peroxidation of PUFAs, the bis-allylic C–H bonds are much more reactive than the mono-allylic C–Hs (reflecting their respective bond dissociation enthalpies) [51–53].

More recently, we determined the hydrogen atom transfer rate constants of four additional cholestadienols, including cholesta-6,8(9)-dienol [6,8(9)-dienol], 8-dehydrocholesterol (8-DHC or 5,8-dienol), cholesta-5,8(14)-dienol [5,8(14)-dienol], and cholesta-6,8(14)-dienol [6,8(14)-dienol], to be 1370, 994, 911, and $412 \text{ M}^{-1}\text{s}^{-1}$, respectively [16]. There is an apparent trend that the reactivity of the unsaturated sterols are better hydrogen atom donors than their acyclic fatty acid analogs and their more flexible cyclic analogs, such as cyclohexene or cyclohexadienes (Table I) [50,54].

We suggest that three factors may collectively contribute to the high reactivity of sterols toward hydrogen atom abstraction:

1. Sterols normally have more alkyl substituents on double bond(s) than those in fatty acids. Substituents can stabilize the transition state and the resulting radical intermediate via hyperconjugation, thus lowering the activation energy of the hydrogen atom transfer process. For example, the allylic radicals formed from cholesterol have three substituents while oleate only has two; the pentadienyl radicals derived from 7-DHC have five substituents while the one derived from linoleate has two (Scheme 3).
2. The dihedral angles between the reactive C–H bond and the adjacent double bond and the planarity the two double bonds (if applicable) (Table I and Figure 1). In 7-DHC, molecular mechanics modeling suggests that the two double bonds are close

to being planar (the C5–C6–C7–C8 dihedral angle = 5.7°) and the dihedral angles between the reactive C–H bonds (at C9 and C14) and the double bond plane are 92.3 and 99.4°, respectively, both being close to the perpendicular geometry [16] (Figure 1). The planarity of the double bonds and the orthogonality of the C–H bonds make the reactant to resemble the transition state for H atom removal at C9 or C14, where maximum overlapping between the π -orbitals and the reactive C–H bond is expected. Thus, a minimum amount of molecular reorientation is required to reach the transition state, that is, there is less entropy demand. On the other hand, for an acyclic system such as linoleate, all the σ -bonds around the double bonds can freely rotate and a significant amount of entropy is lost in order to reach the transition state. For the same number of double bonds and reactive C–H bonds, the less molecular reorientation required, the more reactive the molecule. This could partially account for the reactivity trend of 7-DHC > 6,8(9)-dienol > 8-DHC as seen in Table I, all being dienes on ring-B but all being much more reactive than the acyclic linoleate. In 8-DHC, while the two double bonds are close to planar, the bis-allylic C–H bonds are distorted from the perpendicular with H7 β being the relatively more orthogonal one than H7 α . This rationale, along with the *substituent factor* discussed above, could also account for the reactivity difference between cholesterol and oleate, both being monounsaturated but cholesterol is 10 times more reactive.

3. Dienes that adopt *cisoid* conformations tend to be more reactive than those adopting *transoid* conformations. It is known that the *cisoid* conformation of a conjugated diene has higher enthalpy than the *transoid* conformation [55], which would imply smaller activation energy of hydrogen atom transfer from the allylic positions of the *cisoid* conformation. The high reactivity of *cisoid* could provide a reasonable explanation for the reactivity trend of the conjugated dienols: 5,7-dienol (7-DHC) > 6,8(9)-dienol $\gg$ 6,8(14)-dienol.

Overall, the cholestadienols leading to an *endo* radical (within the same sterol ring) tend to be more reactive than those that give an *exo* radical (spanning multiple rings), which could largely be rationalized by *factors* 2 and 3 [16].

