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PHOTODYNAMICALLY GENERATED 3-β-HYDROXY-5α-CHOLEST-6-ENE-5-HYDROPEROXIDE: TOXIC REACTIVITY IN MEMBRANES AND SUSCEPTIBILITY TO ENZYMATIC DETOXIFICATION

PETER G. GEIGER[1], WITOLD KORYTOWSKI[2] and ALBERT W. GIROTTI[1]*
[1]Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53221, USA and
[2]Institute of Molecular Biology, Jagiellonian University, Krakow, Poland

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Abstract—Singlet oxygen ($^1O_2$)-mediated photooxidation of cholesterol gives three hydroperoxide products: 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). These species have been compared with respect to photogeneration rate on the one hand and susceptibility to enzymatic reduction/detoxification on the other, using the erythrocyte ghost as a cholesterol-containing test membrane and chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) as a $^1O_2$ sensitizer. Peroxide analysis was accomplished by high-performance liquid chromatography with mercury cathode electrochemical detection (HPLC-EC[Hg]). The initial rate of 5α-OOH accumulation in $AlPcS_4$/light-treated ghosts was found to be about three times greater than that of 6α-OOH or 6β-OOH. Membranes irradiated in the presence of ascorbate and ferric-8-hydroxyquinoline ($Fe[HQ]_2$, a lipophilic iron complex) accumulated lesser amounts of 5α-OOH, 6α-OOH and 6β-OOH but relatively large amounts of another peroxide pair, 3β-hydroxycholest-5-ene-7α- and 7β-hydroperoxide (7α,7β-OOH), suggestive of iron-mediated free radical peroxidation. When photoperoxidized membranes containing 5α-OOH, 6α,6β-OOH and 7α,7β-OOH (arising from 5α-OOH rearrangement) were incubated with glutathione (GSH) and phospholipid hydroperoxide glutathione peroxidase (PHGPX), all hydroperoxide species underwent HPLC-EC(Hg)-detectable reduction to alcohols, the relative first order rate constants being as follows: 1.0 (5α-OOH), 2.0 (7α,7β-OOH), 2.4 (6α-OOH) and 3.2 (6β-OOH). Relatively rapid photogeneration and slow detoxification might make 5α-OOH more cytotoxic than the other peroxide species. To begin investigating this possibility, we inserted 5α-OOH into ghosts by transferring it from 5α-OOH-containing liposomes. When exposed to $Fe(HQ)_2$/ascorbate, these ghosts underwent GSH/PHGPX-inhibitable chain peroxidation, as indicated by the appearance of 7α,7β-OOH, phospholipid hydroperoxides and thiobarbituric acid reactive substances. Liposomal 5α-OOH also exhibited a strong, $Fe(HQ)_2$-enhanced, toxicity toward L1210 leukemia cells, an effect presumably mediated by damaging chain peroxidation. This appears to be the first reported example of eukaryotic cytotoxicity attributed specifically to 5α-OOH.

INTRODUCTION

Cholesterol (cholest-5-en-3β-ol) is a prominent neutral lipid in eukaryotic cells, comprising 40–45 mol% of the plasma membrane lipid.[1] Like all unsaturated lipids, cholesterol is susceptible to oxidative modification when exposed to prooxidant conditions.[2–4] Oxidation products generated by exposure of cholesterol to photodynamic action, ionizing radiation, ozone, or Fenton reagents have been identified in relatively simple milieux such as organic solvents[5–9] and liposomes[10,11] and also in more complex systems such as isolated cell membranes,[12–15] low-density lipoprotein[16] and cultured cells.[17] In free radical-mediated reactions, including Type I photoreactions, a variety of hydroperoxides can be produced, the epimeric pair 3-β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH)† and 3β-hydroxycholest-5-ene-7β-hydroperoxide (7β-OOH) typically being the most prominent. Formation of theses species begins with H-abstraction at C-7 of cholesterol; the abstracting agent may either be a strong initiating oxidant such as hydroxyl radical or a propagating species such as lipid oxyl or peroxyl radical.[2,7] In singlet oxygen ($^1O_2$)-mediated reactions, *e.g.* Type II photooxidations, only three primary species are formed, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3β-hy-

*To whom correspondence should be addressed.
†*Abbreviations:* $AlPcS_4$, chloroaluminum phthalocyanine tetrasulfonate; ChOOH, cholesterol hydroperoxide; DCP, dicetylphosphate; DFO, desferrioxamine; $Fe(HQ)_2$, 2:1 (mol/mol) complex of 8-hydroxyquinoline and ferric ion; GPX, glutathione peroxidase; GSH, glutathione; HPLC-EC(Hg), high-performance liquid chromatography with mercury drop electrochemical detection; LOOH, lipid hydroperoxide; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 6α-OOH, 3β-hydroxycholest-4-ene-6α-hydroperoxide; 6β-OOH, 3β-hydroxycholest-4-ene-6β-hydroperoxide; 7α,7β-OOH, mixture of 3β-hydroxycholest-5-ene-7α-hydroperoxide and 3β-hydroxycholest-5-ene-7β-hydroperoxide; PBS, Chelex-treated phosphate-buffered saline (125 m*M* NaCl, 25 m*M* sodium phosphate, pH 7.4); PC, phosphatidylcholine; PHGPX, phospholipid hydroperoxide glutathione peroxidase; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; TBARS, thiobarbituric acid-reactive substances; TLC, thin-layer chromatography.

droxycholest-4-ene-6α-hydroperoxide (6α-OOH) and 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH), each of which arises *via* an ene-addition reaction.[5,6,8] The 6-OOH epimers are generated more slowly than 5α-OOH, both in homogeneous solution[5,6] and in membranes,[18] although the rate difference is much smaller in membrane systems. Typically, $^1O_2$ does not give rise to 7α- or 7β-OOH directly, but in low polarity microenvironments, 5α-OOH can undergo allylic rearrangement to 7α-OOH, which then epimerizes to 7β-OOH.[2,19] Thus, although 5α-OOH, 6α-OOH and 6β-OOH cannot be generated by reactions that are purely free radical in nature, 7α- and 7β-OOH may appear secondarily in purely $^1O_2$ reactions, thereby confusing mechanistic deductions based on product identification.[2-4]

