Enzymatic Reducibility in Relation to Cytotoxicity for Various Cholesterol Hydroperoxides†

Witold Korytowski,‡ Peter G. Geiger,§ and Albert W. Girotti*,§

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, and Institute of Molecular Biology, Jagiellonian University, Krakow, Poland

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ABSTRACT: Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is a selenoenzyme that can catalyze the direct reduction of various membrane lipid hydroperoxides and by so doing could play a vital role in cytoprotection against peroxidative damage. The activity of purified testicular PHGPX on several photochemically-generated cholesterol hydroperoxide (ChOOH) species was investigated, using high-performance liquid chromatography with electrochemical detection for peroxide analysis and thin-layer chromatography with ^{14}C-radiodetection for diol product analysis. The following ChOOH isomers were monitored: 5α-OOH, 6α-OOH, 6β-OOH (singlet oxygen adducts), and unresolved 7α,7β-OOH (derived from 5α-OOH rearrangement). Apparent first-order rate constants for GSH/PHGPX-induced peroxide loss (or diol accumulation) in Triton X-100 micelles, unilamellar liposomes, or erythrocyte ghost membranes increased in the following order: 5α-OOH < 6α-OOH ≈ 7α,7β-OOH < 6β-OOH. A similar trend was observed when the peroxides were incubated with Triton lysates of Se-replete L1210 or K562 cells, implicating PHGPX in these reactions. Consistent with this, there was little or no ChOOH reduction if GSH was omitted or if lysates from Se-deprived cells were used. Liposomal 5α-OOH was found to be much more cytotoxic than equimolar liposomal 6β-OOH, producing a 50% loss of L1210 clonogenicity at ∼$^1/_5$ the concentration of the latter. Faster uptake of 5α-OOH was ruled out as the basis for greater cytotoxicity, suggesting that relatively inefficient metabolism by the GSH/PHGPX system might be the reason. As supporting evidence, it was found that cells accumulate the diol reduction product of 5α-OOH more slowly than that of 6β-OOH during incubation with the respective peroxides. Slow detoxification coupled with rapid formation makes 5α-OOH potentially the most damaging ChOOH to arise in cells exposed to singlet oxygen.

Unsaturated phospholipids, glycolipids, and cholesterol are prominent targets of oxidative damage in biological structures such as cell membranes and lipoproteins. This damage, known as lipid peroxidation, can be triggered by a wide variety of physical and chemical agents, including ionizing radiation, ultraviolet radiation, and activated oxygen species such as ozone, hydroxyl radical, and singlet oxygen (Kappus, 1985; Halliwell & Gutteridge, 1989; Girotti, 1985). Lipid peroxidation can be mutagenic as well as cytotoxic and has been linked to pathological conditions such as atherosclerosis, ischemia-reperfusion injury, inflammatory arthritis, and carcinogenesis (Halliwell & Gutteridge, 1989). Peroxidation may also play a role in the cytotoxic effects of various chemotherapeutic and phototherapeutic agents (Halliwell & Gutteridge, 1989; Girotti, 1985, 1990). During the peroxidative process, lipid hydroperoxides (LOOHs)[1] can arise as characteristic end-products or intermediates, depending on the availability of electron donors and redox metal ions (Kappus, 1985; Halliwell & Gutteridge, 1989; Girotti, 1985). For example, in the presence of ascorbate and active chelated iron, LOOHs undergo one-electron reduction to oxyl radicals, which, either directly or after conversion to epoxyallylic peroxyl radicals (Gardner, 1989), can trigger chain reactions that exacerbate peroxidative damage (Girotti, 1990; Bachowski et al., 1991). Cells cope with these effects by reducing LOOHs to less toxic alcohol derivatives, which are eventually replaced via repair processes (Ursini et al., 1991). One or more GSH-dependent enzyme(s) in the selenoperoxidase (SePX) family play(s) a key role in the reductive detoxification of LOOHs. Two well-characterized SePXs are expressed by most eucaryotic cells (Flohe, 1989),

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* To whom correspondence should be addressed at the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226. Tel: (414) 456-8432. FAX: (414) 266-8497.

‡ Jagiellonian University.

§ Medical College of Wisconsin.

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[1] Abbreviations: $AlPcS_4$, chloroaluminum phthalocyanine tetrasulfonate; Ch, cholesterol; ChOOH, cholesterol hydroperoxide; ChOH, hydroxycholesterol; DCP, dicetylphosphate; DFO, desferrioxamine; GPX, glutathione peroxidase; HPLC-EC(Hg), high-performance liquid chromatography with mercury cathode electrochemical detection; K·Se(+), selenium-replete K562 cells; K·Se(−), selenium-deficient K562 cells; LC_{50}, concentration that reduces cell survival by 50%; LOOH, lipid hydroperoxide; L·Se(+), selenium-replete L1210 cells; L·Se(−), selenium-deficient L1210 cells; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 5α-OH, 5α-cholest-6-ene-3β,5-diol; 6α-OOH, 3-hydroxycholest-4-ene-6α-hydroperoxide; 6β-OOH, 3β-hydroxycholest-4-ene-6β-hydroxyperoxide; 6α-OH, cholest-4-ene-3β,6α-diol; 6β-OH, cholest-4-ene-3β,6β-diol; 7α,7β-OOH, unresolved mixture of 3β-hydroxycholest-5-ene-7α-hydroperoxide and 3β-hydroxycholest-5-ene-7β-hydroperoxide; 7α-OH, cholest-5-ene-3β,7α-diol; 7β-OH, cholest-5-ene-3β,7β-diol; PBS, Chelex-treated phosphate-buffered saline (125 mM NaCl, 25 mM sodium phosphate, pH 7.4); PC, phosphatidylcholine; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLOOH, phospholipid hydroperoxide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SePX, selenoperoxidase; TLC, thin-layer chromatography.