Peroxyl radical addition

Addition of a peroxyl radical to a double bond, followed by an intramolecular homolytic substitution ($S_{\text{H}}1$), generally gives an epoxide as the main product (addition of another oxygen molecule could compete with the epoxidation, particularly under high oxygen tension) [48]. Analogous to the carbon radical addition reaction [56] (although carbon radical is more nucleophilic), the reactivity of a double bond toward peroxyl radical addition largely depends on the stability of the resulting radical (β -effect) and the steric effect at the carbon center of the reaction (α -effect). As such, three general guidelines for understanding addition reactions can be derived: (a) a double bond more substituted at the center remote from the site of addition would be more reactive than a less substituted structure as the stability of the resulting radical would follow the order of tertiary > secondary primary (e.g., cholesterol > oleate); (b) a conjugated diene would be more reactive than a non-conjugated diene since a stabilized allylic radical would be formed from the former (e.g., 7-DHC > 8-

DHC and linoleate) (Scheme 4) and; (c) if similar product radicals are formed, a peroxy radical would preferentially add to the less hindered carbon center. Thus, it is reasonable to suggest that the unsaturated sterols would be more reactive toward peroxy radical addition than their acyclic counterparts in fatty acids because the resulting radicals are generally more stable (with more substituents). In particular, the conjugated dienyl cholesterol precursors, such as 7-DHC and the 4,4-dimethylcholesta-8(9), 14-dien-3 β -ol [8(9),14-dienol], would be prone to undergo addition reactions.

In the peroxy radical addition reaction of 7-DHC, steric factors play a major role in the product selectivity, only the 5 α ,6 α -epoxide was observed. Addition to the β -face of the sterol is relatively hindered by the axial methyl groups while the α axial H-9 and H-14 atoms effectively block the peroxy addition at the α -face of C8. For the addition to C5, again, the top face is shielded by the axial methyl group (C19), leaving the α -face at C5 the default site of attack. These factors control the site and face of addition. In fact, an oxysterol derived from 5 α ,6 α -epoxide of 7-DHC has been found to be a major peroxidation biomarker in cell and animal models of SLOS (*vide infra*) [19].

Based on the above-proposed principles governing the reactivities of sterols toward hydrogen atom transfer and peroxy radical addition, other cholesterol precursors that might be prone to free radical peroxidation are the 8(9),14-dienol, zymostenol, and lathosterol (and their counterparts in the Bloch pathway with an additional C24 double bond). Addition at C15 of the 8(9),14-dienol would be kinetically favorable since that carbon is only monosubstituted while the allyl radical formed upon addition would be an *endo* radical that bears five alkyl substituents. For zymostenol and lathosterol, the allylic C-Hs at C14 (zymostenol) and at both C9 and C14 (lathosterol) are axially positioned and stable allyl radicals would be formed after hydrogen atom transfer (Figure 2).

Mechanisms of sterol oxidation and the effect of α -tocopherol on product distribution

Oxygen addition to radical intermediates

Allylic or pentadienyl radicals are formed from cholesterol or its precursors upon loss of a hydrogen atom (Schemes 3). Oxygen addition to similar radicals derived from oleate or linoleate has been well established [54,57,58], and the knowledge gained from those studies can be applied to understand the reactions of the sterol-derived radicals (Scheme 5). For the pentadienyl radicals derived from 7-DHC, 8-DHC, and the other sterol dienes shown in Table I, oxygen can potentially add to three positions, but the bis-allylic peroxy radical resulting from the addition at the middle carbon will undergo fragmentation rapidly, giving back to the pentadienyl radical (i.e., β -fragmentation; $k_{\beta} = 2.6 \times 10^6 \text{ s}^{-1}$ for the linoleate-derived bis-allylic peroxy radical) [54]. Only in the presence of an excellent hydrogen atom donor that can compete with the β -fragmentation (e.g., α -tocopherol with a $k_{\text{H}} = 3.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [58–60] can products derived from the bis-allylic oxygen addition be observed. The same has been observed in the oxidation of 7-DHC (discussed below). For the allylic radical derived from oleate, the two potential addition sites lead to allylic products that differ only by the geometry of the double bond [57]. On the other hand, the major radical derived from

cholesterol (*endo* radical formed from loss of H-7) [26] should give peroxy radicals derived from oxygen addition at C5 and C7. But the C5 hydroperoxide products have not been observed from free radical reactions although they are formed from photooxidation. It seems likely that the rearrangement of the peroxy radical from the C5 position to C7 is fast since the C5 is in a more stabilized position (with more substituents) [61]. One can speculate that a good hydrogen donor such as *tert*-butyl hydroperoxide should trap the kinetic products from cholesterol peroxidation, just as the kinetic products are trapped by this reagent in the peroxidation of oleate [57].

