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Cholesterol Peroxidation as a Special Type of Lipid Oxidation in Photodynamic Systems†

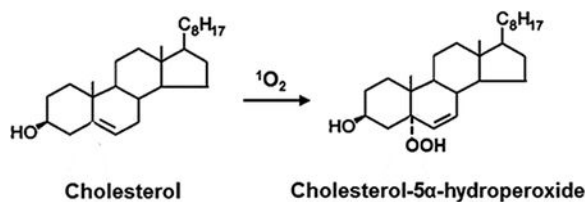
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Abstract

Like other unsaturated lipids in cell membranes and lipoproteins, cholesterol (Ch) is susceptible to oxidative modification, including photodynamic oxidation. There is a sustained interest in the pathogenic properties of Ch oxides such as those generated by photooxidation. Singlet oxygen (¹O₂)-mediated Ch photooxidation (Type II mechanism) gives rise to three hydroperoxide (ChOOH) isomers: 5α-OOH, 6α-OOH, and 6β-OOH, the 5α-OOH yield far exceeding that of the others. 5α-OOH detection is relatively straightforward and serves as a definitive indicator of ¹O₂ involvement in a reaction, photochemical or otherwise. Like all lipid hydroperoxides (LOOHs), ChOOHs can disrupt membrane or lipoprotein structure/function on their own, but subsequent light-independent reactions may either intensify or attenuate such effects. Such reactions include (a) one-electron reduction to redox-active free radical intermediates, (b) two-electron reduction to redox-silent alcohols, and (c) translocation to other lipid compartments, where (a) or (b) may take place. In addition to these effects, ChOOHs may act as signaling molecules in reactions that affect cell fates. Although processes a-c have been well studied for ChOOHs, signaling activity is still poorly understood compared with that of hydrogen peroxide. This review focuses on these various aspects Ch photo-peroxidation and its biological consequences.

Abstract



Cholesterol-5α-hydroperoxide (5α-OOH) is the major product of singlet oxygen (¹O₂)-mediated photosensitized peroxidation of cholesterol, a monoenoic lipid in cell membranes and lipoproteins. In the presence of redox-active metal ions and reductants, 5α-OOH undergoes one-electron reduction to free radical species that can induce light-independent chain peroxidation reactions

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Conflict of Interest

The authors declare that they have no conflicts of interest with any of the work described.

which exacerbate oxidative damage/dysfunction. Selenoperoxidase GPx4-catalyzed two-electron reduction of 5 α -OOH opposes these effects, yet 5 α -OOH remains one of the most noxious of the known natural lipid hydroperoxides. These and related aspects of photodynamic cholesterol peroxidation are discussed in this review.

Keywords

cholesterol; cholesterol oxidation; lipid peroxidation; singlet oxygen; free radicals; translocation; glutathione peroxidase

Introduction

Cholesterol (cholest-5-en-3 β -ol; abbreviated as Ch) is a sterol lipid found naturally in all animal cells and lipoproteins. Most of the Ch (>80%) in mammalian cells is located in the plasma membrane, where it comprises 45–50 mol % of the total lipid (1,2). In low density lipoprotein (LDL), free Ch and esterified Ch (i.e. Ch with a fatty acid at the C3 position) account for ~19 mol % and ~55 mol %, respectively, of the total lipid. As a monounsaturated lipid with a double bond at the 5–6 position, Ch is susceptible to non-enzymatic oxidation (autoxidation), but less readily than a polyunsaturated phospholipid such as 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine. Nevertheless, the relatively abundant Ch in plasma membranes compared with other cellular membranes makes it an important oxidative target in the former. Unlike natural phospholipids, Ch exists as a single molecular species, and its relatively few oxidation products are easier to separate and characterize than those of phospholipids (3,4). Cholesterol oxides (ChOX) generated by chemical oxidants, ionizing radiation, or photodynamic action have been well studied in a variety of reaction systems, ranging from liposomal membranes and lipoproteins to mammalian cells (3–7). The authors review their extensive work in this area, with a special emphasis on photodynamically generated ChOX species.

Cholesterol Hydroperoxides Generated by Photodynamic Action

Lipid hydroperoxides (LOOHs) are important intermediates in the non-enzymatic oxidation of all unsaturated lipids, including phospholipids and Ch (4,8,9). In free radical-mediated reactions, including Type I photooxidation reactions, where O₂ levels are relatively low, Ch gives rise to two epimeric ring hydroperoxides (ChOOHs): 3 β -hydroxycholest-5-ene-7 α -hydroperoxide (7 α -OOH) and 3 β -hydroxycholest-5-ene-7 β -hydroperoxide (7 β -OOH) (3,9–11) (**Fig. 1**). Both of these species arise via abstraction of a C7 allylic hydrogen from the Ch molecule. Abstraction could be accomplished by a strong oxidant such as the radical anion of a proximal photosensitizer (**Fig. 3**) or a reactive oxygen species (ROS) that it generates, e.g. hydroxyl radical (HO \cdot) (12). Rapid addition of O₂ to the resulting C7 radical, followed by hydrogen abstraction from another Ch or proximal other lipid gives 7 α -OOH. The latter gradually isomerizes to 7 β -OOH, which is thermodynamically more stable (11). Redox-active 7 α -OOH and 7 β -OOH are typically accompanied by their downstream diol reduction products: cholest-5-ene-3 β ,7 α -diol (7 α -OH) and cholest-5-ene-3 β ,7 β -diol (7 β -OH), along with 3 β -hydroxycholest-5-ene-7-one (7-ketone) and cholestan-5 α /5 β ,6 α /6 β -epoxy-3 β -ol

(epimeric 5,6-epoxides) (**Fig. 1**). All of these species can potentially arise during Type I photoperoxidation of Ch, 7 α -OOH and 7 β -OOH as unstable intermediates.

In $^1\text{O}_2$ singlet oxygen ($^1\text{O}_2$)-mediated Type II photooxidation reactions, where O_2 levels are relatively high, three primary ChOOH species are generated: 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide (5 α -OOH), 3 β -hydroxycholest-4-ene-6 α -hydroperoxide (6 α -OOH), and 3 β -hydroxycholest-4-ene-6 β -hydroperoxide (6 β -OOH) (**Fig. 1**). Each arises via the ene addition of $^1\text{O}_2$ at the Ch double bond, with all atoms in the target molecule being retained (13–15). The generation rate of 5 α -OOH is usually much greater than that of 6 α - or 6 β -OOH. This is probably due to steric hindrance which results in less favorable $^1\text{O}_2$ attack at the C6 position (16). While 7 α - and 7 β -OOH do not arise directly from $^1\text{O}_2$ attack, they may appear indirectly via allylic rearrangement of 5 α -OOH, which becomes more favorable as the extent of lipid peroxidation increases in organized systems. Cholesteryl esters bearing an unsaturated fatty acyl group would be susceptible to oxidation in this group as well as in the Ch A/B ring portion. If an esterified polyunsaturated fatty acid such as linoleic or linolenic were present, its oxidation rate would be expected to exceed that of the monoenoic Ch moiety; however, it appears that no confirmation of this has been reported, at least for photodynamic oxidation.