Like other lipid hydroperoxides, cholesterol-derived species (ChOOH) exhibit mutagenic and cytotoxic properties. Smith *et al.* [20,21] have reported that 5α-OOH and 7α-OOH are weakly mutagenic toward *Salmonella typhimurium* and that this effect is antagonized by metabolism of these compounds. With regard to cytotoxicity, we have observed that unilamellar liposomes containing a mixture of photochemically generated ChOOH are lethally damaging to L1210 leukemia cells.[22] Loss of viability was much more pronounced in cells that had been made glutathione (GSH) or selenium deficient, implicating GSH and selenoperoxidases (glutathione peroxidase [GPX] and/or phospholipid hydroperoxide glutathione peroxidase [PHGPX]) in cytoprotection against these peroxides. Using noncellular model systems, *e.g.* photoperoxidized liposomes or erythrocyte ghosts,[23,24] we have shown that ChOOH are unreactive with GPX but can be reduced by PHGPX, suggesting that the latter enzyme may be specifically involved in the reductive detoxification of these species. Using high-performance liquid chromatography with mercury drop electrochemical detection (HPLC-EC[Hg]), a newly developed technique for the separation and ultrasensitive detection of peroxides,[25] we were recently able to monitor the reduction rates of individual ChOOH species during incubation of photoperoxidized ghost membranes with GSH/PHGPX. We have determined that 5α-OOH is reduced much more slowly than all other ChOOH, including 6α-OOH, 6β-OOH and the unresolved 7α,7β-OOH pair. These findings, along with the results of experiments designed to test the cytotoxicity of 5α-OOH, are described in this report.

MATERIALS AND METHODS

General materials. Cholesterol, egg phosphatidylcholine (PC), dicetylphosphate (DCP), GSH, sodium ascorbate, 8-hydroxyquinoline, Chelex-100 (50–100 mesh), RPMI-1640 medium, insulin, transferrin and antibiotics (penicillin, streptomycin) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was from Hyclone Laboratories (Logan, UT); 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) from Avanti Polar Lipids (Birmingham, AL); desferrioxamine (DFO) from Ciba-Geigy Corp. (Suffern, NY); and chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) from Porphyrin Products (Logan, UT). The HPLC-grade solvents were obtained from Burdick and Jackson Corp. (Muskegon, MI). Authentic cholesterol hydroperoxides (5α-OOH, 6α-OOH, 6β-OOH and 7α,7β-OOH) were prepared by $AlPcS_4$-sensitized photooxidation of cholesterol-containing liposomes, isolated by means of semipreparative HPLC and characterized as described.[6,19,26] Phospholipid hydroperoxide glutathione peroxidase was isolated from rat testes and purified as described.[16,27] Enzymatic activity was determined by coupled assay, using NADPH, GSH, glutathione reductase and photoperoxidized egg PC.[24] All aqueous solutions, including phosphate-buffered saline (PBS) solutions, were prepared with deionized, glass-distilled water and then treated with Chelex-100 in order to remove trace metal ions that might catalyze peroxide decomposition.[28]

Preparation of liposomal membranes and erythrocyte ghosts. Large unilamellar liposomes consisting, for example, of 1.0 m*M* POPC, 0.5 m*M* cholesterol, 0.05 m*M* DCP, and 0.1 m*M* 5α-OOH in bulk phase were fabricated with an extrusion device (Lipex Biomembranes, Vancouver, BC) using two polycarbonate filters of 100 μm pore size (Nucleopore Corp., Pleasanton, CA).[18] The liposomes were prepared in PBS and stored under argon at 4°C.

Isolated membranes from human erythrocytes (white ghosts) were prepared by conventional hypotonic lysis, followed by extensive washing.[13-15] The ghosts were incubated with 0.1 m*M* DFO for ~1 h to remove non-heme Fe^{3+}, then washed with and resuspended in PBS and stored under argon at 4°C. Membrane protein was determined by the Lowry assay.[29]

Incorporation of 5α-OOH into ghost membranes. A typical transfer procedure was as follows. An aliquot of ghost membranes (2.0 mg protein/mL; ~2.8 m*M* total lipid/mL) was incubated with an equal volume of 5α-OOH-containing liposomes (~1.7 m*M* total lipid/mL) for 24–48 h at 4°C. After centrifugation and removal of liposome-containing supernatant fractions, the pelleted ghosts were washed once with PBS and resuspended to the original volume in this buffer. Extent of transfer was determined by subjecting the lipid fraction of a sample extracted with chloroform/methanol (2:1, vol/vol) to HPLC-EC(Hg) analysis. Timed analyses have indicated that 5α-OOH is progressively taken up by the ghosts, reaching an equilibrium level of 28 ± 4% (mean ± SD, n = 5) incorporated within 24 h. The transferred 5α-OOH was quite stable for several additional days under the conditions described, *i.e.* there was little, if any, reduction to the 5α-alcohol or rearrangement to 7α-OOH.

Cultured cells. Murine leukemia L1210 cells were grown at 37°C under a humidified atmosphere of 95% air/5% CO_2. The RPMI-1640 growth medium contained 1% fetal calf serum plus the following supplements: insulin (10 μg/mL), transferrin (5 μg/mL), penicillin (100 units/mL), streptomycin (0.1 mg/mL) and sodium selenite (10 ng/mL). As needed, the cells were made selenium (selenoperoxidase) deficient by switching to a medium containing all of the indicated supplements except sodium selenite.[30] Cells were reseeded into fresh medium every 2 days. All experiments were carried out on logarithmically growing cells and viability was assessed by 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on mitochondrial succinic dehydrogenase activity.[31] Additional details are provided elsewhere.[30]

Membrane peroxidation. Photoreactions for determining kinetics of lipid hydroperoxide (LOOH) accumulation were carried out at 25°C in thermostatted stirrer bath chambers.[14,15] Ghost membrane suspensions (1.0 mg protein/mL in PBS) were sensitized with 10 μ*M* $AlPcS_4$ and irradiated as such or in the presence of ferric 8-hydroxyquinoline ($Fe[HQ]_2$) and ascorbate (typically 5 μ*M* and 100 μ*M*, respectively). Stock solutions of $Fe(HQ)_2$ in 50% methanol and ascorbate in PBS were prepared as described[32]; ascorbate was added to reaction mixtures immediately after preparation. The light source was a quartz–halogen lamp positioned above the reaction vessels. A cut-off filter was used to exclude wavelengths below ~300 nm. Fluence rate at suspension surfaces was measured with a radiometer and maintained at ~150 mW/cm^2. After addition of EDTA (~1 m*M*) to bind adventitious metal ions, irradiated samples were extracted with chloroform/methanol (2:1, vol/vol). Aliquots from the LOOH-containing organic layers were dried under a stream of argon and stored at −20°C until analyzed by iodometric assay or HPLC-EC(Hg).