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glutathione peroxidase (GPX; EC 1.11.1.9) and the so-called phospholipid hydroperoxide glutathione peroxidase (PHGPX; EC 1.11.1.12).[2] GPX, a homotetramer of molecular mass ~82 kDa, exists in the cytosol and mitochondrial matrix and acts on relatively polar peroxides such as H_2O_2 and fatty acid hydroperoxides, but not on LOOHs. PHGPX, a monomer of molecular mass ~20 kDa, is found in cytosolic and membrane-associated forms (Ursini et al., 1985; Roveri et al., 1992) and can act on a wide variety of peroxides, including LOOHs (Ursini et al., 1991, 1985). In previous work, we demonstrated SePX involvement in cytoprotection against peroxidative stress by showing that selenium deficiency makes leukemia cells more sensitive to the lethal effects of photodynamic action (Thomas & Girotti, 1989; Lin et al., 1992) or exogenous peroxides such as *tert*-butyl hydroperoxide, phospholipid hydroperoxides (PLOOHs), and cholesterol hydroperoxides (ChOOHs) (Geiger et al., 1991, 1993). Other studies showed that purified PHGPX, unlike GPX, can catalyze the direct reduction of PLOOHs and ChOOHs in isolated membranes and lipoproteins (Thomas et al., 1990a,b). The relative reactivity of different molecular species within a given peroxide family, e.g., ChOOH, was not assessed in that work because of the limited resolving power and sensitivity of the TLC analytical system used. Taking advantage of a new analytical approach recently developed in this laboratory, high-performance liquid chromatography with mercury cathode electrochemical detection, HPLC-EC(Hg) (Korytowski et al., 1993, 1995), we have been able to monitor and compare the PHGPX reactivity of several photodynamically-generated ChOOHs, including 5α-OOH, 6α-OOH, 6*β*-OOH, and unresolved 7α,7*β*-OOH. The first three derive exclusively from singlet oxygen attack on cholesterol, and the latter derives from free radical reactions or 5α-OOH rearrangement (Smith, 1981). Results of novel experiments comparing the enzymatic reducibility of these peroxides on the one hand and their cytotoxicity on the other are described. Our findings that 5α-OOH is the least PHGPX-reactive and most cytotoxic of these species have important implications on the cytopathological effects of singlet oxygen.

EXPERIMENTAL PROCEDURES

General Materials. The following chemicals, enzymes, and tissue culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO): cholesterol; 7-ketocholesterol; egg phosphatidylcholine; 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; dicetylphosphate; Chelex-100 (50−100 mesh); GSH, NADPH, and bovine glutathione peroxidase; yeast glutathione reductase; RPMI-1640 medium; insulin, transferrin, penicillin, and streptomycin. Hyclone Laboratories (Logan, UT) provided the fetal calf serum; total Se content varied between 25 and 30 ng/mL in the different batches of 100% serum used. Desferrioxamine was obtained from Ciba-Geigy Corp. (Suffern, NY), chloroaluminum phthalocyanine tetrasulfonate was from Porphyrin Products (Logan UT), and [4-^{14}C]cholesterol (53 mCi/mmol in toluene) was from Amersham Life Sciences Inc. (Arlington Heights, IL). Burdick and Jackson Corp. (Muskegon, MI) supplied the HPLC-grade solvents. All aqueous solutions except those used in cell culture were prepared with deionized, glass-distilled water and then treated with Chelex-100 to remove trace metal ions that might otherwise catalyze peroxide decomposition.

Cell Culture. Murine L1210 cells and human K562 cells were grown at 37 °C under a humidified atmosphere of 95% air/5% CO_2. The growth medium consisted of 10% fetal calf serum in RPMI medium supplemented with insulin (10 μg/mL), transferrin (5 μg/mL), streptomycin (100 μg/mL), penicillin (100 U/mL), and sodium selenite (10 ng/mL or ~60 nM). Cells were reseeded into fresh medium every 2 days. As needed, serum (the major source of Se) was gradually decreased from 10% to 1%, everything else remaining constant (Lin et al., 1992). One population of L1210 or K562 cells, designated L·Se(+) or K·Se(+), was maintained under these conditions. Another population, designated L·Se(−) or K·Se(−), received everything except sodium selenite. Cells were used from five days up to about one month after withholding selenite. All experiments and determinations were performed on logarithmically growing cells.

Cellular Selenoperoxidase Activity. Se-supplemented cells expressed maximal GPX and PHGPX activity, as measured by coupled enzymatic assay (Thomas et al., 1990), whereas >5-day Se-deprived cells were deficient in these activities (Lin et al., 1992; Geiger et al., 1991, 1993). Typical GPX values were as follows (units/mg of cell protein): 235 ± 25 [L·Se(+)]; 14.0 ± 3.0 [L·Se(−)]; 9.3 ± 1.7 [K·Se(+)]; 9.5 ± 1.2 [K·Se(−)] (means ± SD; $n = 4$). PHGPX values measured on the same cell samples were as follows (units/mg of cell protein): 4.4 ± 1.0 [L·Se(+)]; 1.7 ± 0.7 [L·Se(−)]; 7.2 ± 3.1 [K·Se(+)]; 1.5 ± 0.6 [K·Se(−)] (means ± SD; $n = 4$). In agreement with previous findings (Lin et al., 1992; Maiorino et al., 1991), these data indicate that L1210 cells express both GPX and PHGPX, whereas K562 cells express only PHGPX.

