Cholesterol Oxidation: Mechanisms and Signature Products

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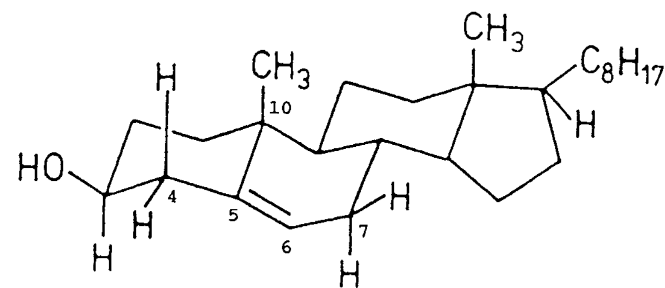
Cholesterol Oxidation: Contents

- Introduction
- Cholesterol oxidation in membranes and lipoproteins
  - key reactions, intermediates and products
- Cholesterol as a mechanistic probe
- Analysis of oxidation products (ChOX)
  - cholesterol hydroperoxides (ChOOHs)
  - other ChOX species
- Redox reactivity of ChOOHs
  - one-electron reduction
  - two-electron reduction
- Translocation of ChOOH species
- 7-Dehydrocholesterol oxidation and SLOS
- Summary and Conclusions

Introduction

- The sterol lipid cholest-5-en-3β-ol (cholesterol, Ch) is found naturally in all mammalian cells and lipoproteins. Most of the cellular Ch (>80%) is located in the plasma membrane, where it comprises ~45 mol % of the total lipid. In low density lipoprotein (LDL), free Ch and cholesteryl ester (CE, *i.e.* Ch esterified with a fatty acid at the 3-OH position) account for ~19 mol % and ~55 mol %, respectively, of the total lipid.



- As a monounsaturated lipid (double bond at 5,6-position), Ch is susceptible to spontaneous oxidation, albeit less so than polyunsaturated phospholipids. Unlike metabolic oxidation products (bile acids, steroid hormones), spontaneous oxidation products of Ch, including epoxides, peroxides and diols, are potentially cytotoxic and mutagenic.

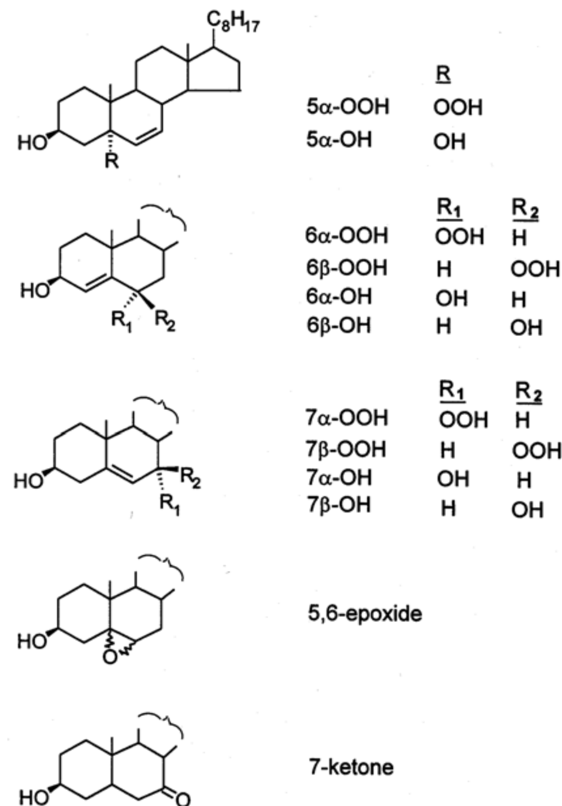
Cholesterol Oxidation: Key Intermediates/Products

- **Free radical-mediated reactions**

  Two epimeric ChOOHs are generated during free radical oxidation of Ch in cell membranes or lipoproteins: 3β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH) and 3β-hydroxycholest-5-ene-7β-hydroperoxide (7β-OOH). These are typically accompanied by the hydroxy analogues, cholest-5-ene-3β,7α-diol (7α-OH) and cholest-5-ene-3β,7β-diol (7β-OH), the 5,6-epoxide epimers, and the 7-ketone. Trace levels of other species may also be observed. 7α- and 7β-OOH are subject to redox turnover, making them reactive intermediates, whereas the other species are usually end-products. CE oxidation can give rise to species modified in either the cholesteryl or fatty acyl moiety, or both.