Relatively large amounts of photoperoxidized ghosts for GSH/PHGPX experiments (see below) were prepared in large (45 mm i.d.) thermostatted beakers.[24] Except for the fluence rate (~225 mW/cm^2), reaction conditions were similar to those described in the preceding section.

Susceptibility of 5α-OOH-primed ghosts to free radical-mediated lipid peroxidation was assessed by incubating the membranes with $Fe(HQ)_2$ and ascorbate (*e.g.* 10 μ*M* and 250 μ*M*, respectively) at

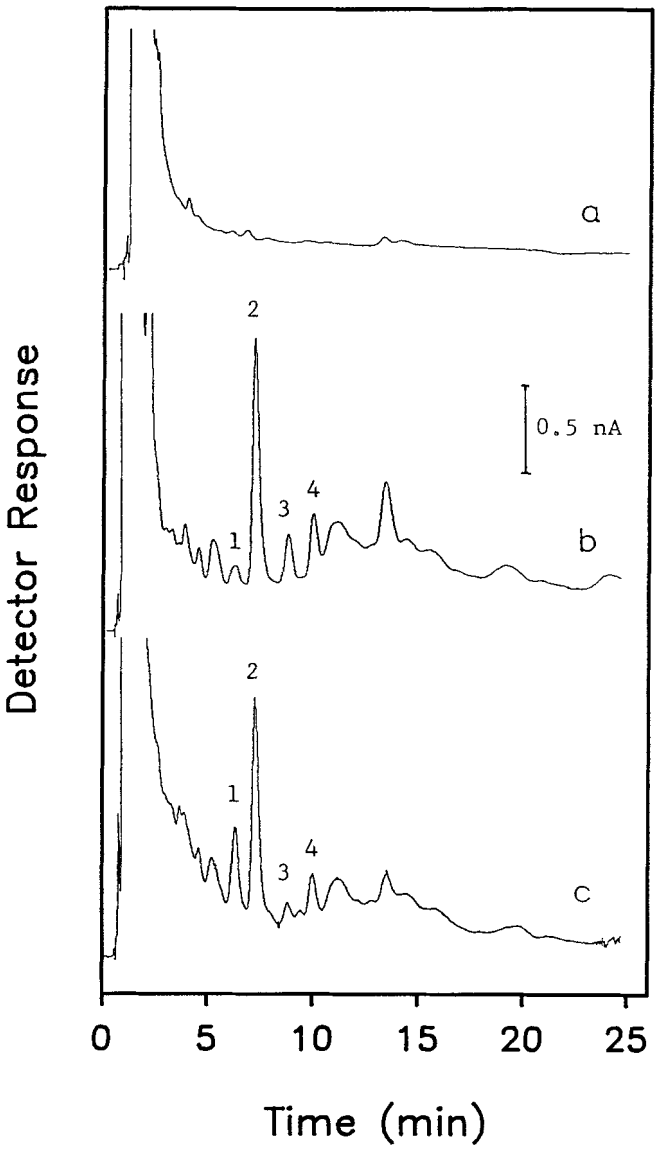

Figure 1. The HPLC-EC(Hg) of lipid hydroperoxides generated by photooxidation of erythrocyte ghosts in the absence or presence of iron/ascorbate. Ghost membranes (1.0 mg protein/mL in PBS) were sensitized with 10 μM $AlPcS_4$ and exposed to a light fluence of ~180 J/cm^2 in the absence and presence of 5 μM $Fe(HQ)_2$/100 μM ascorbate. Extracted lipid fractions were subjected to HPLC-EC(Hg), using the mobile phase described in the Materials and Methods and an elution rate of 1.8 mL/min. The chromatographic scans represent the following samples: (a) ghosts irradiated in the absence of added sensitizer, iron and ascorbate: (b) ghosts irradiated in the presence of $AlPcS_4$ alone; (c) ghosts irradiated in the presence of $AlPcS_4$, $Fe(HQ)_2$ and ascorbate. The ChOOH assignments and retention times are as follows: (1) 7α,7β-OOH (6.7 min); (2) 5α-OOH (7.5 min); (3) 6α-OOH (9.1 min); (4) 6β-OOH (10.3 min); peaks observed over the 11–15 min range represent phospholipid hydroperoxides. Full-scale detector sensitivity was 2.0 nA for all scans. Sample load per injection: 55 μg total lipid.

25°C. Samples were removed periodically and analyzed by HPLC-EC(Hg) or by thiobarbituric acid assay (see below).

Enzymatic reduction of membrane cholesterol hydroperoxides. A typical protocol for examining PHGPX-catalyzed reduction of membrane ChOOH was as follows. Ghosts that had been photoperoxidized in the presence of $AlPcS_4$ were adjusted to a total starting peroxide concentration of ~200 μM in PBS, and then incubated in the presence of 50 μM DFO, 5.0 mM GSH and 0.05 unit/mL PHGPX. Control mixtures lacking enzyme or GSH were incubated alongside. Samples were removed periodically and extracted; the recovered lipid fractions were subsequently analyzed by HPLC-EC(Hg).