One-Electron Reduction of ChOOHs: Exacerbation of Oxidative Damage/Dysfunction

If catalytic metal ions and/or reductants are absent or inaccessible, Type II $^1\text{O}_2$ -derived ChOOHs, like phospholipid counterparts (PLOOHs), will accumulate linearly with increasing sensitizer concentration or light fluence (4,6,17). The same may apply for Type I ChOOH formation, although a light-generated sensitizer radical anion might reduce the ChOOH to an oxyradical which begets new LOOHs/ChOOHs via chain peroxidation (see below), although this has not been rigorously tested for Type I chemistry. If a properly chelated metal ion (e.g. iron) and a reductant such as ascorbate or cysteine are available, the primary ChOOH (like LOOHs in general) can undergo one-electron reduction to an oxyl radical (ChO \cdot), which may directly abstract an allylic hydrogen from a neighboring lipid, thereby triggering a wave of free radical-mediated (chain) peroxidation (**Fig. 2**). Alternatively, the ChO \cdot might undergo rapid one-electron rearrangement with O_2 addition to give an epoxyallylic peroxy radical (OChOO \cdot) which initiates chain peroxidation. Although the latter mechanism has been demonstrated for relatively simple fatty acid model systems (18), there appears to be no similar existing evidence for Ch or fatty acids in phospholipid form. Chain peroxidation induced by one-electron reduction of primary or “priming” ChOOHs in a membrane bilayer would produce new ChOOHs/LOOHs that feed into the overall process (**Fig. 2**). These secondary reactions would exacerbate the damaging effects of primary peroxidation alone, e.g. $^1\text{O}_2$ -mediated 5 α -OOH formation in a photodynamic system. **Fig. 3** illustrates this by showing that liposomes containing an unsaturated phospholipid (POPC), Ch, and priming 5 α -OOH underwent a rapid loss of the latter during incubation with ascorbate and a catalytic amount of lipophilic iron chelate, ferric-8-hydroxyquinoline [Fe(HQ) $_3$]. 7 α /7 β -OOH accumulated concurrently, reached a steady state level after ~15 min, and declined thereafter, evidently because it also began to be consumed by one-electron reduction and free Ch became limiting. Thus, 7 α /7 β -OOH served as a reporter of free radical-mediated lipid peroxidation triggered by 5 α -OOH one-electron

turnover. Consistent results were obtained when cultured cells were monitored after a photodynamic challenge. For example, exposure of merocyanine 540-sensitized leukemia L1210 cells to increasing fluences of broad-band visible light, followed by dark incubation, resulted in a >3-log loss of clonogenicity (6). When introduced immediately after irradiation, the chain-breaking antioxidant butylated hydroxytoluene (BHT) inhibited the cell killing in substantial, dose-dependent fashion. However, di-*tert*-butyltoluene (DBT), a non-phenolic analogue used as a control, had no effect. Importantly, BHT also suppressed post-irradiation chain peroxidation in these cells, as indicated by increasing 7-OOH/5 α -OOH ratio (5). Of related interest, treating cells with an iron chelator/redox inhibitor (desferrioxamine) before a photodynamic challenge strongly attenuated the extent of photokilling and increase in 7-OOH/5 α -OOH ratio, whereas addition of a lipophilic iron chelate (ferric-8-hydroxyquinoline) in trace amounts did the opposite (5). Collectively, these findings support the notion that while primary LOOHs/ChOOHs can be deleterious on their own (depending on levels attained), they may induce secondary, light independent reactions that are potentially more damaging. In other words, there could be an after-light continuum in the cytotoxic activity initiated by photodynamic events, primary peroxide formation being the main driving force. This possibility needs to be better recognized in photo-cytotoxicity studies, e.g. those involving anti-cancer photodynamic therapy (PDT).

The preceding comments about damaging peroxide turnover require some qualification, based on a more recent comparison of the chain-initiating potency of different ChOOH isomers. When unilamellar liposomes containing POPC, Ch, and 5 α -OOH, 6 β -OOH, or 7 α -OOH (each at the same starting level) were incubated with Fe(HQ)₃ and ascorbate, all three hydroperoxides decayed at the same rate (16,19). This implied equal ability to undergo one-electron reduction. However, when chain-initiating potency was assessed, using [¹⁴C]Ch as an endogenous sensor (see below), 5 α -OOH and 7 α -OOH exhibited nearly equal potency, whereas 6 β -OOH showed very little activity (19). Because of its scarcity, 6 α -OOH was not tested, but is expected to mimic 6 β -OOH as a relatively poor initiator. The contrasting initiation ability of 5 α -OOH and 6 β -OOH was also observed at the cellular level. Thus, when L1210 cells were challenged with 5 α -OOH- vs. 6 β -OOH-containing liposomes, chain peroxidation and cytotoxicity caused by the latter were substantially less than those by the former (19). Since 5 α -OOH and 6 β -OOH decay equally rapidly in the presence iron and ascorbate, their oxyl radicals should appear at the same rate. Why, then, is the chain-initiating potency of 6 β -O \cdot so low compared with that of 5 α -O \cdot ? Two possible explanations have been offered (19): (i) The oxidizing potential of 6 β -O \cdot , i.e. its ability to abstract an allylic hydrogen from another lipid, might be much lower than that of 5 α -O \cdot ; (ii) Improper orientation of 6 β -O \cdot in the bilayer may substantially slow its access to neighboring chain-propagating lipids. The latter kinetic argument is more plausible because 6 β -OOH can induce at least some chain peroxidation after prolonged membrane incubation with iron and a reductant (19). One should note that even if 6 β -OOH were as efficient as 5 α -OOH in triggering chain reactions, 6 β -OOH would still have a relatively small effect, given its low yield in Type II photooxidation reactions.

Two-Electron Reduction of ChOOHs: Alleviation of Oxidative Damage/Dysfunction

Unlike superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are inactivated by superoxide dismutases and catalase/peroxidases, respectively, 1O_2 has no known primary enzymatic scavengers. In the case of cytotoxicity due to photogenerated 1O_2 , therefore, secondary or back-up defenses need to be engaged. This would typically involve enzymatic reduction or removal of oxidized groups at damage sites, followed by repair or replacement processes (4,6,8). Four intracellular enzymes with glutathione (GSH)-dependent peroxidatic activity have been implicated in LOOH reduction/detoxification: glutathione peroxidase-1 (GPx1), glutathione peroxidase-4 (GPx4), glutathione-S-transferase type- α (GST α), and peroxiredoxin-6 (Prx6) (4). GPx1 and GPx4 (formerly known as phospholipid hydroperoxide glutathione peroxidase, PHGPx) have been studied far more extensively than the others. Each GPx isotype has an active site selenocysteine residue (Sec), which participates in the two-electron reduction of peroxides to redox-inactive alcohols. Monomeric GPx4 (~20 kDa) is functionally quite distinct from homotetrameric GPx1 (~82 kDa), the most abundant seleno-peroxidase in mammalian cells. Whereas GPx4 with GSH can act directly on LOOHs in membrane and lipoprotein environments, GPx1 lacks this ability (4,20–22). Instead, GPx1 acts on H_2O_2 and relatively polar LOOHs such as fatty acid hydroperoxides, typically after they are released from peroxidized membrane phospholipids by phospholipase- A_2 (PLA $_2$) hydrolysis (23). Consistent with this, two general enzymatic mechanisms have been proposed for elimination of PLOOH peroxide groups in membranes or lipoproteins: (i) excision/reduction/repair, where sequential action of PLA $_2$ and GPx1 is followed by acyltransferase-catalyzed re-acylation of the lysolipid; and (ii) reduction/excision/repair, where sequential action of GPx4 and PLA $_2$ is followed by re-acylation (4,23). Existing evidence favors the latter mechanism over the former, although studies in this important area are ongoing.