- **Singlet oxygen-mediated reactions**

  Singlet oxygen ($^1O_2$) attack on Ch gives three ChOOHs *via* ene-type addition: 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3β-hydroxycholest-4-ene-6α-hydroperoxide (6α-OOH), and 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH). 5α-OOH yield always predominates. Like the 7-hydroperoxides, 5α-OOH, 6α-OOH and 6β-OOH can be chemically or enzymatically reduced to diol analogues: 5α-OH, 6α-OH and 6β-OH, respectively. 7α/7β-OOH cannot arise from direct $^1O_2$ attack, but 5α-OOH generated in a pure $^1O_2$ reaction might partially rearrange to 7α-OOH, thereby confusing mechanistic deductions based on product analysis. However, this is more likely to occur during lipid extraction than *in* situ, and can be minimized with due precaution.



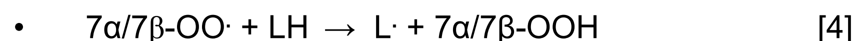
Cholesterol Oxidation: Key Reactions

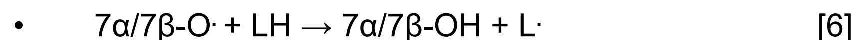
- **Free radical reactions**

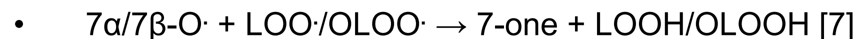
  Propagative (chain) lipid peroxidation can be triggered by a wide variety of chemical and physical agents. Reactive intermediates include lipid peroxyl radicals (LOO·) and lipid epoxyallylic peroxyl radicals (OLOO·). LOO·/OLOO· attack on the Ch double bond gives the epimeric 5,6-epoxides [Eq. 1]. Abstraction of a C-7 hydrogen by LOO·/OLOO· gives a cholesteryl radical [Eq. 2], which reacts rapidly with O_2 to give the 7α- or 7β-peroxyl radical [Eq. 3]. This abstracts a lipid (LH) allylic hydrogen to give 7α- or 7β-OOH [Eq. 4], the latter being thermodynamically more stable. Iron-mediated one-electron reduction of 7α/7β-OOH gives 7α/7β-oxyl radical [Eq. 5], which reacts with LH to give 7α/7β-diol [Eq. 6]. In the presence of LOO·/OLOO·, 7α/7β-oxyl radical can also undergo β-hydrogen scission to give 7-ketone [Eq. 7].

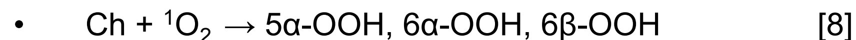
- **Singlet oxygen reactions**

  Singlet oxygen ($^1O_2$) generated in sensitized photodynamic reactions, for example, can also peroxidize lipids. $^1O_2$ adds to Ch with an allylic shift of the double bond to give 5α-OOH, 6α-OOH and 6β-OOH, the latter two in relatively low yield [Eq. 8].









[Korytowski *et al.* (1999) *Anal. Biochem.* **270:** 123-132]
[Girotti and Korytowski (2000) *Methods Enzymol.* **319:** 85-100]

Cholesterol as a Mechanistic Probe

- Lipid peroxidation and other oxidative damage can be "diagnosed" for free radical or $^1O_2$ involvement in various ways, including use of exogenous probes, *e.g.* EPR spin traps, fluorophores, and chemical antioxidants.

- Exploiting endogenous lipids for this purpose *via* detection of characteristic "signature" products avoids possible structural perturbations in the host membrane or lipoprotein by administered agents.

- Unsaturated phospholipids are oxidized to characteristic hydroperoxides (PLOOHs) in their *sn*-2 fatty acyl positions. [Frankel (1985) *Prog. Lipid Res.* **23:** 197-221] However, PLOOH molecular species are difficult to separate and analyze as such. When hydrolyzed off, the fatty acid hydroperoxides can be separated from one another, but are often degraded in the process.

- By comparison, use of Ch as an *in situ* probe has the following advantages: *(i)* unlike phospholipids, Ch exists as a single molecular species, making product analysis much less complicated; *(ii)* Ch's oxidation products are ready for analysis without the need for potentially artifactual hydrolysis steps; *(iii)* unlike phospholipids, Ch can be transfer-radiolabeled spontaneously in cells or lipoproteins, *i.e.* without a transfer protein requirement; *(iv)* Ch preponderance in the plasma membrane allows preferential probing of oxidative damage in this compartment. [Korytowski *et al.* (1999) *Anal. Biochem.* **270:** 123-132]

Analysis of Oxidation Products (ChOX)

- **Hydroperoxide species (ChOOHs)**

The following cutting-edge techniques have been developed for high sensitivity separation and quantitation of ChOOHs and other LOOHs:

(i) HPLC with chemiluminescence (CL) detection

[Miyazawa (1989) *Free Radic. Biol. Med.* **7**:209-217]

(ii) HPLC with mercury cathode electro-chemical [EC(Hg)] detection.