Iodometric assay. The total LOOH content of standards and sufficiently peroxidized experimental samples was measured by iodometric assay, as described.[15] Hydroperoxides were reduced anaerobically in the presence of excess iodide, with stoichiometric formation of triiodide, which was measured spectrophotometrically at 353 nm. Quantitation was accomplished by using an extinction coefficient of 22.5 mM^{-1} cm^{-1}.[15]

Thiobarbituric acid assay. Aldehyde by-products of free radical lipid peroxidation triggered by iron/ascorbate in photooxidizing ghosts or by iron/ascorbate treatment of 5α-OOH-primed ghosts were measured by thiobarbituric acid assay. The conventional procedure[33] was modified slightly in order to improve sensitivity. A membrane sample (300 μL) was mixed with 50 μL of 5% (wt/vol) trichloroacetic acid. After centrifugation to pellet precipitated proteins, a 250 μL aliquot of the supernatant solution was mixed with 50 μL of warm 1.65% (wt/vol) 2-thiobarbituric acid. The mixture was heated at 100°C for 15 min, cooled rapidly to room temperature and absorbance at 532 nm was recorded, using a Hewlett-Packard diode-array spectrophotometer equipped with a 50 μL microcell. Absorbance readings were converted to thiobarbituric acid-reactive substance (TBARS) values by using an extinction coefficient of 157 mM^{-1} cm^{-1}.[33]

HPLC. Analytical HPLC-EC(Hg) was accomplished using a C18 Ultrasphere column (4.6 × 150 mm; 5 μm particles) from Beckman Instruments (San Ramon, CA), an Isco HPLC system (Isco Inc., Lincoln, NE) and an EG&G Princeton model 420 mercury drop electrochemical detector. The mobile phase consisting of (by volume) 80.5% methanol, 10.5% acetonitrile and 9.0% of an aqueous solution containing 10 mM ammonium acetate and 0.25 mM sodium perchlorate was sparged continuously with high purity argon and delivered isocratically at a flow rate of 1.7 or 1.8 mL/min. Injection volume for all samples (as isopropanol solutions) was 10 μL. The mercury cathode was set at −300 mV *vs* a Ag/AgCl reference for all measurements. Data collection, storage and manipulation were accomplished with an on-line IBM 486 clone and Isco Chem-Research software. Quantitation was based on the EC(Hg) characteristics of authentic standards; 5α-OOH and 7α,7β-OOH were found to have the same EC(Hg) response, which was ~30% greater than the 6α-OOH or 6β-OOH response. Additional details are provided elsewhere.[17,25]

RESULTS

Photoperoxidation of cholesterol and phospholipids in ghost membranes

Exposure of nonsensitized erythrocyte ghosts to a light fluence of ~180 J/cm^2 produced few, if any, HPLC-EC(Hg)-detectable products (Fig. 1, scan a). However, similar irradiation of $AlPcS_4$-sensitized ghosts resulted in the appearance of several distinguishable EC(Hg) peaks, which are attributed to lipid hydroperoxides (LOOH). All of the peaks observed in the 3–25 min retention time range disappeared upon treatment of a photooxidized sample with triphenylphosphine or GSH/PHGPX (data not shown), consistent with LOOH identity.[17] Peaks 1, 2, 3 and 4 (ranging from 6 to 11 min) have been assigned as cholesterol hydroperoxide species, *i.e.* 7α,7β-OOH, 5α-OOH, 6α-OOH and 6β-OOH, respectively, based on coelution of authentic standards. Because both 7α-OOH and 7β-OOH (in varying proportions) can arise *via* free radical processes or by rearrangement of 5α-OOH,[2] we believe that both species are represented in peak 1 and so refer to it as 7α,7β-OOH. Although the 6α- and 6β-OOH epimers were well separated from one another

under the chromatographic conditions used, 7α- and 7β-OOH were not, and therefore their individual concentrations could not be determined. Quantitation based on standard EC(Hg) responses showed that the order of abundance of ChOOH observed in Fig. 1b was as follows: 5α-OOH > 6α-OOH = 6β-OOH > 7α,7β-OOH, indicating that singlet oxygen (Type II) photochemistry was predominant in this system. Whereas peaks with retention times <6 min have not been identified, those above 11 min are attributed mainly to phospholipid hydroperoxides, based not only on susceptibility to reduction with GSH/PHGPX but also selective hydrolysis with phospholipase A_2 (*cf.* Bachowski *et al.*[17]). The time course for ChOOH build-up is shown in Fig. 2A. Linearity out to at least 30 min indicates that there was no significant depletion of substrate cholesterol under the reaction conditions used. (The 5α-OOH level at 30 min corresponds to ~0.33% of total membrane cholesterol.) Rates of peroxide accumulation (nmol/h/mg protein) were as follows: 3.90 (5α-OOH); 1.38 (6α-OOH or 6β-OOH); 0.33 (7α,7β-OOH). Relative rate values (*e.g.* 11.8 for 5α-OOH/7-OOH; 2.8 for 5α-OOH/6β-OOH) approximate those determined previously for $AlPcS_4$/light-treated liposomes.[18] As in those experiments, the small amount of 7α,7β-OOH seen in Fig. 1b and Fig. 2 is attributed to 5α-OOH rearrangement rather than free radical (Type I) photochemistry.

Membrane photoperoxidation in the presence of ascorbate and a lipophilic iron complex

The LOOH profile of $AlPcS_4$-sensitized ghosts was distinctly different when irradiation was carried out in the presence of a lipophilic iron complex ($Fe[HQ]_2$, 5 μ*M*) and an electron donor (ascorbate, 100 μ*M*) (compare scan c with scan b in Fig. 1). Most obvious was the large increase in the magnitude of peak 1 (7α,7β-OOH) with parallel decreases in peaks 2, 3 and 4 (5α-OOH, 6α-OOH and 6β-OOH, respectively). A significant decrease in some of the phospholipid hydroperoxide signals was also apparent, *e.g.* the peak at ~13.5 min. Based on previous qualitative evidence,[14] the observed effects of $Fe(HQ)_2$/ascorbate are attributed to mechanistic switching from a $^1O_2$-dominated reaction system (Fig. 1b) to one in which light-independent, one-electron reduction of $^1O_2$-derived peroxides triggers free radical reactions, which amplify production of 7α,7β-OOH (Fig. 1c). Figure 2B shows time courses for the accumulation of different ChOOH species during photooxidation on ghosts in the presence of $Fe(HQ)_2$/ascorbate. Peroxide levels increased in apparent linear fashion out to at least 40 min of irradiation, the rates of accumulation (nmol/h/mg protein) being as follows: 3.08 (5α-OOH); 0.90 (6α-OOH or 6β-OOH); 1.47 (7α,7β-OOH). Thus, inclusion of iron and ascorbate increased the 7α,7β-OOH rate nearly five-fold while decreasing the 5α-OOH rate by ~20% and the 6α- or 6β-OOH rate by ~45% (compare Fig. 1B with Fig. 1A). The TBARS content of membrane samples was substantially elevated when photooxidation was carried out in the presence of added iron and ascorbate, providing further evidence for the stimulation of free radical activity. In the Fig. 2 experiments, for example, the TBARS level was 0.086 nmol/mg protein after 30 min of irradiation in the absence of $Fe(HQ)_2$/ascorbate and 0.247 nmol/mg protein after 30 min of irradiation in the presence of $Fe(HQ)_2$/ascorbate.