As discovered by the authors and collaborators (24,25), GPx4, but not GPx1, can readily catalyze not only the two-electron reduction of PLOOHs, but ChOOHs as well. To date, GPx4 appears to be the only enzyme capable of catalyzing ChOOH reductive detoxification, and regardless of whether these peroxides are membrane-bound or in Triton-X100 micellar form. Studies in the authors' laboratory have revealed a broad range of reactivity with GSH/GPx4 for various ChOOH isomers; collectively, however, they reacted much more slowly than PLOOHs. The first-order rate constants for hydroperoxide decay were found to increase in the following order: 5α -OOH \ll $7\alpha/\beta$ -OOH $<$ 6β -OOH $<$ PLOOH (26). This pattern was observed for purified GPx4 from rat testis and also for GPx4 in homogenates of selenium-replete L1210 cells, whereas selenium-starved cells showed little activity (**Fig. 4A**). Importantly, the order of cytotoxicity for selenium-replete cells under similar incubation conditions (**Fig. 4B**) was diametrically opposite to that of GPx4-catalyzed detoxification. Thus, 5α -OOH with the slowest two-electron reduction rate was the most cytotoxic, whereas POPC-OOH with the fastest rate was the least cytotoxic. Note that 6β -OOH was the least toxic among the ChOOHs; although it was reduced relatively rapidly by GSH/GPx4, this probably mattered little compared with its sluggish one-electron reduction (see preceding section). It should be emphasized that very slow 5α -OOH detoxification, combined with rapid generation under 1O_2 stress conditions (e.g. UVA radiation, PDT, etc.), would make 5α -OOH the most physiologically deleterious of the LOOHs described. A plausible

explanation for slow inactivation is that 5 α -OOH is a tertiary hydroperoxide, and as such might not interact with GPx4 as readily as PLOOHs, 6 α /6 β -OOH, or 7 α / β -OOH, which are all secondary hydroperoxides (16).

Cholesterol as a Mechanistic Probe and Chain Peroxidation Reporter

Mechanistic information based on detection of 5 α -OOH as a $^1\text{O}_2$ reporter and 7 α / β -OOH as a free radical reporter has been acquired for a variety of isolated membrane and cellular systems (5,27). High sensitivity/specificity analytical techniques are essential for monitoring these ChOOHs, the yields of which are typically quite low. An key example is reverse-phase high performance liquid chromatography with mercury cathode electrochemical detection [HPLC-EC(Hg)], which was developed in the authors' laboratory (28,29). Using an appropriate column and deoxygenated mobile phase, one can separate and detect all of the ChOOHs described at very high sensitivity; for example, 7 α / β -OOH has a detection limit of ~0.2 pmol (29). With suitable care in sample preparation, HPLC-EC(Hg) is amenable to analysis of complex systems, e.g. lipid extracts from photodynamically-stressed cells. Mechanistic assignment based on 5 α -OOH for $^1\text{O}_2$ and 7 α / β -OOH for free radicals is usually very dependable, although some uncertainties may exist. For example, 5 α -OOH can isomerize to 7 α -OOH, but this is usually very slow in moderately peroxidized membranes (28,29). Also, 5 α -OOH might possibly derive from $^1\text{O}_2$ arising via downstream Russell-type reactions, i.e. peroxy radical disproportionation reactions (30,31). Detecting both reporters in such cases could lead to confusion, e.g. co-existence of Type I and Type II photooxidation mechanisms. Up to now, however, no *de novo* 5 α -OOH has been detected in membranes undergoing chain peroxidation (16), so we believe that it strictly reports on primary $^1\text{O}_2$ formation in such systems.

In addition to using specific ChOOHs as diagnostics, the authors have used cholesterol oxide intermediates/products called "ChOX" to obtain additional mechanistic information. A novel approach was devised whereby radiolabeled Ch is exploited as a natural probe for assessing overall chain peroxidation or the chain-initiating potency of pre-existing primary PLOOHs/ChOOHs. In this approach, [^{14}C]Ch is first incorporated into a lipid matrix such as liposomal membrane, cell membrane, or lipoprotein. During chain reactions induced by an oxidative challenge in the presence of reducible metal ions or reductive turnover of pre-existing LOOHs, the probe gives rise to [^{14}C]ChOX species. The latter are conveniently detected and quantified by high performance thin layer chromatography with phosphorimaging detection (HPTLC-PI) (32,33). Individual ChOX species include redox-active 7 α / β -OOH as well as redox-inactive 7 α / β -OH, 7-ketone, and 5,6-epoxide. The overall levels attained can reflect the chain-initiating potency of a primary peroxide such as PDT-generated 5 α -OOH as well as the oxidizability of surrounding lipids and availability of metal ions and reductants. There are many advantages of using [^{14}C]Ch as a natural sensor. First of all, oxidative degradation of Ch has been studied extensively for many years and most of its oxidation products (oxides/oxysterols) have been well characterized (3,7,13,14,34). This was facilitated by the fact that Ch, unlike natural phospholipids, exists as a single molecular species, making separation and identification of its oxidation products much more straightforward than for phospholipids, each of which can exist in multiple molecular forms. A second advantage of [^{14}C]Ch is that it translocate spontaneously and

relatively rapidly from a liposome or serum albumin carrier to an acceptor, e.g. cell membrane. Transfer rate can be increased using a protein transporter such as recombinant sterol carrier protein-2 (SCP-2), which is easily removed sufficient transfer uptake is achieved. For mammalian cells, most of the [^{14}C]Ch transferred in this way will reside in the plasma membrane, allowing chain peroxidation activity to be probed specifically in this compartment (32,33). Another plus about using [^{14}C]Ch is that it avoids the potential artifacts associated with exogenous probes such as spin traps and fluorophores (34,35), which might perturb lipid organization or act as antioxidants in systems being studied. On the other hand, such probes have the advantage of being non-invasive, i.e. signals are obtained directly without having to solubilize and extract lipids. Nevertheless, the [^{14}C]Ch approach is highly sensitive and straightforward in application and has been successfully applied to a number of reaction systems, ranging from liposomes and lipoproteins to membranes of living cells (33).