Cholesterol Hydroperoxides. Authentic 5α-OOH and 6*β*-OOH were prepared by $AlPcS_4$-sensitized photooxidation of Ch in pyridine, and, after HPLC-EC(Hg) separation/detection using a semipreparative column, characterized by NMR (Korytowski et al., 1991). For preparing radiolabeled 5α-OOH and 6*β*-OOH, [^{14}C]Ch (1 mM; ~10 μCi/μmol) in pyridine was photooxidized and chromatographed similarly. 7α-OOH and any unresolved 7*β*-OOH (from spontaneous epimerization of 7α-OOH) were prepared by photooxidizing Ch in liposomal form, where rearrangement of 5α-OOH to 7α-OOH is more favorable (Beckwith et al., 1989). HPLC separation and NMR identification were carried out as described (Korytowski et al., 1991). ChOOHs were quantified by iodometric assay (Bachowski et al., 1991).

Membrane Preparation. Unilamellar liposomes (100 nm diameter) containing (i) POPC/[^{14}C]Ch/DCP (for incubation with GSH/PHGPX after photoperoxidation); (ii) POPC/Ch/5α-OOH or POPC/Ch/6*β*-OOH (for cytotoxicity experiments); and (iii) POPC/Ch/[^{14}C]5α-OOH or POPC/Ch/[^{14}C]6*β*-OOH (for peroxide uptake experiments) were prepared by lipid extrusion in PBS (Mayer et al., 1986).

Erythrocyte ghosts were prepared by hypotonic lysis of human erythrocytes and resuspended in PBS after extensive washing (Bachowski et al., 1991). Ghost membranes were

[2] This enzyme can act on a wide variety of lipid hydroperoxides, including species derived from phospholipids, cholesterol, and cholesteryl esters. Therefore, the trivial name "phospholipid hydroperoxide glutathione peroxidase" (PHGPX) is too restrictive; a better designation would be simply "lipid hydroperoxide glutathione peroxidase" (LHGPX). In order to avoid confusion in this publication, we retain the abbreviation "PHGPX", despite its shortcomings.

radiolabeled with [^{14}C]Ch by incubating with egg PC/[^{14}C]-Ch/DCP (5:4:1 mol/mol/mol) liposomes (Bachowski et al., 1994). After being washed, typical preparations contained 0.5−1.0 μCi of [^{14}C]Ch/mL (∼2 mg protein/mL).

Preparation of PHGPX. PHGPX was prepared from rat testes as described (Roveri et al., 1992) and stored at −80 °C. The purified enzyme migrated as a single band of molecular mass ∼20 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific activity was determined by coupled enzymatic assay, using NADPH, GSH, GSSG-reductase, and photoperoxidized egg PC (Thomas et al., 1990a). The assay was carried out immediately before using PHGPX in an experiment.

Membrane Photooxidation. [^{14}C]Ch-labeled liposomes and ghost membranes were peroxidized by irradiation with broad-band visible light (fluence rate ∼150 mW/cm^2) in the presence of 10 μM $AlPcS_4$ (Bachowski et al.,1994). Total Ch- and PL-derived LOOH was determined by iodometric analysis of chloroform/methanol (2:1) extracts (Bachowski et al., 1991; Girotti et al., 1985).

Enzymatic Reduction of ChOOHs in Cell-Free Systems. Three different systems were monitored: (i) artificial mixture of 5α-OOH, 6β-OOH, and 7α,7β-OOH standards; (ii) photoperoxidized POPC/[^{14}C]Ch/DCP liposomes; (iii) photoperoxidized [^{14}C]Ch-labeled erythrocyte ghosts. System i was incubated in the presence of 5 mM GSH and either purified PHGPX (0.01−0.05 units/mL) or an extract of Triton-lysed L1210 or K562 cells (∼2 × 10^8/mL) in PBS containing 0.1% Triton X-100 and 50 μM DFO. Intact membranes in systems ii and iii were incubated with 5 mM GSH and 0.05−0.1 units of PHGPX/mL. During incubation at 37 °C, samples were removed, extracted with chloroform/methanol (2:1), and recovered lipid fractions analyzed for ChOOH loss by HPLC-EC(Hg) and for ChOH (diol) formation by TLC with radioimaging. Details about the extraction procedure are provided elsewhere (Bachowski et al., 1991; Girotti et al., 1985).

ChOOH Uptake and Metabolism by Cells. Uptake was typically assessed by mixing L·Se(+) cells in 1% serum/RPMI with 0.4 vol of liposomal [^{14}C]5α-OOH or [^{14}C]6β-OOH to give ∼10^7 cells/mL and 50 μM initial ChOOH (0.5−1.0 μCi/mL). Samples collected during incubation at 37 °C were centrifuged; after they were washed, cell pellets were extracted and recovered lipid fractions were subjected to scintillation counting. For assessing conversion of ChOOH to ChOH, lipid extracts from cellular and liposomal fractions were analyzed by silica-gel TLC, using radioimaging for detection and quantitation. Analyte identification was based on ChOOH and ChOH standards run alongside.

Peroxide Cytotoxicity. This was typically assessed by incubating L·Se(+) or K·Se(+) cells (∼10^6/mL in 1% serum/RPMI) with increasing amounts of 5α-OOH/Ch/POPC or 6β-OOH/Ch/POPC liposomes (0.3/0.3/1.0, mol/mol/mol; 1.6 mM total lipid) in 12-well plates. Peroxide concentrations up to 150 μM in bulk suspension were used. In any given experiment, all mixtures were standardized to the same concentration of total liposomal lipid such that any background toxicity due to lipid alone was the same throughout. After addition of peroxides, plates were returned to the incubator; 20 h later, cell survival was determined by clonal assay.