[Korytowski *et al.* (1995) *J. Chromatogr.* **670:** 189-197]

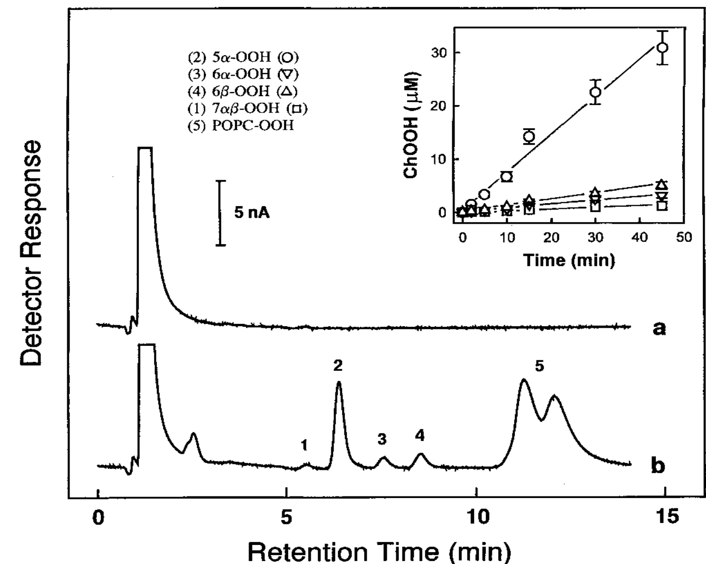
Reverse-phase HPLC-EC(Hg) is well-suited for ChOOH analysis, as demonstrated with peroxidized liposomes, red cell membranes, LDL and leukemia cells.

[Bachowski *et al.* (1994) *Lipids* **29:**449-45]

[Thomas *et al.* (1994) *Arch. Biochem. Biophys.***315:** 244-254]

The ChOOH detection limit with HPLC-EC(Hg) is 0.1-0.2 pmol or at least 10-times lower than that with HPLC-CL.

In HPLC-EC(Hg) profile *b* (representing a 10-min photooxidized membrane sample), several ChOOH molecular species are well-separated from one another and from a PCOOH family. $^{1}O_2$ was the dominant oxidant in the photoreaction because 5α-OOH accumulated much more rapidly than 7-OOH (inset).



[Korytowski and Girotti (1999) *Photochem. Photobiol.* **70:** 484-489]

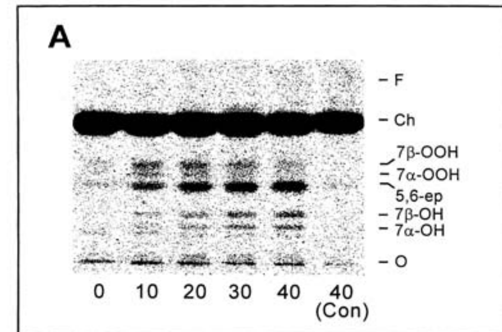
Analysis of Oxidation Products (ChOX)

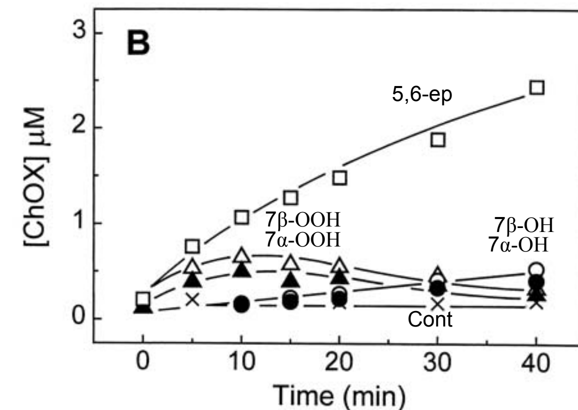
- **Other ChOX species**

In addition to "diagnostic" ChOOHs, one may also measure non-peroxide ChOX and thus obtain additional mechanistic information. In this case, a novel approach involving [^{14}C]Ch-labeling of the host membrane or lipoprotein can be used. Here, Ch is exploited as a natural "sensor" of free radical activity in its surroundings. Samples are analyzed by high performance normal-phase thin layer chromatography with phosphorimaging detection (HPTLC-PI) to determine radiolabeled 7α-OOH and 7β-OOH as well as 7α-OH, 7β-OH, 5,6-epoxide, and 7-ketone. The technique can be applied to living cells, where the [^{14}C]Ch probes mainly for free radical peroxidation taking place in the plasma membrane.