Spontaneous and Protein-Mediated ChOOH Translocation

As first demonstrated by the authors and associates about 20 years ago, one-electron or two-electron reduction of a primary ChOOH such as 5 α -OOH is not necessarily limited to its membrane of origin, but can extend to other membranes via a translocation process (37–39). Transfer from lipoproteins to membranes and vice-versa has also been shown to occur (39). All LOOHs are capable of spontaneous translocation, but ChOOHs move from a donor to acceptor membrane/lipoprotein far more rapidly than PLOOHs with the same peroxy group content, i.e. one per molecule (39). The parent lipids can also translocate, Ch much more rapidly than any given PL; however, peroxidation increases each rate substantially due to increased hydrophilicity of the peroxide products (40). An experiment with photoperoxidized erythrocyte membrane (ghost) donors and small unilamellar liposome acceptors in ~10-fold lipid molar excess showed that the rate constant for overall ChOOH transfer exceeded that of parent Ch by >60-fold (38). Transfer occurred via an aqueous pool, departure from the donor membrane being the rate-limiting step (40). For HPLC-EC(Hg)-assessed individual ChOOHs, the first-order transfer rate constants were found to decrease in the following order: 7 α /7 β -OOH > 5 α -OOH > 6 α -OOH > 6 β -OOH, which correlated with their elution rate (or hydrophilicity) order on reverse-phase HPLC (38,39). ChOOH or PLOOH translocation to an acceptor membrane could put the latter at greater risk of chain peroxidative damage if ferric iron and reductants are more available there. On the other hand, if the GSH/GPx4 system is more available in the acceptor compartment, translocation might become beneficial by promoting LOOH detoxification.

Although pro-oxidant ChOOH translocation may play a significant role in various types of oxidative challenge, it could be particularly important for $^1\text{O}_2$ -mediated photodynamic reactions. Special attention should be directed to 5 α -OOH translocation in this regard. In a cell under $^1\text{O}_2$ attack, e.g. a skin fibroblast exposed to UVA (320–400 nm) radiation, 5 α -OOH would be generated much more rapidly than other ChOOHs, but detoxified relatively slowly by GSH/GPx4 (see Fig. 4A). PLOOHs would also be generated rapidly, e.g. in a plasma membrane exposed to $^1\text{O}_2$, but detoxified rapidly by GSH/GPx4 (24,25). Moreover, PLOOHs translocate far more slowly than all ChOOHs, including 5 α -OOH (39). This being the case, intermembrane transfer of 5 α -OOH, whether spontaneous or protein-mediated (see

below), would clearly be favored over that of other LOOHs, and would thus greatly expand its range as a cytotoxic oxidant. Up to now, such possibilities for 5 α -OOH have not been well recognized in the photobiology field.

Lipid transfer proteins are known to play key roles in lipid metabolism, membrane biogenesis, and membrane function (41). A well-known example is sterol-carrier protein-2 (SCP-2), a 13.2 kDa protein that can facilitate intracellular translocation of relatively low polarity lipids such as Ch and other sterols, along with various phospholipids (42,43). Because of its broad ligand recognition, SCP-2 is often referred to as a non-specific lipid transfer protein. Seminal studies in the authors' laboratory revealed that the rate of intermembrane ChOOH or PLOOH transfer could be substantially increased over the spontaneous rate when SCP-2 was present. As shown in **Fig. 5**, recombinant SCP-2 significantly accelerated the movement of Ch and various ChOOHs from photoperoxidized red cell ghosts to unilamellar liposomes (44). The first-order rate constants (corrected for spontaneous transfer) decreased in the following rank order: 7 α /7 β -OOH > 5 α -OOH > 6 α -OOH > 6 β -OOH > Ch, which was the same as that for spontaneous transfer (see above). This was the first known evidence for enhanced ChOOH transfer by a lipid transfer protein. Subsequent work revealed that a transfectant clone of hepatoma cells expressing ~10-fold more SCP-2 than vector controls, imported liposomal 7 α -OOH more rapidly than the latter and delivered it preferentially to mitochondria over other subcellular compartments (45). Such delivery resulted in relatively rapid peroxidation of mitochondrial lipids, membrane depolarization, and apoptotic cell death (45). These effects were primarily attributed to damaging free radical (chain) reactions triggered by one-electron reduction of SCP-2-delivered 7 α -OOH. Mitochondria are rich in redox iron, which would have favored these reactions. No significant damage or cytotoxicity was observed when redox-inactive 7 α -OH (a SCP-2 ligand) or redox-active *tert*-butyl hydroperoxide (a non-SCP-2 ligand) were used, demonstrating the necessity for both protein recognition and redox activation. When attached to the sterol binding pocket of SCP-2, 7 α -OOH was probably protected from redox turnover in transit, allowing it to survive delivery through the cytosol to/into mitochondria. The molecular basis for mitochondrial targeting of SCP-2-borne ChOOH is unknown, but presumably reflects the existence of some outer membrane recognition site for the protein.

Proteins of the steroidogenic acute regulatory (StAR) family have also been implicated in potentially deleterious ChOOH transfer (46–49). These proteins contain a unique sterol binding (START) domain and play a crucial role in steroid hormone synthesis by steroidogenic cells (50) as well as reverse cholesterol transport (RCT) by vascular macrophages for maintenance of Ch homeostasis (51). Most StAR proteins bind a single Ch or similar sterol molecule with high specificity, and thus are distinct from SCP-2 or other non-specific lipid transporters (52). StarD4 is a cytosolic member of the StAR family whose proposed function is to deliver Ch to the mitochondrial outer membrane, where it is recognized by StarD1 and transferred to the inner membrane for metabolism to pregnenolone in steroidogenic cells or 27-hydroxycholesterol (27-OH) in macrophages (53,54). We (the authors) postulated that under pathological conditions associated with oxidative stress, including stress from certain therapeutic regimens, oxidized LDL (oxLDL)-derived ChOOHs might be caught up in StAR-mediated trafficking to/into mitochondria,

thereby triggering peroxidative damage that impairs Ch metabolism. Korytowski *et al.* (55) provided the first supporting evidence by using testicular MA-10 cells, which exhibited a strong upregulation of StarD4 and StarD1 after cAMP stimulation. When incubated with 7 α -OOH-containing liposomes, stimulated cells trafficked more of the peroxide to mitochondria than non-stimulated controls, resulting in a greater loss of membrane potential and progesterone output, along with more extensive apoptotic cell death (55). StarD1 knockdown using siRNA strongly blunted 7 α -OOH uptake and membrane depolarization, confirming StarD1's role in the mitochondrial damage/dysfunction. Similarly to SCP-2, StarD4/D1 would be expected to protect ligated ChOOH against premature reductive turnover in transit, allowing the bulk of this turnover to occur after release in mitochondria.