Intramolecular homolytic substitution ($S_{\text{H}}\text{i}$) and peroxy radical cyclization

$S_{\text{H}}\text{i}$ typically occurs when a peroxy radical adds to a double bond as illustrated in Scheme 4, leading to formation of epoxides [48]. This reaction is particularly common for sterols for the above-outlined reasons.

5-*Exo* peroxy radical cyclization is an important transformation observed in the free radical oxidation of PUFAs with three or more double bonds [62 – 64]. These types of reactions account for the formation of numerous prostaglandin-like compounds, for example, isoprostanes or neuroprostanes, from the oxidation of arachidonic acid and docosahexaenoic acid, respectively [65–67]. In the oxidation of 7-DHC, two peroxy radical intermediates formed are well positioned for a 5-*exo* cyclization (Scheme 6), giving the same cyclic peroxide product [17]. Subsequent $S_{\text{H}}\text{i}$ on the peroxide or addition of another oxygen leads to some of the major products found in the peroxidation of 7-DHC. The peroxy radical derived from loss of H14 is not well positioned for 5-*exo* cyclization, but the hydroperoxide products can undergo homolytic peroxy bond cleavage, 3-*exo* cyclization, and addition of another oxygen, to give the observed products [10].

Effect of α -tocopherol on product distribution

Peroxidation in the presence of α -tocopherol significantly changes the profile of product derived from the cholestadienols [16]. Thus, peroxy radical addition to the double bond is suppressed when α -tocopherol is present because H-atom transfer from the antioxidant to propagating peroxy radicals is faster than the addition of those radicals to the diene. Products derived from bis-allylic oxygen addition are found to be the major products because of the rapid trapping of the bis-allylic peroxy radical by α -tocopherol (competing with β -fragmentation). It is also noteworthy that products containing the “enone” moiety are preferentially formed in dienol peroxidations carried out in the presence of α -tocopherol (Scheme 7).

Although ketone formation has been observed in radical termination reactions in the oxidation of fatty acids (Scheme 7A) [68–70], the formation of ketone products seems more common in sterol free radical oxidation [16]. We have suggested that the key step to the formation of sterol ketones is loss of the remaining bis-allylic or allylic hydrogen atom at the α -position of the hydroperoxide, followed by elimination of a hydroxyl radical. The bis-allylic addition to the 7-DHC-derived *endo* radical eventually leads to the formation of 7-oxo-5,8-dien-3 β -ol (**7-keto-8-DHC**) (Scheme 7B). On the other hand, the endoperoxyenone (from addition at C5 or C9) formed in Scheme 7C was found to be unstable and was further

reduced by α -tocopherol to give $3\beta,5\alpha,9\alpha$ -trihydroxycholest-7-en-6-one (**THCEO**), which can further dehydrate at C9 and C11 to give $3\beta,5\alpha$ -dihydroxycholesta-7,9(11)-dien-6-one (**DHCDO**). Notably, all three enones discussed here have been identified in the brain of a mouse model for SLOS [23,24].

Tocopherol-mediated peroxidation (TMP) can occur under the conditions used in the above studies on 7-DHC, where the radical initiation rate is low and the concentration of α -tocopherol is high [16]. TMP has been suggested to play an important role in the oxidation of LDL [71,72]. Recently, large kinetic isotope effects (KIE; >20) have been observed during TMP of PUFAs and 7-DHC [73], suggesting that tunneling is involved in the step of hydrogen atom transfer to the chain-carrying tocopheryl radical. This would be particularly important for SLOS pathophysiology as the plasma of SLOS patients is enriched with the highly oxidizable 7-DHC.