[Hurst *et al.* (2001) *Free Radic. Biol. Med.* **31:** 1051-1065]

In a relatively simple model, [^{14}C]Ch-labeled, 5α-OOH-primed unilamellar liposomes accumulated HPTLC-PI-detectable [^{14}C]ChOX during incubation with iron and ascorbate (panel A). 5,6-Epoxide, 7α-OH and 7β-OH levels increased progressively, whereas 7α-OOH and 7β-OOH increased to a steady state after ~10 min and then declined, consistent with one-electron turnover (panel B).





(Vila *et al.* (2000) *Arch. Biochem. Biophys.* **380:** 208-218)

Redox Reactivity of ChOOHs

- **One-electron reduction**

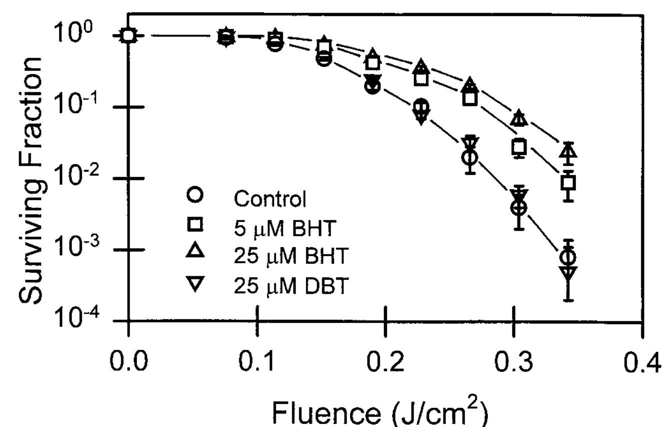
In the presence of reductants and catalytic iron, membrane or lipoprotein ChOOHs can undergo one-electron reduction to oxyl radicals, which trigger or propagate peroxidative damage [Eqs. 5, 6]. Model studies have indicated that liposomal 5α-OOH and 7α-OOH are reduced at the same rate during iron/ascorbate treatment. Moreover, these hydroperoxides are equally efficient in inducing [^{14}C]ChOX-assessed chain reactions.

[Korytowski *et al.* (1999) *Anal.* Biochem. 270: 123-132]

This suggests that in cells the relative toxicities of 5α-OOH and 7α-OOH would depend on differences in detoxification susceptibility rather than in chain initiation potency. Lethal injury can result if free radical reactions enhanced by peroxide pressure and iron availability outpace detoxification reactions.

An experiment with photodynamically stressed leukemia cells has provided evidence that supports this hypothesis.

Dye-sensitized cells accumulated $^1O_2$-derived LOOHs (including 5α-OOH and 6-OOH) and lost viability when exposed to increasing light fluences. When added immediately after irradiation, the chain-breaking antioxidant BHT (but not DBT, a non-phenolic analogue) reduced lethality, assessed after a 24 h dark delay. Thus, after-light chain peroxidation contributed significantly to the lethal response.



[Girotti (2001) *J. Photochem. Photobiol. B*. **63**: 103-113]

Redox Reactivity of ChOOHs

- **Two-electron reduction**

Selenoperoxidases (SePxs) catalyze the GSH-dependent two-electron reduction of hydroperoxides to alcohols, thus protecting cells against damaging one-electron reduction. For a $^1O_2$ challenge, this is the only known defense because $^1O_2$ has no primary enzymatic scavengers. Two intracellular SePxs have been implicated in LOOH detoxification: GPx1 and GPx4 (a.k.a. PHGPx). GPx4 can act directly on PLOOHs in membranes, whereas GPx1 is unreactive unless the *sn*-2 fatty acyl hydroperoxide moiety is liberated by hydrolysis. GPx4 can also detoxify membrane or lipoprotein ChOOHs, but these species are inert to GPx1. Studies with purified GPx4 and ChOOH-containing model membranes revealed the following rank order of first-order decay constants for individual ChOOHs: 5α-OOH<<7α/7β-OOH~ 6α-OOH<6β-OOH. (NB: 5α-OOH is a tertiary peroxide; the others are secondary peroxides.) The same trend was observed with homogenates of Se-replete L1210 cells (panel A), whereas Se-deficient counterparts had little activity (panel B), consistent with GPx4 involvement in (A). The order of cytotoxicity of these ChOOHs was diametrically opposite to that of GPx4-mediated detoxification (Table 1). Thus, 5α-OOH, with the same chain-initiating potency as the others, but longest metabolic lifetime, was the most toxic.