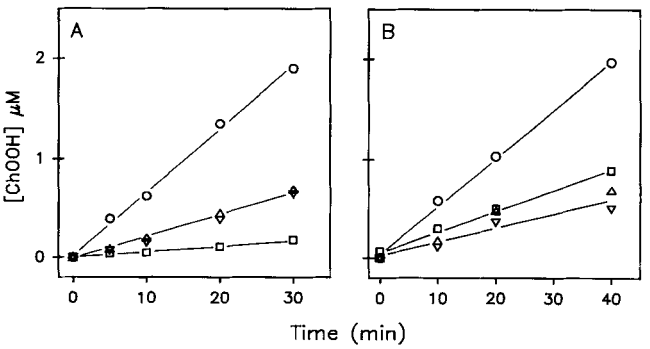

Figure 2. Time course of ChOOH accumulation during dye/light treatment of erythrocyte membranes. Ghost membranes (1.0 mg protein/mL in PBS) were irradiated at 25°C in the presence of 10 μ*M* $AlPcS_4$ alone (A) or 10 μ*M* $AlPcS_4$ plus 10 μ*M* $Fe(HQ)_2$ and 100 μ*M* ascorbate (B). A quartz–halogen source was used (fluence rate ~150 mW/cm^2). Lipid fractions from samples extracted at the indicated time points were analyzed for ChOOH by means of HPLC-EC(Hg). The ChOOH concentrations in the bulk phase of reaction mixtures are indicated. Measured species are designated as follows: (○) 5α-OOH; (△) 6α-OOH; (▽) 6β-OOH; (□) 7α,7β-OOH.

Enzymatic reduction of ChOOH in membranes

In view of the relatively rapid accumulation of 5α-OOH in photooxidizing membranes, it was of interest to ask whether this species is subject to enzymatic (reductive) inactivation and, if so, how rapidly, compared with other ChOOH. In previous studies,[23,24] we determined that membrane ChOOH, like phospholipid counterparts, are unreactive with the classical selenoperoxidase, GPX, but can be reduced by PHGPX, a more recently discovered homologue.[27] In that work, LOOH reactivity with PHGPX was assessed qualitatively, using normal phase thin-layer chromatography (TLC). In the case of ChOOH, TLC analysis during GSH/PHGPX treatment was restricted to diol reduction products, since the ChOOH themselves are poorly resolved from one another by TLC.[7,14,24] These limitations have now been circumvented through the use of HPLC-EC(Hg), which has allowed us to examine the kinetics of PHGPX-catalyzed reduction for individual ChOOH. Results of a typical experiment in which HPLC-EC(Hg) was used to analyze photooxidized ghost membranes during GSH/PHGPX treatment are shown in Fig. 3. (Note that the starting ratio of 7α,7β-OOH to 5α-OOH was much higher in this experiment than in the one described in Fig. 1. We attribute this to the fact that the membranes in Fig. 3 were much more extensively peroxidized, making 5α-OOH to 7α-OOH rearrangement more favorable.[18]) Whereas membranes incubated only with GSH or PHGPX in the presence of DFO showed no peroxide decay over a 1 h period, membranes incubated with both GSH and PHGPX showed apparent first order losses for all identifiable ChOOH (Fig. 3). Under the specified reaction conditions, the apparent first order rate constants for these losses were as follows: 0.87 h^{-1} (5α-OOH); 1.73 h^{-1} (7α,7β-OOH); 2.05 h^{-1} (6α-OOH); 2.77 h^{-1} (6β-OOH). Clearly, 5α-OOH was the most slowly reduced ChOOH, its decay rate being only ~30% that of 6β-OOH.

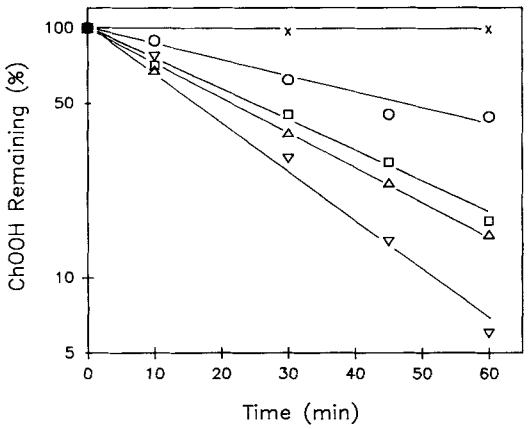

Figure 3. Enzymatic reduction of ChOOH in photooxidized erythrocyte ghosts. Ghost membranes (1.0 mg protein/mL in PBS) were sensitized with 10 μM $AlPcS_4$ and exposed to a light fluence of ~1 kJ/cm^2. The peroxidized membranes (329 μM total LOOH, 43 μM 5α-OOH, 31 μM 7α,7β-OOH, 14 μM 6α-OOH, 14 μM 6β-OOH in bulk suspension) were incubated at 37°C in the presence of 50 μM DFO, 5.0 mM GSH and 0.05 units/mL PHGPX. At the indicated time points, residual amounts of ChOOH in lipid extracts were determined by HPLC-EC(Hg). Peroxide species are represented as follows: (○) 5α-OOH; (△) 6α-OOH; (▽) 6β-OOH; (□) 7α,7β-OOH. Also shown (×) is total ChOOH in a control that contained GSH, but not PHGPX.