High-Performance Liquid Chromatography. Reverse-phase HPLC-EC(Hg) analysis of ChOOHs was accomplished as described (Korytowski et al., 1993, 1995) 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 consisted of (by volume) 81% methanol, 11.5% acetonitrile, and 7.5% aqueous solution containing 10 mM ammonium acetate/1 mM sodium perchlorate. It was sparged continuously with high-purity argon that had been passed first through an OMI-1 O_2 scrubber (Supelco, Bellefonte, PA) to reduce O_2 contamination to <10 ppb and then through a presaturating mobile phase scrubber. The mobile phase was delivered isocratically at a flow rate of 1.0−2.0 mL/min. Dried extracts were dissolved in 2-propanol, sparged with helium when necessary (Korytowski et al., 1993), and injected in 20−μL aliquots. The mercury cathode was typically set at an operating potential of −300 mV vs a Ag/AgCl reference. 5α-OOH and 7α,7β-OOH were found to have the same EC(Hg) response, which was ∼30% greater than that of 6α- or 6β-OOH. Additional details are available elsewhere (Korytowski et al., 1993, 1995; Bachowski et al.,1994).

Thin-Layer Chromatography. TLC separation of ChOOHs and ChOHs was carried out according to published methods (Smith et al., 1967), with slight modifications. Dried lipid extracts from samples recovered during incubation of [C^{14}]-ChOOH-containing membranes with GSH/PHGPX or with cells were dissolved in chloroform/methanol (2:1) and applied to Silica Gel-60 plates (EM Science, Cherry Hill, NJ). The mobile phase was benzene/ethyl acetate (1:1). Radiolabeled ChOOH and ChOH standards were chromatographed alongside as markers. The relative amounts of resolved ChOOHs and ChOHs were determined by radioimaging using an Ambis 4000 system (Ambis, San Diego, CA). On some occasions when sufficiently large amounts of material were analyzed, ChOOHs were visualized by spraying with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, and ChOHs were visualized by spraying with 50% sulfuric acid, followed by warming (Smith et al., 1967).

RESULTS

PHGPX-Catalyzed Reduction of ChOOHs. Three different systems were analyzed for ChOOHs by HPLC-EC(Hg): detergent micelles containing peroxide standards (Figure 1A); photooxidized POPC/[^{14}C]Ch/DCP liposomes (Figure 1B); and photooxidized [^{14}C]Ch-labeled erythrocyte ghosts (Figure 1C). Well-resolved peaks representing 5α-OOH, 6α-OOH, 6β-OOH, and 7α,7β-OOH were observed for each system. After a 30-min incubation in the presence of GSH/PHGPX, there was a substantial decrease in intensity for most of the ChOOH peaks (compare scans c and a). An unassigned broad peak at 3−6 min in Figure 1C was also greatly diminished after GSH/PHGPX treatment, suggesting that it represented some type(s) of hydroperoxide. Incubation with GSH only (scans b) or PHGPX only (not shown) had no significant effect on peak intensity. Similarly, when GPX (0.5 units/mL) was substituted for PHGPX in the presence of GSH, there were no detectable losses (data not shown). DFO was present in all reaction mixtures represented in Figure 1; without this chelator, GSH alone caused significant peroxide loss over a 30-min period, probably via iron-catalyzed reduction (Halliwell & Gutteridge, 1989). Among the ChOOHs examined in the different systems, 5α-OOH