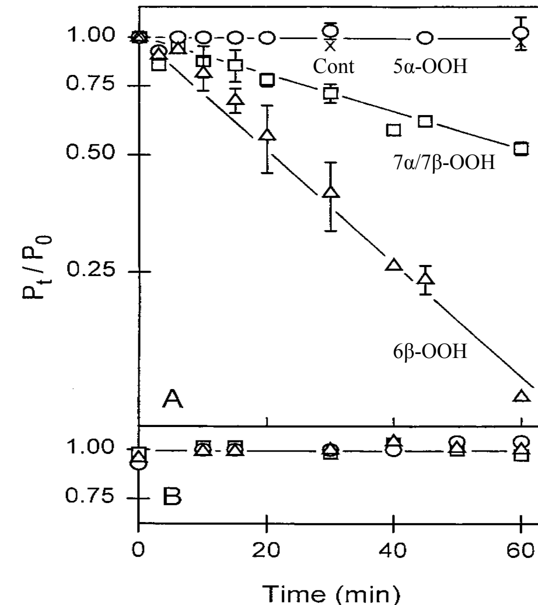


Table 1. Cytotoxic effects of various cholesterol-derived hydroperoxides

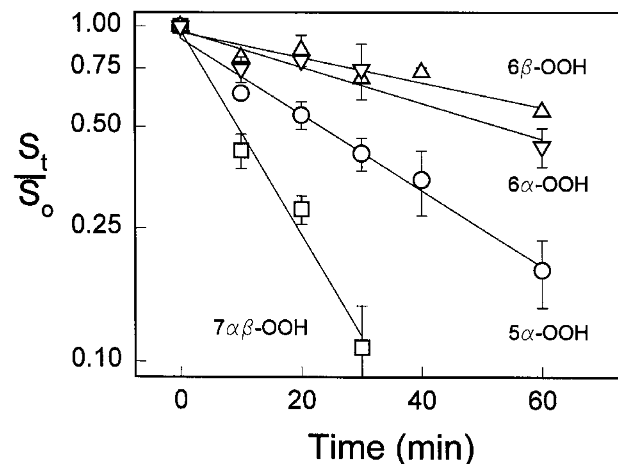
ChOOH	Log$_{10}$ reduction value† 50 μ*M*	100 μ*M*	150 μ*M*
5α-OOH	1.27 ± 0.03	2.64 ± 0.18	ND‡
6β-OOH	0.13 ± 0.02	0.33 ± 0.09	0.48 ± 0.09
7αβ-OOH	0.24 ± 0.05	0.50 ± 0.04	1.06 ± 0.02

[Korytowski *et al.* (1996) *Biochemistry* **35:** 8670-8679]
[Korytowski and Girotti (1999) *Photochem. Photobiol.* **70:** 484-489]

ChOOH Translocation

- **Intermembrane transfer**

Recent studies indicate that chain induction by a plasma membrane ChOOH may not be limited to its immediate environment, but can extend to other membranes or lipoproteins *via* spontaneous or protein-facilitated transfer. Model studies with photooxidized erythrocyte ghost donors and small liposome acceptors in ~10-fold lipid molar excess indicate that the rate constant for overall ChOOH transfer exceeds that of parent Ch by ~65-fold. Transfer occurs *via* an aqueous pool, desorption from the donor compartment being rate-limiting. Individual ChOOHs translocate with different first-order rate constants, the rank order being as follows: 7α/7β-OOH>5α-OOH>6α-OOH>6β-OOH. Reverse-phase HPLC elution rates of these species decrease in the same order; thus, faster transfer correlates with greater hydrophilicity. ChOOH transfer is accelerated by sterol carrier protein (SCP-2), suggesting involvement of the latter in ChOOH as well as Ch intracellular movement. Intra- and intercellular transfer of ChOOHs and other LOOHs could disseminate peroxidative injury, but in some instances might provide for more efficient detoxification.