In more recent work, mitochondrial impairment due to inauspicious StAR-mediated ChOOH trafficking was studied in the context of cardiovascular disease, specifically atherogenesis. Using human THP-1 monocyte-derived macrophages, Korytowski *et al.* (49) showed that cAMP stimulation resulted in a robust upregulation of StarD1 and also the ABCA1/G1 transporters, which localize to the plasma membrane and mediate Ch efflux to extracellular acceptors such as apoA1 and HDL (49). The major functional consequences of exposing stimulated cells to liposome-borne 7 α -OOH were (i) enhanced mitochondrial uptake of this ChOX; (ii) greater chain lipid peroxidation localized to mitochondria; (iii) significant activity loss of 27-hydroxylase (CYP27A1), which generates 27-OH, a key agonist for ABCA1/G1 expression and RCT operation; (iv) diminished 27-OH yield from Ch; (v) diminished ABCA1/G1 expression; and (vi) increasing cell loss to intrinsic (mitochondrial-initiated) apoptosis during exposure to 7 α -OOH (49). Collectively, these findings with MA-10 cells and THP-1 macrophages were the first to show that a ChOOH like 7 α -OOH can impair crucial mitochondrial functions by integrating into a natural trafficking pathway (46–49). Although most of this work was done with free-radical-derived 7 α -OOH, similar damage/dysfunction would be expected if 5 α -OOH were used, since this ChOX can also be trafficked by the StAR system. A long lifetime because of low susceptibility to enzymatic detoxification and protection against reductive turnover when StarD1/D4-bound (see above), would favor “stealthy” 5 α -OOH delivery to mitochondrial targets. Chronic skin exposure to ¹O₂-generating UVA might conceivably generate sufficient 5 α -OOH *in vivo* to act in this fashion, but this remains to be documented.

ChOOHs as Stress Signaling Mediators

It is now clear that many LOOHs, including ChOOHs, may not only induce/propagate oxidative damage in biological systems, but at relatively low levels may act in a more subtle manner characterized by reversible signaling processes. As oxidative pressure intensifies, the outcome may change accordingly, e.g. (i) no net damage if antioxidant capacity is sufficient; (ii) relatively minor damage that induces cytoprotective or pro-survival responses; (iii) more extensive damage that exceeds cytoprotective antioxidant capacity and triggers programmed (apoptotic or autophagic) cell death; or (iv) extensive membrane damage that results in necrotic cell death because programmed responses are no longer possible (8). The tipping point between cell survival and death would occur at some point between responses (ii) and (iii). This often involves a signaling cascade induced by oxidation of a low pK_a cysteine thiol group to a sulfenic acid (-CySOH) form, followed by reaction (resolution) with another

cysteine residue to give a disulfide link. The modified protein would either gain or lose activity, and this could be reversed by a reductant such as glutathione (GSH) or thioredoxin, qualifying this event as part of a signaling pathway (55,56). Hydrogen peroxide (H_2O_2) is a well-established initiator of redox signaling cascades, and much more is known about cysteine-mediated H_2O_2 signaling than that of most other natural peroxides, including PLOOHs and ChOOHs. The discovery that these LOOHs can translocate from sites of origin (40) has suggested new possibilities for disseminated redox signaling which could enhance our understanding of this phenomenon beyond what is known about H_2O_2 signaling via simple diffusion. One important distinguishing factor is that amphiphilic LOOHs should move mainly between low polarity compartments in cells, i.e. membranes, whereas hydrophilic H_2O_2 , which has no known protein transporters, would be confined to aqueous compartments. Accordingly, one can postulate that sensor proteins on or near membranes would be the preferred targets of mobilized LOOHs. By contrast, H_2O_2 would preferentially target relatively hydrophilic, non-membrane-associated sensors. While there are several well-known examples of the latter, including thioredoxins, peroxiredoxins, and protein tyrosine phosphatases (55–57), membrane-associated analogues specifically activated by LOOHs are yet to be identified. Because PLOOH and ChOOH molecules are larger than H_2O_2 , they would diffuse more slowly to sensor targets. LOOH transit would be even slower if mediated by transfer proteins. However, there would be distinct advantages for signaling in the latter case, e.g. (i) longer peroxide lifetime due to sequestration against reductive turnover in transit, and (ii) site-specific LOOH delivery dictated by precise transfer protein-sensor protein interactions. PLOOHs and ChOOHs may also have a longer lifetime in transit because only one enzyme, GPx4, could potentially inactivate them, whereas H_2O_2 can be rapidly decomposed by much more abundant GPx1 as well as catalase and peroxiredoxins. Largely due to difficulties in establishing it experimentally, genuine LOOH-mediated signaling remains largely unexplored except for a few well qualified examples (58,59).

As indicated in the preceding section, 5 α -OOH is the most rapidly generated ChOOH in photodynamic systems and the slowest to be redox-inactivated by GPx4. Thus, 5 α -OOH's relatively long lifetime, combined with its ability to be dispersed by protein transporters, would make it one of the most active and far-reaching LOOHs in terms of cytotoxicity as well as redox signaling. One intriguing possibility regarding 5 α -OOH signaling activity relates to peroxide modification/inactivation of PTEN, the tumor-suppressor enzyme that down-regulates PI3K/Akt-promoted survival/expansion of many tumor cells (60,61). Most studies have associated PTEN inactivation with H_2O_2 produced, for example, by NADPH oxidase (NOX) enzymes (61,62). However, in cells irradiated in the presence of a membrane-associated 1O_2 -generating sensitizer (e.g. 5-aminolevulinate-induced protoporphyrin IX), high yield 5 α -OOH might reach a sensor like PTEN via translocation, resulting in its inactivation. This is one of many signaling possibilities for this ChOOH. Our understanding of 5 α -OOH's unique characteristics along these lines is still far from complete, and much more remains to be learned in an effort to better understand its role in pathologic as well as therapeutic photobiology.

Summary and Perspectives

We have discussed the special features of Ch peroxidation and the ChOOH species produced, with an emphasis on photoperoxidation reactions. The hydroperoxide most associated with oxidative stress damage/dysfunction or oxidative signaling is H₂O₂. This ROS is generated by NADPH-dependent oxidase (NOX) enzymes and during mitochondrial electron transport (54,55), but is usually not a prominent intermediate in phototoxidation reactions, with the possible exception of free radical Type I reactions (12). When O₂ is sufficient, most photooxidations proceed via ¹O₂-generating Type II reactions. Unlike H₂O₂, ¹O₂ has no enzymatic scavengers, so protection against this ROS must occur at the secondary level, e.g. GPx4-catalyzed reduction of ¹O₂ adducts such as 5α-OOH and 6α/6β-OOH (25,26). Like these ChOOHs, H₂O₂ may undergo toxic one-electron reduction at/near its site of origin or diffuse to other sites where this may occur. Such movement would be a random process, since H₂O₂ has no known protein transporters. In contrast, ChOOHs can be trafficked intracellularly by protein carriers such as SCP-2 and StARs, as discussed. In mammalian cells under oxidative pressure, ChOOHs arising in the Ch-rich plasma membrane might be transported to endoplasmic reticulum or mitochondria, thus permitting dissemination of one-electron oxidative damage. Unlike H₂O₂, which could encounter potent scavengers (catalase, various peroxidases), protein-bound ChOOHs would be effectively shielded from degradation until released at target sites. Similar arguments would hold for more subtle ChOOH vs. H₂O₂ signaling activity, where ChOOH “sensor” protein analogous to those involved in H₂O₂ signaling would be engaged. Based on peroxide polarity, one can predict that ChOOH sensors would be localized at relatively hydrophobic sites compared with H₂O₂ sensors (56). These arguments about ChOOH signaling are still largely hypothetical, given that specific receptors/sensors for such species have yet to be identified. An import goal for future investigation is to achieve a better mechanistic understanding of the diverse ChOOH activities described in this review.