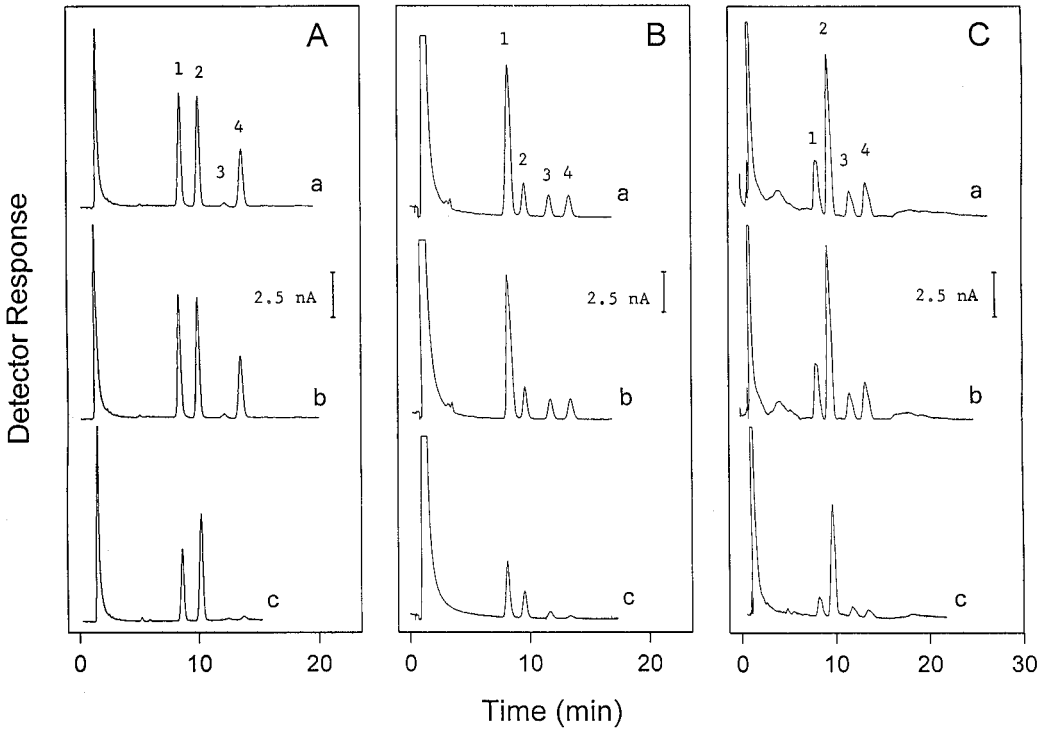

FIGURE 1: HPLC-EC(Hg) profiles of cholesterol hydroperoxides in various organized systems before and after exposure to GSH/PHGPX. ChOOH assignments were as follows: (1) 7α,7β-OOH (8.5 min); (2) 5α-OOH (9.9 min); (3) 6α-OOH (12.2 min); (4) 6β-OOH (13.9 min). (A) Detergent micelles. A synthetic mixture of 5α-OOH, 6β-OOH, and 7α,7β-OOH, each at 40 μM in PBS containing 0.1% Triton X-100, 50 μM DFO, and 1% ethanol (the ChOOH vehicle), was extracted and analyzed before (a) and after a 30-min incubation at 37 °C in the presence of 5 mM GSH alone (b) or 5 mM GSH plus 0.02 units of PHGPX/mL. (c). Total ChOOH per injection (a, b) was 0.96 nmol. The trace amount of 6α-OOH (peak 3) was a contaminant of 6β-OOH. (B) Lipid vesicles. Suspensions of unilamellar POPC/[^{14}C]Ch/DCP (1:1:0.4, mol/mol/mol) liposomes (2 mM [^{14}C]Ch; ~1 μCi/mL) were photoperoxidized in the presence of $AlPcS_4$. The suspensions containing 350 μM total LOOH in PBS/50 μM DFO were analyzed before (a) and after a 30 min incubation at 37 °C in the presence of 5 mM GSH (b) or 5 mM GSH plus 0.05 units of PHGPX/mL (c). Total peroxide per injection (a, b) was 3.5 nmol; this included POPC-OOH (not represented). (C) Erythrocyte ghosts. Ghost membranes were transfer-radiolabeled by incubation with [^{14}C]Ch-containing liposomes. After they were washed free of liposomes, the ghosts (1.0 mg of protein/mL; 0.3−0.4 μCi of [^{14}C]Ch/mL) in PBS/50 μM DFO were photoperoxidized to a total LOOH level of 200 ± 24 μM and analyzed before (a) and after a 15-min incubation at 37 °C in the presence of 5 mM GSH (b) or 5 mM GSH plus 0.05 units of PHGPX/mL (c). Total peroxide per injection (a, b) was 7.6 nmol. Identity of material eluting at 3−6 min in a and b is unknown. Full-scale detector sensitivity was 10 nA for all scans in A−C.

appeared to be least reactive with GSH/PHGPX over a single time interval. To corroborate this, we compared time courses for the enzymatic reduction of the different peroxides. Figure 2 shows that all ChOOHs decayed with apparent first-order kinetics in the different systems studied. However, within any given system, there were large differences in the decay rates, which typically increased in the following order: 5α-OOH < 6α-OOH ≈ 7α,7β-OOH < 6β-OOH (see Table 1 for kinetic parameters). In studying peroxidized liposomes (Figure 2B), we found that whereas rate constants increased proportionately with [PHGPX], they were unaffected by changing the membrane concentration, e.g., halving or doubling it, at constant [GSH] and [PHGPX] (data not shown). We have not determined whether rate constants might be altered by varying the peroxide density at a fixed membrane concentration. The observed rate differences were greatest for the micellar system (Figure 2A) and smallest for the erythrocyte ghost system (Figure 2C). Increasing reactivity of 5α-OOH on going from micelles to liposomes to ghosts (currently unexplained) had the greatest effect on these trends; the 6β-OOH/5α-OOH rate ratios were 27.5, 7.5, and 3.5 in micelles, liposomes, and ghosts, respectively. We asked whether ChOOH losses during GSH/PHGPX treatment could be accounted for entirely by formation of hydroxycholesterol (ChOH) products. EC-silent ChOHs can be readily separated from one another and from ChOOHs by normal phase TLC (Bachowski et al., 1991; Smith et al., 1967). Therefore, we monitored formation of [C^{14}]ChOHs by TLC, using high-sensitivity radioimaging for detection. A chromatogram representing the liposome experiment (Figure 2B) is shown in Figure 3. Note that as intensity of the partially resolved ChOOH zone decreased during GSH/PHGPX incubation, intensity of well-resolved ChOHs (5α-OH, 6β-OH, 7α-OH, 7β-OH) increased. Each process (representing the entire population of ChOOHs or ChOHs) exhibited apparent first-order kinetics, the rate constants being 2.88×10^{-2} min^{-1} for ChOOH decay and 2.72×10^{-2} min^{-1} for ChOH accumulation. Thus, a tight substrate−product relationship existed for the overall reaction catalyzed by PHGPX. Similar observations were made on the erythrocyte ghost system (cf. Figure 2C). Good kinetic agreement was also observed when HPLC-EC(Hg)-detectable ChOOHs and TLC-radiodetectable ChOHs in individual families were compared in this manner for both membrane systems. As shown in Figure 4, 5α-OH, 6β-OH, and 7α,7β-OH accumulated in apparent first-order fashion during incubation, with rate constants that agreed quite well with those for decay of the respective peroxides (Table 1).