Oxidation of sterols by other oxidants

Cholesterol reacts with singlet oxygen ($^1\text{O}_2$) *via* an “ene” type reaction, leading to 5α -hydroperoxy-6-en- 3β -ol (5α -OOH-Chol) as the major product, and 6α - and 6β -hydroperoxy-4-en- 3β -ol as the minor products (Scheme 8A) [25,74,75]. 7-DHC undergoes a similar “ene”-type reaction as well as a [4 + 2] cycloaddition, giving 7-hydroperoxy-5-,8-dien- 3β -ol (7-OOH-8-DHC) and 5,8-endoperoxy-6-en- 3β -ol [EnP(5,8)], respectively, in a ratio of 1:3 (Scheme 8B) [76,77]. Interestingly, the hydroperoxides formed from photooxidation are actually the kinetic products found in the free radical oxidation of cholesterol and 7-DHC. These hydroperoxides rearrange to the thermodynamic products [61] and/or initiate free radical processes upon thermal or transition metal-catalyzed decomposition. Therefore, caution has to be exerted to avoid photooxidation and subsequent transformations that may perturb the endogenous product profile of biological samples [20], where there may be an abundance of chromophores present that can act as photosensitizers.

Ozonolysis of cholesterol has been studied extensively as it has been closely linked to airway inflammation [78,79]. Major ozonolysis products of cholesterol are the reactive electrophiles 5,6-secosterol and its cyclized product *via* aldol condensation (Scheme 9) [78–82], which can form adducts with proteins, modulating protein structures and functions [83,84]. However, in lung surfactant, cholesterol $5\beta,6\beta$ -epoxide was found to be the major product, instead of the ring-opening products [78]. Notably, the photooxidation and free radical oxidation product, 5α -OOH-Chol, can also serve as a precursor to the 5,6-secosterol *via* acid-catalyzed Hock fragmentation [85] (Scheme 9).

The additional double bond at C7 of 7-DHC also makes it an unusual substrate of cytochrome P450 (CYP) 7A1, leading to the formation of 7-ketocholesterol [86], an oxysterol that was normally formed from cholesterol oxidation at C7 (See Scheme 5B). Indeed, elevated levels of 7-ketocholesterol has been observed in tissues and/or fluids of the rat model of SLOS and human patients [20,87,88], suggesting that 7-DHC is the predominant precursor to this oxysterol in these samples since the level of cholesterol is low. Furthermore, 7-DHC was also found to be a good substrate of CYP 46A1, the nervous-system-specific enzyme, leading to the unusual 25-hydroxy-7-DHC, in addition to the expected 24-hydroxy-7-DHC [20,89].

Biological consequences of 7-DHC-derived oxysterols and their role in SLOS

SLOS displays a broad spectrum of phenotypes including multiple congenital malformations, neurological defects, mental retardation, autism-like behavior, and photosensitivity [34,90,91]. Even before the rate constants of 7-DHC and 8-DHC were determined [15,16], the high reactivity of 7-DHC and/or the oxidative stress in SLOS have been suggested in a number of studies. Porter and coworkers reported over 30 years ago that 7-DHC acted as an excellent hydrogen donor to linoleate peroxy radicals in oxidations carried out in liposomes [92], which led to our recent measurement of the k_H of this sterol [15]. In 1996, De Fabiani et al. reported the identification of cholesta-5,7,9(11)-trien-3 β -ol in plasma of SLOS patients, which was suggested to be formed from the decomposition of 7-OOH-8-DHC (see Scheme 8) [93]. However, these plasma samples were not processed under protected conditions (from oxygen and light), and it was recently demonstrated that 7-OOH-8-DHC and EnP(5,8) (also identified in that study) can be formed from *ex vivo* photooxidation of 7-DHC [20]. Nevertheless, this study did support the high reactivity of 7-DHC toward oxidation in a biological environment. In 2006, Fliesler and coworkers suggested that retinal degeneration in a rat model for SLOS was caused by elevated levels of lipid peroxides, likely cytotoxic oxysterols derived from 7-DHC, which was intensified by light [94,95]. They also found that supplementation of an antioxidant, dimethylthiourea, protected retina from light damage in this model [94]. In the same year, Kochevar and coworkers reported that 7-DHC enhanced ultraviolet A-induced oxidative stress in keratinocytes [96,97], also consistent with the high oxidizability of 7-DHC.