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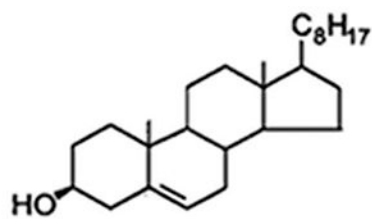
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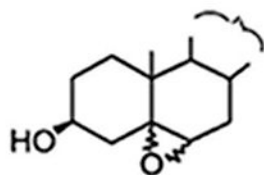
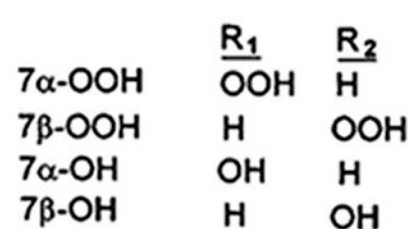
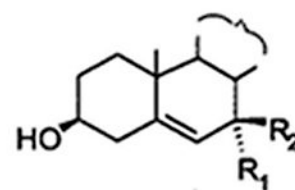
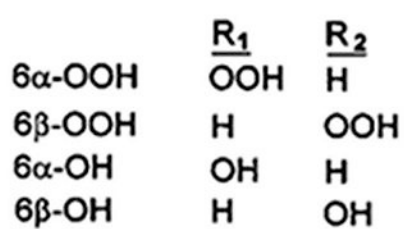
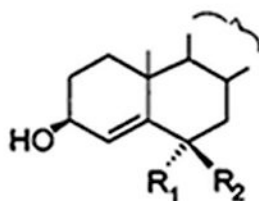
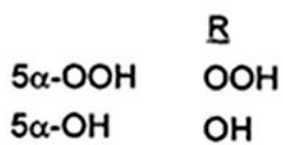
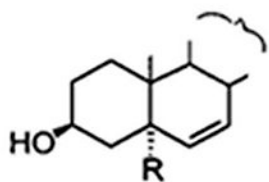
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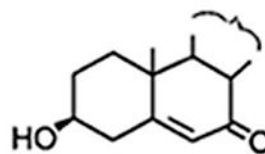
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Cholesterol

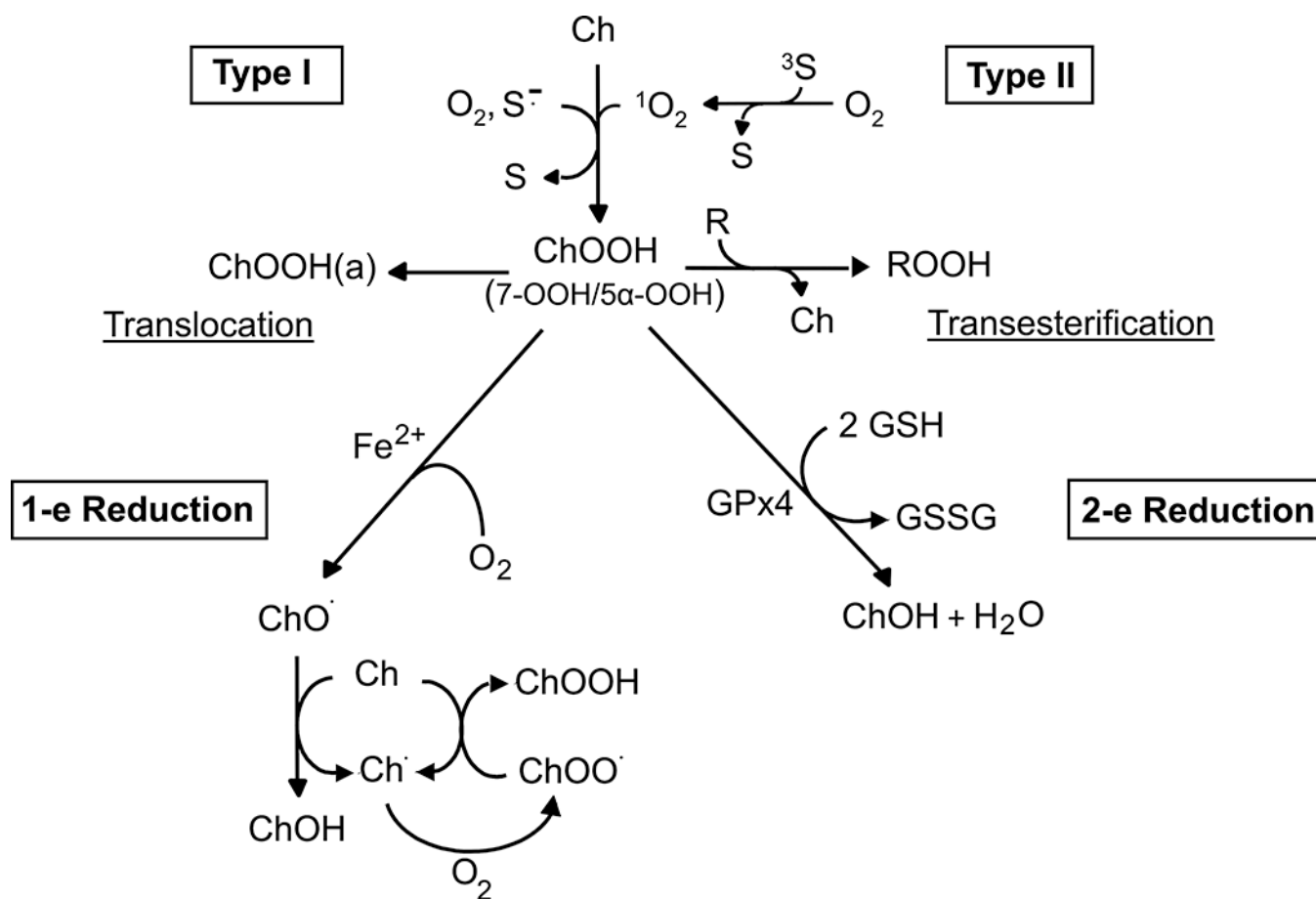


5,6-epoxide



7-ketone

Fig. 1. Structures of cholesterol, its relevant redox-active hydroperoxides (ChOOHs), and corresponding redox-inactive alcohols (ChOHs).