ChOOH Reduction by GSH-Supplemented Cell Lysates: Effects of Selenium Deficiency. As observed with GSH/PHGPX treatment, ChOOHs decayed in apparent first-order fashion when incubated in the presence of GSH and Triton

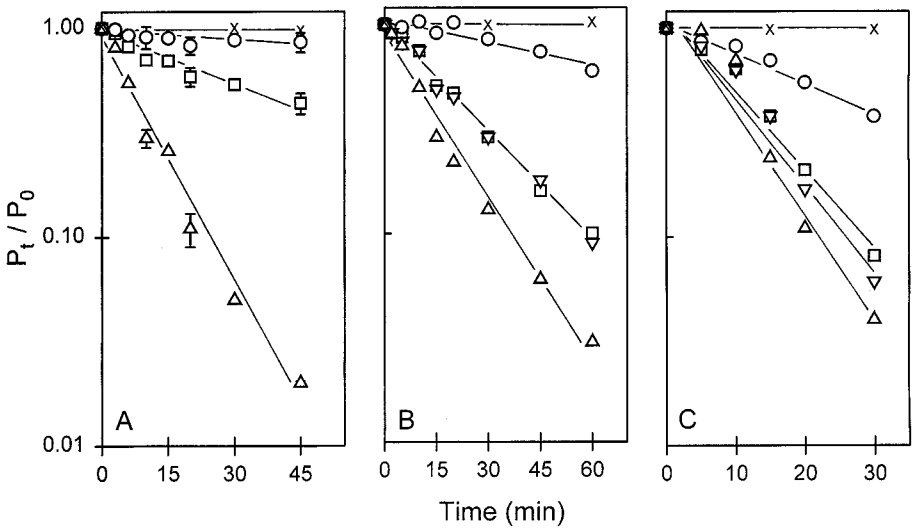

FIGURE 2: Time courses for PHGPX-catalyzed reduction of cholesterol hydroperoxides in different organized systems: (A) mixture of 5α-OOH, 6β-OOH, and 7α,7β-OOH in Triton X-100 suspension; (B) photoperoxidized POPC/[^{14}C]Ch/DCP liposomes; (C) photoperoxidized [^{14}C]Ch-labeled erythrocyte ghosts. Details of reaction conditions are provided under Figure 1. During incubation of each system in the presence of GSH/PHGPX, samples were removed periodically and extracted; recovered lipids were subjected to HPLC-EC(Hg) analysis to determine residual ChOOHs. In systems B and C, sample extracts were also subjected to TLC with radioimaging detection in order to assess formation of ChOH products (see Figures 3 and 4). The experiment with system A was carried out in duplicate; error bars show range of measured values for any given peroxide. P_0 and P_t denote peroxide concentrations at zero time and time t, respectively. Designations are as follows: (○) 5α-OOH; (▽) 6α-OOH; (△) 6β-OOH; (□) 7α,7β-OOH; (×) total ChOOH in a control that contained GSH but not PHGPX.

Table 1: Rate Constants for ChOOH Loss and ChOH Formation in GSH/PHGPX-Treated Peroxidized Membranes[a]

	$k \times 10^2$ (min^{-1})[b]			
	5α	6α	6β	7α,7β
lipid vesicles				
ChOOH	0.80 ± 0.13	3.98 ± 0.14	5.99 ± 0.42	3.96 ± 0.12
ChOH	0.90 ± 0.05	nd[c]	4.81 ± 0.23	3.50 ± 0.13[d]
erythrocyte ghosts				
ChOOH	3.21 ± 0.01	9.75 ± 0.95	11.24 ± 1.38	8.69 ± 0.74
ChOH	2.38 ± 0.13	nd[c]	10.40 ± 0.78	6.70 ± 0.24[d]

[a] See Figure 1 for details of reaction conditions. [b] ChOOH values were obtained from slopes of regression lines in Figure 2B (vesicles) or Figure 2C (ghosts). ChOH values were obtained from slopes of regression lines in Figure 4A (vesicles) or Figure 4B (ghosts). Error limits are represented as standard deviations of regression coefficients. [c] Not detected [d] Composite of separately determined 7α-OH and 7β-OH values.

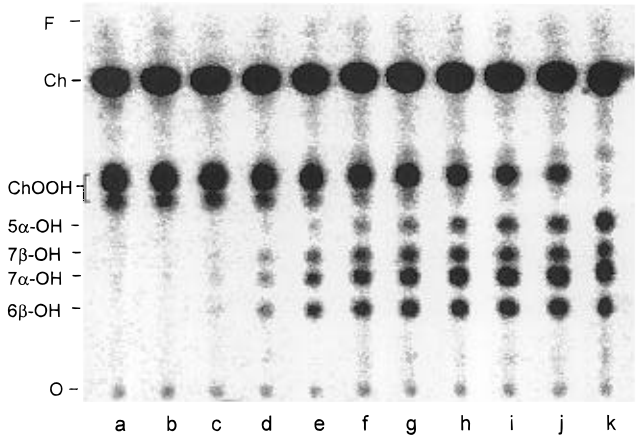

FIGURE 3: Thin-layer chromatogram depicting loss of [^{14}C]ChOOHs and formation of [^{14}C]ChOHs during incubation of photoperoxidized liposomes with GSH/PHGPX. Samples for TLC analysis were taken from the same experiment as described in Figure 2B. Extracted lipids were chromatographed on a silica gel plate, using benzene/ethyl acetate (1:1) as the solvent system. After separation, radiolabeled species were detected by radioimaging, using an Ambis 4000 system. Peroxidized membranes were analyzed before (a) and after incubation with GSH alone for 60 min (b) or GSH plus PHGPX for 2 min (c); 5 min (d); 10 min (e); 15 min (f); 20 min (g); 30 min (h); 45 min (i); 60 min (j). Lane k represents photooxidized starting material after complete reduction with triphenylphosphine. Assignments were based on the locations of authentic standards (run separately), ChOOHs being detected by spraying with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and ChOHs by spraying with 50% H_2SO_4. Sample load (as total cholesterol) was ∼150 nmol per lane.