Similar kinetic trends were observed in repeat experiments, using either the same or a different enzyme concentration (results not shown). Preliminary experiments with photooxidized, ^{14}C-cholesterol-labeled ghosts have shown that the decay rates for individual ChOOH during GSH/PHGPX treatment closely approximate the formation rates of corresponding diol reduction products; EC(Hg)-silent diols were separated by TLC and determined by radiometric scanning (data not shown). Thus, for PHGPX-catalyzed reduction of each of the peroxides described, a tight substrate/product relationship is apparent.

Free radical-mediated lipid peroxidation in 5α-OOH-primed ghosts

Compared with the other ChOOH examined, 5α-OOH is potentially more cytotoxic because it can be photogenerated more rapidly on the one hand, and enzymatically inactivated more slowly on the other. To begin investigating this possibility, we prepared ghosts that contained 5α-OOH as the predominant LOOH species. This was accomplished by means of translocation from 5α-OOH-containing liposomes. An HPLC-EC(Hg) profile of a lipid extract from a typical preparation is shown in Fig. 4 (scan b). The major peak centered at 8.3 min (peak 2) represents 5α-OOH, while the minor one at 7.2 min (peak 1) represents 7α,7β-OOH, which amounts to ~7% of 5α-OOH in this particular preparation. (The retention times in this chromatogram were longer than those in the Fig. 1 chromatograms because the elution rate was lower.) Two other minor peaks are observed (5.0 min and 5.9 min), neither of which is assigned. It appears that the minor components, including 7α,7β-OOH, were mainly generated during the transfer incubation period, since they were barely detectable in the 5α-OOH-containing liposomes. When 5α-OOH-primed ghosts were exposed to $Fe(HQ)_2$ and ascorbate, there was a gradual decay in the 5α-OOH peak area over a 10 min reaction period and a corresponding increase in the 7α,7β-OOH peak area (Fig. 4, scans d–f). For this experiment, we estimate that [5α-OOH] in bulk suspension decreased from 4.0 μM to 0.9 μM after 10 min, whereas [7α,7β-OOH] increased from 0.3 μM to 3.1 μM. There was no change in the HPLC-EC(Hg) profile when ascorbate was included but $Fe(HQ)_2$ omitted from the system (Fig. 4, compare scans c and b), indicating that endogenous [iron] was too low to support any significant reaction. Furthermore, no 7α,7β-OOH could be detected when non-5α-OOH-primed ghosts were exposed to $Fe(HQ)_2$/ascorbate (Fig. 4, scan a), indicating that preexisting peroxide (5α-OOH most importantly in this case) was essential for the observed effects. Although barely evident in Fig. 4 (>10 min in scans d–f), phospholipid hydroperoxides (undetectable in starting membranes) accumulated steadily during iron/ascorbate treatment. These species were clearly evident at higher sensitivity settings, suggesting that free radical chain peroxidation triggered mainly by one-electron reduction of 5α-OOH consumed cholesterol as well as phospholipids in the host membrane. In keeping with the results shown in Fig. 4, TBARS were not detected when 5α-OOH-primed ghosts were incubated with ascorbate alone or $Fe(HQ)_2$ alone, but were clearly evident when both agents were present in the reaction mixture, accumulating to a level of ~3.8 nmol/mg protein after 30 min (Fig. 5). TBARS were also produced when nonprimed ghosts were incubated with $Fe(HQ)_2$/ascorbate

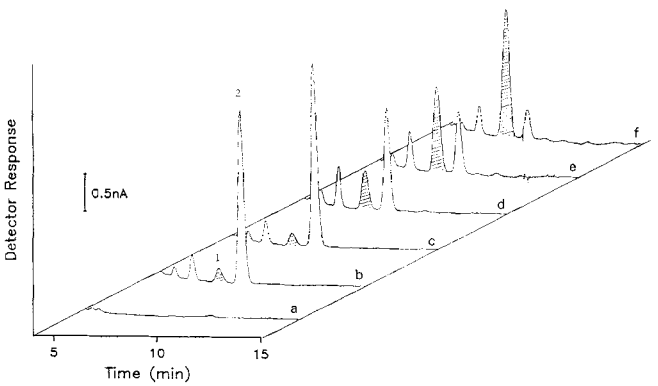

Figure 4. Chromatographic analysis of 5α-OOH-containing erythrocyte membranes before and after exposure to iron and ascorbate. The 5α-OOH-containing liposomes were incubated with freshly prepared ghosts as described in the Materials and Methods. Total (iodometrically determined) peroxide content of the mixture after a 24 h incubation at 4°C was 15.0 μM, essentially all of which was 5α-OOH. After liposome removal, the resuspended ghost membranes (1.0 mg protein/mL in PBS) contained 4.0 ± 0.5 μM 5α-OOH, indicating that 26% transfer had occurred (7α,7β-OOH at a concentration of 0.3 ± 0.1 μM was also detected). The 5α-OOH-primed ghosts were incubated at 25°C in the presence of 10 μM $Fe(HQ)_2$ and 100 μM ascorbate for 0 min, 2 min, 5 min and 10 min, after which lipids were extracted and analyzed by HPLC-EC(Hg): scans b, d, e and f, respectively. Scan a represents control ghosts (non-5α-OOH-primed) after incubation with $Fe(HQ)_2$ and ascorbate for 10 min; scan c represents 5α-OOH-primed ghosts after incubation with 100 μM ascorbate alone for 10 min. Peak 1 (shaded, 7.2 min) represents 7α,7β-OOH and peak 2 (8.3 min) 5α-OOH. Minor peaks at 5.0 min and 5.9 min are unidentified, and no signals were detected at <4 min. The HPLC elution rate was 1.7 mL//min. Each injected sample (a–f) contained 28 ng of total membrane lipid.