A decade before the free radical oxidation products of 7-DHC were fully elucidated, Gaoua et al. found that products generated from photooxidation of 7-DHC induced growth retardation of cultured rat embryos [98] and upon our recent identification of individual 7-DHC-derived oxysterols [17,19], systematic studies of their biological activities have been carried out. Korade et al. reported that these oxysterols exerted differential cytotoxicity to the Neuro2aneuroblastoma cells and primary neurons, with oxysterols possessing the endoperoxide moiety being the most toxic ones (e.g., compounds **2** shown in Scheme 6B) [18]. The cytotoxicity of the oxysterols is likely due to reduced cell proliferation, as suggested by the downregulation of proliferation-related genes, and induced differentiation, as indicated by the changes in cell morphology [18]. The oxysterols were also found to affect expression of gene transcripts related to lipid biosynthesis and cell growth [18], accelerate differentiation and arborization of neuronal cells [21], and induce retinal degeneration in the rat model of SLOS [87]. In the following sections, we will focus on the metabolic fate of the primary oxysterols formed from 7-DHC peroxidation: (a) metabolism of the primary oxysterols to more stable oxysterols in cells and (b) adduction of some electrophilic oxysterols with proteins.

Metabolism of primary peroxidation oxysterols derived from 7-DHC

We define the oxysterols formed from free radical oxidation of 7-DHC in solution as the *primary oxysterols* since they are not metabolized in a biological environment [23]. Upon analysis of samples from cell and animal models for SLOS, the oxysterol profiles in the

SLOS samples are distinctly different from the profile found in solution oxidations [19,20,22]. This prompted a study to investigate the metabolism of the primary oxysterols in cells [23]. Thus, Neuro2a and human fibroblast cells were exposed to the primary oxysterols and the metabolites were analyzed by high-performance liquid chromatography–mass spectrometry. The metabolites of the primary 7-DHC oxysterols were found to be identical to the major oxysterols observed in the SLOS cells and tissues. Typical metabolic transformations include reduction of peroxides to alcohols, ring opening of epoxides to give diols, and oxidation of allylic alcohols to ketones, leading to α,β -enone moieties (Scheme 10) [23]. The structures for the metabolites of the primary oxysterols other than **1**, **2**, and the 5 α ,6 α -epoxide were proposed based on their masses and elution order on normal phase chromatographic separation. Note that some allylic alcohols such as 6 α -tetraol and 7 $\alpha(\beta)$ -tetraol were not oxidized to their corresponding ketones. Whether or not an enzyme is involved in the allylic oxidation remains to be elucidated. Among the metabolites, 3beta, 5alpha-dihydroxycholesta-7,9(11)-en-6-one (DHCEO) and THCEO have been established as two major biomarkers for the peroxidation of 7-DHC *in vivo* [19,23].

As discussed earlier, THCEO can also be formed from free radical oxidation of 7-DHC when α -tocopherol is present, along with 7-keto-8-DHC and DHCDO[16]. However, the formation of the precursor of DHCEO, 7-DHC 5 α ,6 α -epoxide, would be completely suppressed in the presence of α -tocopherol (*vide supra*) [16]. Therefore, the presence of both DHCEO and 7-keto-8-DHC in the brain of the SLOS mouse model suggests that both mechanisms (oxidation with or without α -tocopherol) operate *in vivo*, which collectively contribute to the endogenous oxysterol profile.

Adduction of electrophilic oxysterols with proteins

Lipid electrophiles, such as 4-hydroxynonenal, are formed during lipid peroxidation [48,99], and adduction of lipid electrophiles with proteins play important roles in cell signaling under physiological or pathological conditions [100]. Protein adducts from the ozonolysis products of cholesterol have been suggested to lead to protein misfolding [83,84]. Some of the oxysterols formed from 7-DHC are good electrophiles judging from their structures, such as the 5 α ,6 α -epoxide and those oxysterols containing the α,β -enone moiety. When *Dhcr7*-deficient Neuro2a cells (which cannot effectively convert 7-DHC to cholesterol) were exposed to an alkynylated 7-DHC, a significant amount of protein adduction was observed after the adducted proteins were covalently linked to biotin by “click” chemistry and visualized by streptavidin fluorophore [44] (Figure 3). When the same experiments were carried out in control Neuro2a cells (with intact cholesterol biosynthesis machinery), significantly less adducts were formed. Importantly, exposure to 7-DHC 5 α ,6 α -epoxide alone gave more adducts than was formed from the incubation with the same concentration of 7-DHC. It is notable that adduction by 7-DHC and its epoxide was found to be more extensive than adduction from PUFAs in the same cells and under the same conditions [44].