**Fig. 2.**

Type I (free radical) *versus* Type II (1O_2) photoperoxidation of cholesterol to hydroperoxide (ChOOH) intermediates, which can have different fates in membrane and lipoprotein environments, including transesterification, translocation, iron-catalyzed one-electron reduction to chain-initiating/propagating free radicals, and Gpx4-catalyzed two-electron reduction to redox-inert alcohols.

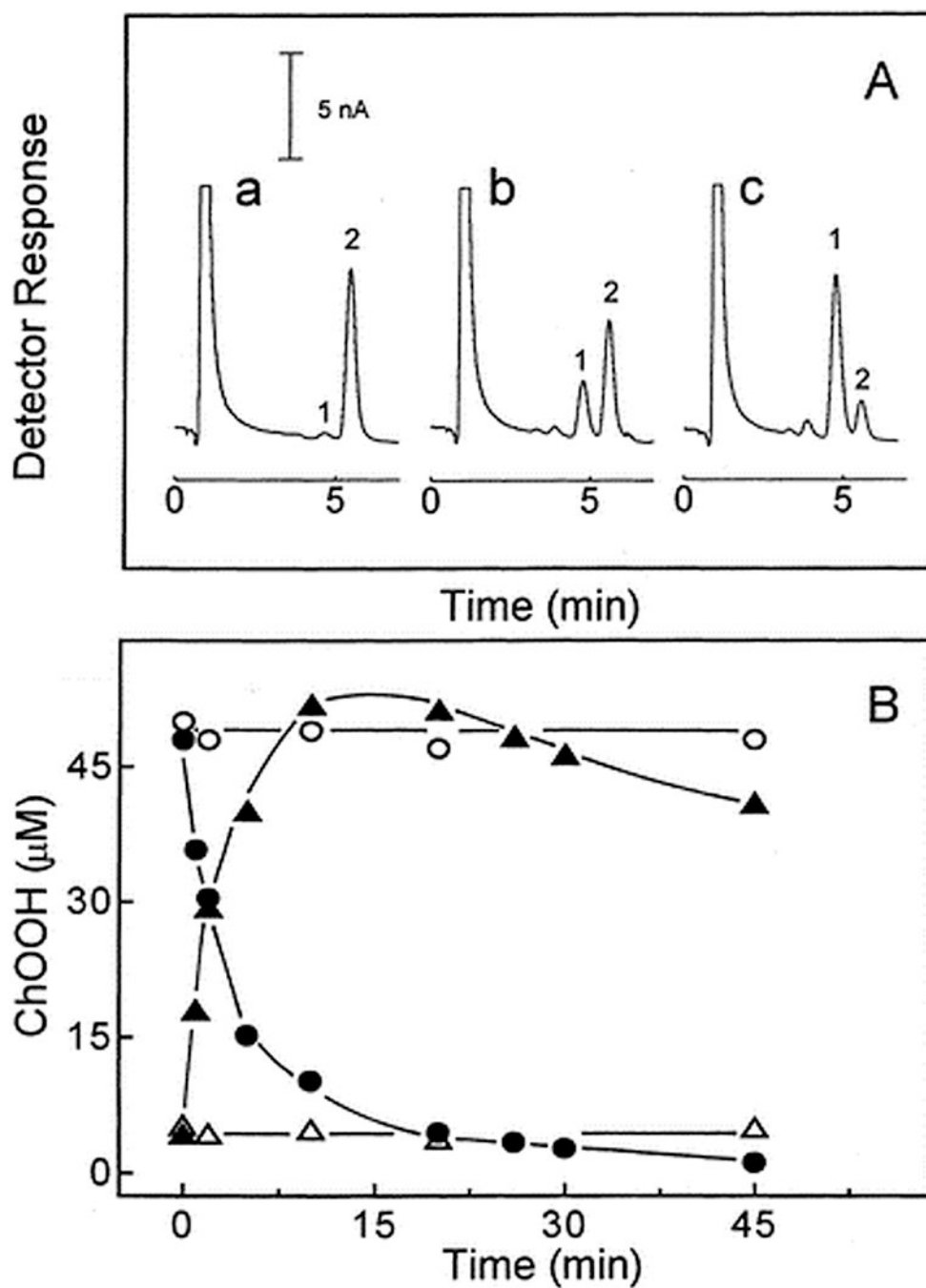


Fig. 3. One-electron degradation of 5 α -OOH with concurrent formation of 7 α /7 β -OOH in unilamellar POPC/Ch/5 α -OOH (100/75/5 by mol) liposomes during incubation with ascorbate (1 mM) and Fe(HQ)₃ (1 μM) in Chelexed PBS at 37°C. (A) HPLC-EC(Hg) profiles of lipid extracts after 0 min (a), 5 min (b), and 30 min (c). (B) 5 α -OOH (●,○) and 7 α /7 β -OOH (π,ρ) levels over time with Fe(HQ)₃ present (●, π) or absent (○, ρ). Reproduced from Korytowski *et al.* (2000) *Chem. Res. Toxicol.* 13:1265 (with permission).