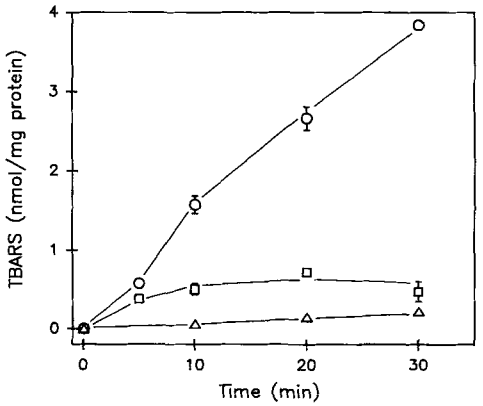

Figure 5. Iron/ascorbate-induced lipid peroxidation in 5α-OOH-containing erythrocyte ghosts. Ghost membranes were charged with 5α-OOH by incubating with liposomes consisting of 1.0 m*M* POPC, 0.4 m*M* cholesterol and 0.1 m*M* 5α-OOH in bulk phase. After 24 h at 4°C, the ghosts were washed free of liposomes and resuspended in PBS to a protein concentration of 1.0 mg/mL. Starting peroxide concentration was found to be 11.0 μ*M*. The membranes were incubated at 25°C in the presence of 10 μ*M* $Fe(HQ)_2$ alone (△) or 10 μ*M* $Fe(HQ)_2$ plus 250 μ*M* ascorbate (○), and samples were analyzed periodically for TBARS content. Non-5α-OOH-containing ghosts that had been manipulated similarly to 5α-OOH-containing counterparts were also exposed to 10 μ*M* $Fe(HQ)_2$/250 μ*M* ascorbate and analyzed for TBARS (□). Experimental values were corrected for the starting TBARS level in each reaction mixture, *i.e.* before addition of iron or iron/ascorbate; absolute starting levels (nmol/mg protein) were as follows: 0.28 (□); 0.43 (○); 0.52 (△). Data points are means ± deviation of values from duplicate determinations.

but at a much lower level than in primed ghosts (~0.5 nmol/mg protein at 30 min). This presumably reflected a low level of preexisting LOOH other than 5α-OOH in the membranes. It is apparent from the results of Figs. 4 and 5 that membrane-bound 5α-OOH, like LOOH in general,[3] can undergo iron-mediated, one-electron reduction to free radical species, which initiate damaging chain peroxidation reactions. Formation of TBARS indicates that polyunsaturated phospholipids (comprising ~45 mol% of the membrane lipid) are important participants in the overall peroxidative process.

Cytotoxicity of 5α-OOH-containing liposomes

To gain further insights into the biological effects of 5α-OOH, we examined its cytotoxicity toward selenium-deficient L1210 cells. Based on previous findings that selenium-deficient cells are more sensitive to LOOH than selenium-satisfied cells,[22] we used the former type in order to minimize consumption of 5α-OOH. As shown in Fig. 6, cells incubated for 20 h in the presence of $Fe(HQ)_2$ at a nontoxic concentration (0.5 μ*M*) and liposomes that lacked 5α-OOH remained essentially as viable as controls not treated with iron or liposomes. However, cells exposed for 20 h to 5α-OOH-containing liposomes (4.0 μ*M* peroxide) showed approximately 75% loss of viability, which increased to nearly 95% when 0.5 μ*M* $Fe(HQ)_2$ was included. Importantly, peroxide-containing liposomes that had been preincubated with GSH and PHGPX (bringing 5α-OOH to <1% of its starting level) exhibited relatively little cytotoxicity, reducing viability by only 10–12% relative to the nontreated control. These results clearly demonstrate that 5α-OOH in liposomal form is highly cytotoxic and that cytotoxicity, which is exacerbated by iron, is strictly peroxide dependent. 3β-Hydroxycholest-5-ene-7-one, a by-product of 5α-OOH rearrangement that might have arisen in our system,[2] was ruled out as a possible contributor to cytotoxicity (data not shown).

DISCUSSION

Singlet oxygen attack on cholesterol generates three hydroperoxide species: 5α-OOH, 6α-OOH and 6β-OOH. Identification of these species in a photodynamic system, *e.g.* dye/light-treated cells, provides unambiguous evidence that $^1O_2$ is a reactive intermediate.[5,6] The initial rate of 5α-OOH photogeneration always exceeds that of 6α-OOH or 6β-OOH but (for unknown reasons) much more so in homogeneous solution than in membrane environments.[18] In a previous study involving $AlPcS_4$/light-treated liposomes,[18] we showed that 5α-OOH accumulated two to three times more rapidly than 6β-OOH until a threshold level was reached (5–10% of starting cholesterol), after which rapid rearrangement of 5α-OOH resulted in a declining 5α-OOH/6β-OOH ratio. The reason for enhanced 5α-OOH rearrangement in heavily photooxidized membranes is unknown but might relate to altered local polarity or destabilized interactions of 5α-OOH with phospholipids.[34] Using $AlPcS_4$-sensitized erythrocyte membranes in the present study, we have confirmed that 5α-OOH accumulates about three times more rapidly than 6β-OOH (and much more so than 7α,7β-OOH) during the early stages of photooxidation. The highest level of 5α-OOH reached was far below the threshold level referred to above.

We have determined that not only is the initial rate of 5α-OOH photogeneration greater than that of 6α,6β-OOH or 7α,7β-OOH, but that its reductive inactivation by the GSH/

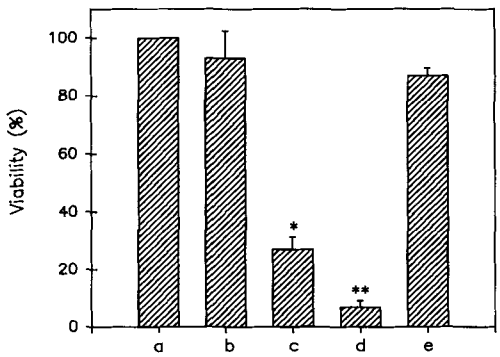

Figure 6. Cytotoxicity of liposomal 5α-OOH. Selenium-deficient L1210 cells (1.0 × 10^6/mL in 1% serum/RPMI medium) were incubated in the presence of the following additives: (a) none; (b) 0.5 μ*M* $Fe(HQ)_2$ plus liposomes lacking 5α-OOH; (c) 4.0 μ*M* liposomal 5α-OOH; (d) 0.5 μ*M* $Fe(HQ)_2$ plus 4.0 μ*M* liposomal 5α-OOH; (e) 0.5 μ*M* $Fe(HQ)_2$ plus 5α-OOH liposomes that had been preincubated with 5.0 m*M* GSH and 0.1 unit/mL PHGPX in the presence of 50 μ*M* DFO for 2 h at 37°C, followed by 5 h of dialysis against PBS. (The HPLC-EC[Hg] analysis indicated that GSH/PHGPX treatment had decreased liposomal 5α-OOH content by >99%.) Reaction mixtures b–e contained the same concentration of total liposomal lipid, *i.e.* 84 μ*M*. After 20 h of incubation at 37°C, cell viability was assessed by MTT assay. Values for systems b–e are expressed relative to system a; each value is the mean ± deviation of measurements from duplicate experiments. *$P < 0.001$ compared with system a or b. **$P < 0.001$ compared with system c.