Conclusions and perspective

Here we proposed that the more rigid and more substituted sterol structure makes cholesterol and its precursors more reactive toward free radical oxidation than the acyclic structures found in fatty acids. Sterols tend to be more reactive than PUFAs containing the same

number of double bonds and this reactivity is found for either hydrogen atom transfer or peroxy radical addition mechanisms. Thus, even a small perturbation on the levels of the reactive cholesterol precursors could result in a significant increase in oxidative stress and a shift of the oxidation product profile (see Table II for an illustration using the levels of different lipids found in human plasma [29,36,101,102]). The free radical oxidation of sterols follows mechanisms that are well established in the oxidation of PUFAs, but there are some unique features such as unusually fast 5-*exo* cyclizations, S_H on a cyclic peroxide structure, and enone formation in the presence of α -tocopherol that is not common in the PUFA systems. The most reactive sterol found, 7-DHC, was closely associated with the pathophysiology of the human disease SLOS. A number of 7-DHC oxysterols identified *in vivo* were found to originate from free radical oxidation although enzymatic oxidation also contributes to the oxysterol profile. Continued investigation of the biological activities of the 7-DHC-derived oxysterols may ultimately lead to new therapies that may counter the effect of these oxysterols. Based on our understanding on the reactivities of sterols toward free radical oxidation, a number of other cholesterol precursors, such as the 8(9),14-dienol, lathosterol, and zymostenol, may also serve as good free radical peroxidation substrates, which suggest that oxidative stress may be associated with other cholesterol biosynthesis disorders.

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Figure 1. Structures of 7-dehydrocholesterol (A) and 8-dehydrocholesterol (B) (optimized by MM2 in ChemBio3D) showing the dihedral angles between the planes containing the allylic C-H bonds and the adjacent planes containing the double bonds.

Figure 2.
Other potentially reactive cholesterol biosynthesis precursors toward free radical oxidation.

Figure 3.

(A) Illustration of the strategy for detecting endogenously formed lipid-protein adducts using alkynylated lipids. (B) Adapted from Figure 7 in ref [44]: comparison of protein adducts of metabolites of 25-alkynyl-7-DHC (*a*-7-DHC) in control Neuro2a *versus* in *Dhcr7*-deficient Neuro2a cells (*a*-Chol = alkynyl cholesterol). This research was originally published in the *Journal of Lipid Research* (ref [44]). © the American Society for Biochemistry and Molecular Biology.

Scheme 1.

Postsqualene cholesterol biosynthesis pathway. Sequence on the left shows the Bloch pathway and the one on the right shows the Kandutsch–Russell pathway.

Scheme 2.

Typical sequence involved in free radical chain oxidation reactions.

Scheme 3.

Radical intermediates formed from sterols and fatty acids.

Scheme 4.
Peroxyl radical addition to sterols and fatty acids.

Scheme 5.

Addition of oxygen to radical intermediates formed during free radical oxidation.

Scheme 6.

Peroxyl radical cyclization and intramolecular homolytic substitution (S_{Hi}) reactions involved in peroxidation of fatty acids and sterols.

Scheme 7.

Proposed mechanisms for the formation of enones in peroxidation of fatty acids and sterols.

TOH = α -tocopherol.

Scheme 8.

$^1\text{O}_2$ oxidation of cholesterol and 7-DHC.

Scheme 9.

Ozonolysis of cholesterol and Hock fragmentation of 5 α -OOH-Chol.

Scheme 10.

Proposed mechanisms for the metabolism of the primary oxysterols of 7-DHC in cells.

Summary of hydrogen atom transfer rate constants of sterols in comparison with flexible molecules and the dihedral angles involving the reactive C–Hs and the double bonds.