[Vila *et al.* (2001) *Biochemistry* **40:** 14715-14726]

- **Membrane-to-LDL transfer**

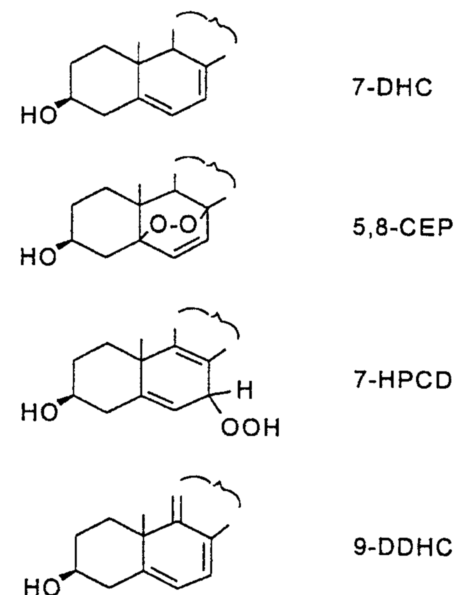
ChOOH transfer from peroxidized RBC ghost membranes to LDL has also been demonstrated and this sensitizes the LDL to Cu(II)-induced free radical peroxidation. Cell-to-LDL ChOOH transfer *in vivo* could be an important source of "seeding" peroxides which induce proatherogenic LDL modification.

7-DHC Oxidation and SLOS

- **Background**

Mental retardation and various congenital abnormalities are associated with the Smith-Lemli-Opitz syndrome (SLOS), which affects 1 in 20,000 infants. In SLOS, 7-dehydrocholesterol (7-DHC), the immediate biosynthetic precursor of Ch, accumulates to abnormal levels due to a deficiency in 7-DHC reductase. The disorder is variously ascribed to insufficient Ch and/or excess 7-DHC or its oxidation products.

[Waterham and Wanders (2000) *Biochim. Biophys. Acta* **1529:** 340-356]



- **Oxidation Mechanisms**

As a diene, 7-DHC is more oxidizable than Ch in both free radical- and $^1O_2$-mediated fashion. In solution, 7-DHC reacts with photogenerated $^1O_2$ to give the endoperoxide 5,8-CEP and the hydroperoxide 7-HPCD. The latter can also derive from free radical (e.g. HO·) attack. 7-HPCD is unstable and decomposes to give the trien-ol 9-DDHC and H_2O_2. 5,8-CEP and 9-DDHC have been identified in SLOS plasma, consistent with *in vivo* oxidative events. 7-HPCD arising from $^1O_2$ or HO· attack might induce damaging chain peroxidation *via* direct one-electron reduction or *via* decomposition and reduction of liberated H_2O_2. There is a skin and eye photosensitivity associated with SLOS that may be linked to 9-DDHC's ability to act as a UVA-absorbing sensitizer. Photogenerated $^1O_2$ converts 7-DHC to 7-HPCD and since the latter can form 9-DDHC, the overall process would be self-intensifying. Based on this information, it would make sense to boost the antioxidant defenses of SLOS patients, *e.g*. with β-carotene or vitamins C and E.

[Albro *et al.* (1997) *Photochem. Photobiol.* **65:** 316-325]

[Gaoua *et al.* (1999) *J. Lipid Res.* **40:** 456-463]

Summary and Conclusions

- As an unsaturated lipid, Ch is susceptible to free radical- and $^1O_2$-mediated oxidation, which contributes to overall membrane and lipoprotein oxidative damage.
- Being a single molecular species, Ch gives rise to relatively few oxidation products, the separation and characterization of which is relatively straightforward.
- Ch can be exploited as an *in situ* mechanistic probe, generating ChOOH intermediates which specify either $^1O_2$ or free radical intermediacy.
- With HPLC-CL or HPLC-EC(Hg), ChOOHs (*e.g*. $^1O_2$-generated 5α-OOH and radical-generated 7α/7β-OOH) are detected at much higher sensitivity than non-peroxide ChOX.
- Cell membranes and lipoproteins can be [^{14}C]Ch-labeled for non-invasive, high-sensitivity monitoring of free radical peroxidation *via* [^{14}C]ChOX formation.
- Various biological fates of ChOOHs include: *(i)* iron-mediated one-electron reduction (damage-enhancement); *(ii)* SePx-mediated two-electron reduction (damage control); and *(iii)* translocation, followed by *(i)* or *(ii)*.
- 7-DHC, the conjugated diene precursor of Ch, accumulates in SLOS and its relatively favorable oxidation is implicated in this disorder
- Cellular ChOOHs, like other LOOHs, may participate in stress signaling, which could either enhance cellular defense and/or growth or affect apoptotic death