X-100 lysates of Se-replete cells. L·Se(+) cells are represented in Figure 5A, and K·Se(+) cells are represented in Figure 5C. The general order of reactivity was maintained, 5α-OOH being reduced most slowly and 6β-OOH most rapidly. The rate constants determined for L·Se(+) cells were as follows ($\times 10^2$ min^{-1}): <0.1 (5α-OOH); 1.07 ± 0.08 (7α,7β-OOH); 3.45 ± 0.13 (6β-OOH); those determined for K·Se(+) cells were as follows ($\times 10^2$ min^{-1}): <0.1 (5α-OOH); 0.72 ± 0.07 (7α,7β-OOH); 1.88 ± 0.11 (6β-OOH). Although there was barely any detectable net loss of 5α-OOH under the conditions represented, a significant (albeit small) loss was observed when the concentration of lysed L·Se(+) material was doubled, the rate constant being (1.3 ± 0.8) $\times 10^{-3}$ min^{-1}, which is ∼1/50 of the value determined for 6β-OOH. Thus, 5α-OOH was found to be poorly reactive but not inert. The similar kinetic trends observed for pure enzyme (Figure 2) and cell lysates (Figure 5A,C) suggest that reactions stimulated by the latter were mediated by PHGPX. Consistent with this, none of the peroxides decayed to any significant extent when GSH was omitted from reaction mixtures. Moreover, peroxide losses were severely depressed when lysates from Se-starved (SePX-deficient) cells were used; results for L·Se(−) cells are shown in Figure 5B, and those for K·Se(−) cells are shown in Figure 5D. This effect was especially striking for the more reactive peroxides, i.e., 6β-OOH and 7α,7β-OOH.

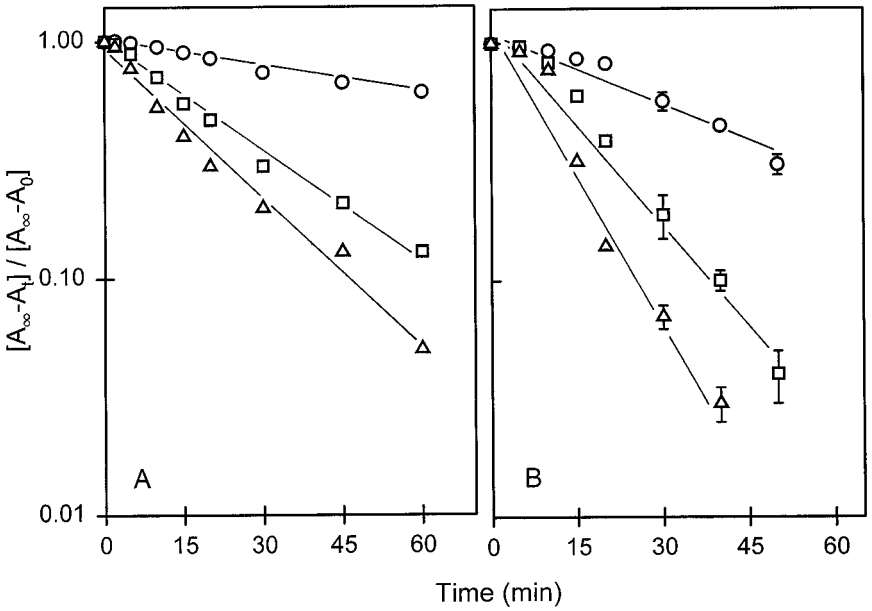

FIGURE 4: Time courses for the formation of alcohol reduction products during incubation of ChOOH-containing membranes with GSH/PHGPX. (A) Photoperoxidized POPC/[^{14}C]Ch/DCP liposomes. Plotted data are taken from the experiment described in Figures 2B and 3. (B) Photoperoxidized [^{14}C]Ch-labeled erythrocyte ghosts. Alcohol (ChOH) data from duplicate experiments are represented; ChOOH data from one of these are shown in Figure 2C. Details about reaction systems are provided in Figures 2 and 3. A_o, A_t, and A_∞ denote alcohol concentration at zero time, time *t*, and infinite time, respectively; the latter value was determined by reducing samples completely with triphenylphosphine. Diol products are denoted as follows: (○) 5α-OH; (□) 7α-OH and 7*β*-OH (composite value for both species); (△) 6*β*-OH.