Table I

Substrate	k_H ($M^{-1}s^{-1}$) ^a	# of Sub ^b	ϕ_{HC}^c	ϕ_{CC}^d	<i>endo/exo</i> ^e	References
Cholesterol	11	3	$H^{4\alpha}-C^{4,5,6}$: -7.9° (82.1°) $H^{4\beta}-C^{4,5,6}$: 109.1° (19.1°) $H^{7\alpha}-C^{5,6,7}$: -106.2° (16.2°) $H^{7\beta}-C^{5,6,7}$: 137.7° (47.7°)	–	<i>endo/exo</i>	[15]
7-Dehydrocholesterol	2260	5	$H^9-C^{7,8,9}$: -92.3° (2.3°) $H^{14}-C^{7,8,14}$: 99.4° (9.4°)	5.7°	<i>endo/exo</i>	[15]
9,8(9)-Dienol	1370	5	$H^5-C^{5,6,7}$: 79.2° (10.8°) $H^{11a}-C^{8,9,11}$: 126.2° (36.2°) $H^{11\beta}-C^{8,9,11}$: -117.7° (27.7°)	9.3°	<i>endo/exo</i>	[16]

Substrate	k_H ($M^{-1}s^{-1}$) ^a	# of Sub ^b	Φ_{HC}^c	Φ_{CC}^d	<i>endo/exo</i> ^e	References
6,8(14)-Dienol	412	5	$H^5-C^{5,6,7}$: 91.7° (1.7°) $H^{15\alpha}-C^{8,14,15}$: 51.8° (38.2°) $H^{15\beta}-C^{8,14,15}$: -69.9° (20.1°)	4.6°	<i>exo</i>	[16]
8-Dehydrocholesterol	994	5	$H^{7\alpha}-C^{5,6,7}$: -133.5° (43.5°) $H^{7\beta}-C^{5,6,7}$: 108.6° (18.6°) $H^{7\alpha}-C^{7,8,9}$: 129.8° (39.8°) $H^{7\beta}-C^{7,8,9}$: -111.5° (21.5°)	3.2° <i>f</i>	<i>endo</i>	[16]
5,8(14)-Dienol	911	5	$H^{7\alpha}-C^{5,6,7}$: -114.3° (24.3°) $H^{7\beta}-C^{5,6,7}$: 128.5° (38.5°) $H^{7\alpha}-C^{7,8,9}$: -87.5° (2.5°) $H^{7\beta}-C^{7,8,9}$: 31.4° (58.6°)	21.5° <i>g</i>	<i>exo</i>	[16]
Oleate	0.88 (0.22)	2	-	-	-	[50]
Conjugated linoleate	14 (3.5) ^h	2	-	-	-	[53] ^h
Linoleate	62 (31)	2	-	-	-	[50]
Cyclohexene	6 (1.5)	2	-	-	<i>endo</i>	[50]
1,3-Cyclohexadiene	220 (55)	2	-	-	<i>endo</i>	[50]
1,4-Cyclohexadiene	265 (66)	2	-	-	<i>endo</i>	[54]

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d $\nu_{\text{H-H}}$ per H-atom is shown in the parentheses.

q Number of substituents on the delocalized radical intermediates.

c Dihedral angles between the reactive C–H and the double bond plane and the values in the parenthesis show the difference from 90° .

d Dihedral angles between the planes containing individual double bond.

e *endo*: radical delocalize within the same ring; *exo*: radical delocalize across rings.

f The angle between the planes C^{5,6,7} and C^{7,8,9}.

g The angle between C^{5,6,7} and C^{7,8,14}.

h value was obtained by extrapolating the rate constants of oleate and linoleate using their computed bond dissociation enthalpy [53].

Table IIComparison of reactivity of unsaturated lipids toward free radical oxidation in human plasma.^a.

	Lipid	k_H (M⁻¹s⁻¹)^b	[L-H] (nmol/mL)^c	$k_H \times [L-H]$ (relative oxidation rates)
Control population	Oleate	0.88	533 ^d	0.5
	Linoleate	62	1820 ^d	113
	Arachidonate	197	237 ^d	47
	Cholesterol	11	3760 ^e	41
	7-DHC	2260	3 ^e	7
SLOS patients	Cholesterol	11	2200 ^f	24
	7-DHC	2260	651 ^f	1471

^a Assuming similar kinetic rate law as in solution oxidation applies.^b Rate constants in solution (see Table I for refs).^c Levels in human plasma.^d Levels of the corresponding cholesteryl esters in human plasma [29].^e Levels of total sterols[29].^f Mean levels found in SLOS patients [102].