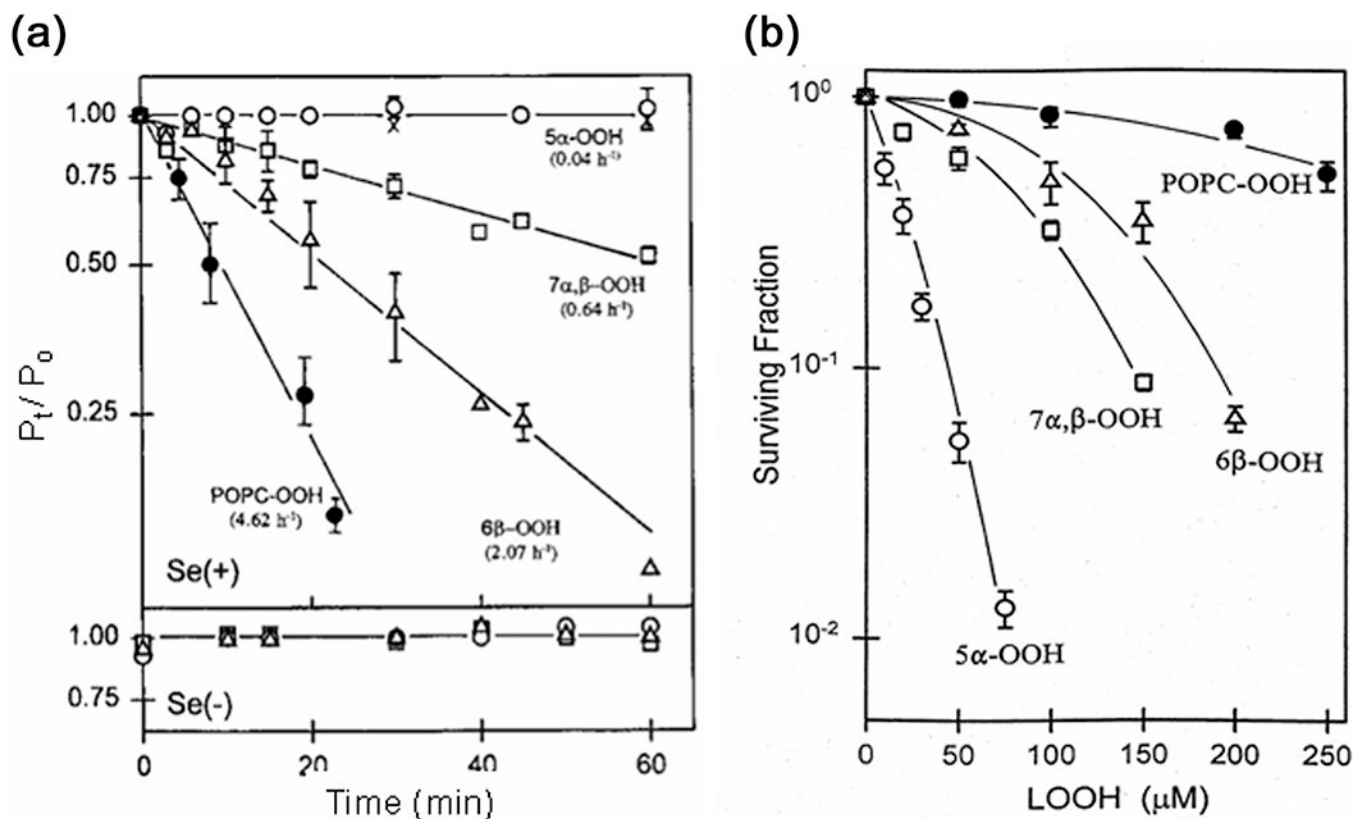


Fig. 4. Ability of GSH/GPx4 in lysates of selenium-replete [Se(+)] L1210 cells to (A) reduce and (B) detoxify different ChOOH species *versus* a PLOOH species. (A) Reaction mixtures contained the same concentration of hydroperoxide (5 α -OOH, 6 β -OOH, 7 α /7 β -OOH, or POPC-OOH), GSH, desferrioxamine, EDTA, and cell lysate in Triton-X100/PBS at 37°C. At the indicated times, lipid were extracted and analyzed by HPLC-EC(Hg). Se(-): lysates of selenium-starved cells; (x): controls lacking GSH or lysate. Plotted data are means \pm SEM (n=3). (B) Se(+) cells treated with ChOOH- or POPC-OOH-bearing liposomes. Lipid extracts were analyzed 20 h after incubation. Data are means \pm deviation (n=2). Reproduced in part from Korytowski *et al.* (1996) *Biochemistry* 35:8670 (with permission).

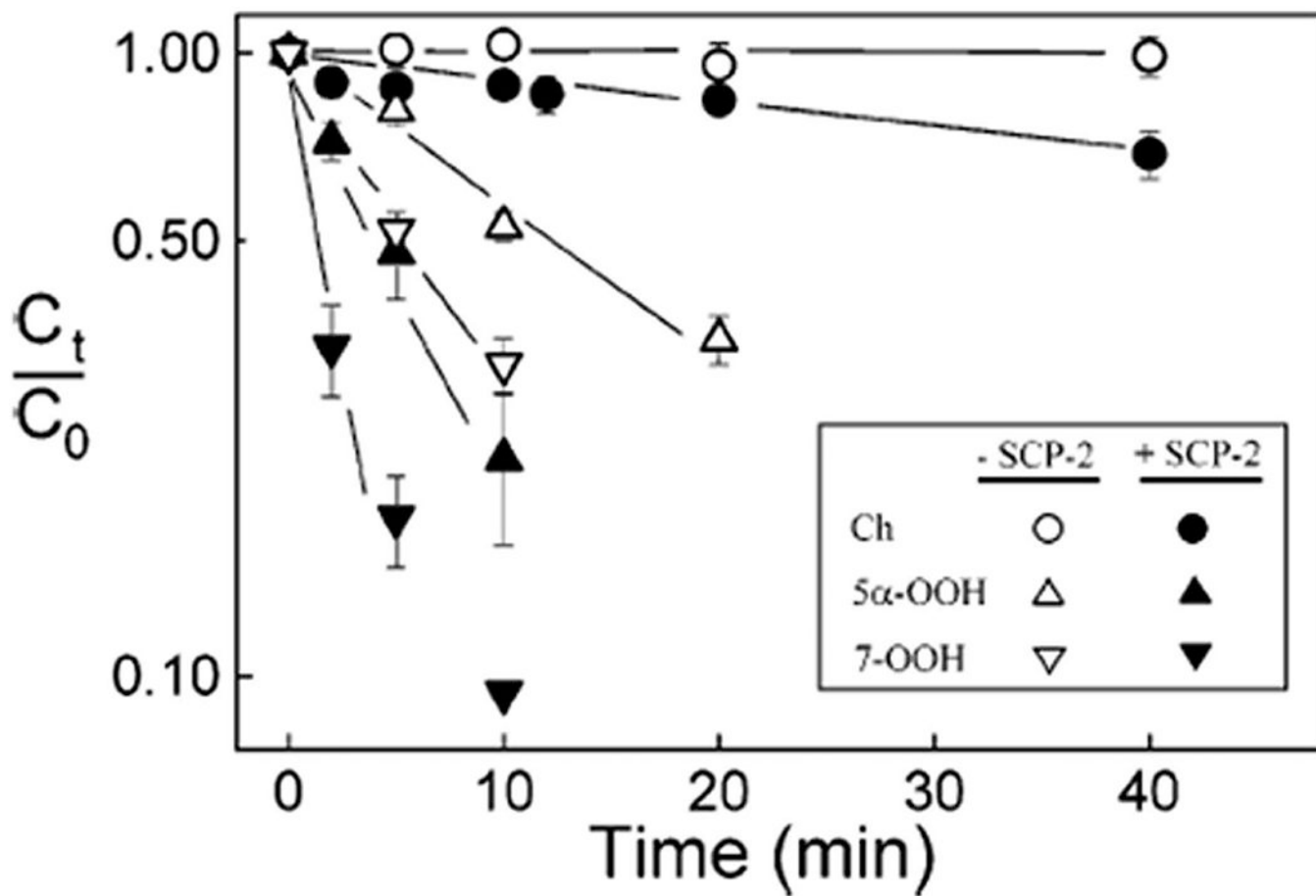


Fig. 5. SCP-2-accelerated intrmembrane transfer of Ch and ChOOHs. Reaction mixtures contained photoperoxidized erythrocyte ghosts, and DMPC/DCP (100:1 by mol) liposome acceptors in redox metal ion-depleted PBS containing or lacking SCP-2. Plots: HPLC-EC(Hg)-assessed Ch and ChOOH departure from ghosts during 37°C incubation. Plotted values are means \pm SEM (n=3). Reproduced from Vila *et al.* (2004) *Biochemistry* 43:12592 (with permission).