PHGPX system is substantially slower. This is the first reported evidence that ChOOH are reduced at different rates by PHGPX. We found that solubilizing photooxidized ghosts with Triton X-100 prior to GSH/PHGPX treatment caused all ChOOH to be reduced more rapidly (data not shown); however, the relative decay rates remained approximately the same, suggesting that 5α-OOH is intrinsically less reactive than the other ChOOH. The low reactivity might relate to the fact that 5α-OOH is a tertiary (inter-ring) hydroperoxide and, as such, might not be recognized by PHGPX as well as 6α,6β-OOH or 7α,7β-OOH, which are secondary hydroperoxides. Based on these observations, we have postulated that among the ChOOH generated by a photooxidative insult, 5α-OOH has the potential of being the most cytotoxic.

Initial testing of this idea involved preparation of a natural membrane, the erythrocyte ghost, in which 5α-OOH was the predominant LOOH species. This was accomplished by intermembrane transfer, using 5α-OOH-containing liposomes as donors. While often used for transfer of cholesterol itself,[35] this approach has not been described previously for a cholesterol hydroperoxide (nor for any lipid hydroperoxide, for that matter). As expected, 5α-OOH was by far the most abundant peroxide in acceptor membranes. Although there were no detectable phospholipid hydroperoxides, a small amount of 7α,7β-OOH (4–5% of 5α-OOH) was typical, most likely reflecting some rearrangement of 5α-OOH during lengthy transfer incubation. Two other minor peroxides arose during incubation, the identities and sources of which are currently under investigation. The 5α-OOH-primed ghosts were found to be highly susceptible to free radical peroxidative damage when challenged with $Fe(HQ)_2$ and ascorbate, mimicking the behavior of membranes that were photoperoxidized in the presence of these agents. The reaction was characterized by a rapid loss of 5α-OOH, with appearance of new 7α,7β-OOH, phospholipid hydroperoxides and TBARS. Although not shown in Fig. 4, the increase in 7α,7β-OOH was transient, *i.e.* its signal maximized after 15–20 min exposure to $Fe(HQ)_2$/ascorbate and then declined, presumably because of a slowdown in propagation reactions in the midst of continued reduction. A plausible mechanism for the overall process is summarized as follows. Upon accessing ligated Fe^{3+} on the ghost membrane, ascorbate (AH^-) reduces it to Fe^{2+} (Eq. 1) while being oxidized to ascorbate radical ($A^{-\cdot}$), which rapidly disproportionates.[3]

$$AH^- + Fe^{3+} \rightarrow A^{-\cdot} + H^+ + Fe^{2+} \quad (1)$$

$$5\alpha\text{-OOH} + Fe^{2+} \rightarrow 5\alpha\text{-O}^\cdot + OH^- + Fe^{3+} \quad (2)$$

$$5\alpha\text{-O}^\cdot + LH \rightarrow 5\alpha\text{-OH} + L^\cdot \quad (3)$$

$$L^\cdot + O_2 \rightarrow LOO^\cdot \quad (4)$$

$$LOO^\cdot + LH \rightarrow LOOH + L^\cdot \quad (5)$$

Interaction of 5α-OOH and Fe^{2+} would result in reduction of the peroxide to an alkoxyl radical (5α-O·) (Eq. 2), which (either directly or after transformation to an epoxyallylic peroxyl radical[36]) initiates chain peroxidation by abstracting allylic hydrogens from unsaturated membrane lipids (LH) (Eq. 3). Subsequent propagation reactions involving peroxyl radicals (LOO·) (Eqs. 4 and 5) would give rise to TBARS and species such as 7α- and 7β-OOH. The 7α,7β-OOH and other LOOH derived from ghost membrane lipids would then react similarly to priming 5α-OOH, resulting in an amplified response. A similar scheme is proposed for photooxidized membranes treated with iron/ascorbate[14] or for membranes photooxidized in the presence of iron/ascorbate (Fig. 2B), except in these cases, many different LOOH (phospholipid- as well as cholesterol-derived) could have triggered chain peroxidation.

The observed lethal effects of 5α-OOH on L1210 cells (Fig. 6) can be accounted for by a mechanism similar to that described in Eqs. 1–5, although for cells, the identity of the putative one-electron donor (*cf.* Eq. 1) is unknown. We reported previously[22] that ChOOH-containing liposomes can cause lethal injury to L1210 cells. However, in that case, the peroxides were inserted into the liposomes by photooxidation and consisted mainly of 7α- and 7β-OOH generated by 5α-OOH rearrangement. The marked sensitivity to 5α-OOH observed in the present study (5 μ*M* peroxide killing ~90% of the cell population) may reflect the fact that our cells were deficient in PHGPX activity. Although we have not determined whether 5α-OOH is more toxic to PHGPX-deficient than PHGPX-sufficient cells, earlier studies carried out with a more heterogeneous population of liposomal ChOOH showed that this is the case.[22] Because 5α-OOH has been shown to be the poorest PHGPX substrate among the ChOOH tested, it seems reasonable to predict that 5α-OOH should be the most cytotoxic of these species. We are in the process of testing this possibility by assessing the relative cytotoxicities of 5α-OOH, 6α-OOH, 6β-OOH and 7α,7β-OOH toward various cell lines.

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