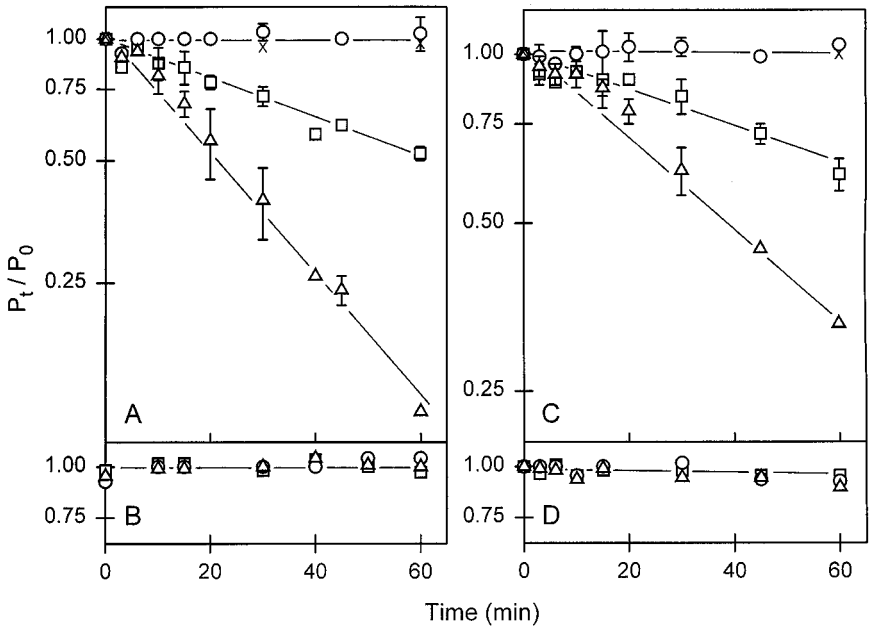

FIGURE 5: Time courses for the reduction of cholesterol hydroperoxides in the presence of GSH and cell lysates obtained from selenium-sufficient and selenium-deficient L1210 or K562 cells. Cell types are represented as follows: (A) L·Se(+); (B) L·Se(−); (C) K·Se(+); (D) K·Se(−). Reaction mixtures contained 40 μM each of 5α-OOH, 6*β*-OOH, and 7α,7*β*-OOH, 1% ethanol (added with the ChOOHs), 5 mM GSH, 50 μM DFO, 100 μM EDTA, and cell lysate corresponding to ~2.3 × 10^7 L1210 or K562 cells per ml in 0.1% Triton X-100/PBS. During incubation at 37 °C, samples were removed periodically and extracted. Residual ChOOHs in lipid fractions were determined by HPLC-EC(Hg): (○) 5α-OOH; (△) 6*β*-OOH; (□) 7α,7*β*-OOH. Controls lacking GSH were also monitored; in this case plotted data represent total ChOOH (×). P_0 and P_t denote peroxide concentrations at zero time and time *t*, respectively. Means ± deviation of values from duplicate experiments are shown.

Comparative Uptake and Cytotoxicity of 5α-OOH and 6β-OOH. We hypothesized that if the ChOOHs described exhibit similar cytotoxic reactivity, then 5α-OOH should be the most deleterious because it is reduced and detoxified more slowly than all the others. We tested this by exposing cells to increasing concentrations of 5α-OOH or 6*β*-OOH in liposomal form and examining survival by clonal assay. As anticipated, 5α-OOH proved to be much more cytotoxic than 6*β*-OOH; the respective LC_{50} values were ~25 and ~120 μM for L·Se(+) cells (Figure 6A) and ~38 and ~172 μM for K·Se(+) cells (Figure 6B). The cytotoxic potency of 7α,7*β*-OOH was not determined, but one would expect it to fall somewhere between that of 5α-OOH and 6*β*-OOH, on the basis of susceptibility to enzymatic detoxifi-

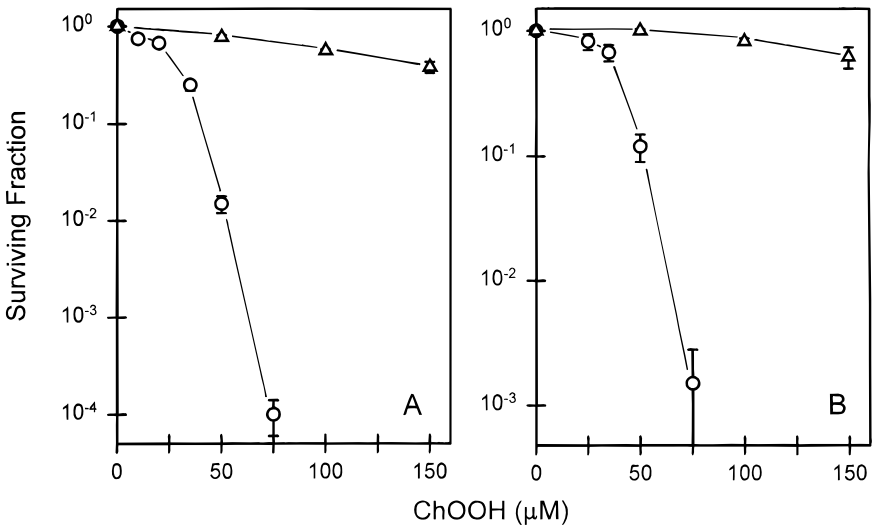

FIGURE 6: Comparison of 5α-OOH and 6β-OOH toxicity toward L1210 cells (A) and K562 cells (B). L·Se(+) cells (8.0×10^5/mL) or K·Se(+) cells (5.0×10^5/mL) in 1% serum/RPMI medium were incubated in the presence of increasing concentrations of POPC/Ch liposomes containing ~19 mol % of either 5α-OOH (○) or 6β-OOH (△). Peroxide concentrations up to 150 μM in bulk suspension were used. Total liposomal lipid (POPC, Ch, and ChOOH) in each reaction mixture for both cell types was normalized to ~1.0 mM by including liposomes that lacked peroxide. After 20 h of incubation at 37 °C, a clonogenic cell survival assay was initiated. Error bars indicate the range of values for duplicate experiments.

cation (Figure 5A,C). In keeping with this, earlier work (Geiger et al., 1991) showed that photooxidized liposomes containing 7α,7β-OOH as the predominant peroxide species inactivated L·Se(+) cells with an LD_{50} of ~70 μM. Significantly, a diol analogue, 7β-OH, was innocuous in concentrations up to at least 100 μM, confirming that the peroxide moiety is required for cytotoxicity.

Using ^{14}C-labeled peroxides at equal starting concentrations, we determined that the rate of accumulation of total radioactivity in L·Se(+) cells was essentially the same for 5α-OOH and 6β-OOH (Figure 7). This rules out the possibility that 5α-OOH was more cytotoxic than 6β-OOH because it was taken up more rapidly.

Reductive Metabolism of 5α-OOH and 6β-OOH. Results of an experiment aimed at comparing the reductive metabolism of 5α-OOH and 6β-OOH by L·Se(+) cells are shown in Table 2. ChOOH/ChOH distribution in cell and liposome fractions was assessed 13 h after adding liposomal [^{14}C]5α-OOH or [^{14}C]6β-OOH to a cell suspension, at which point ~11% of the radioactivity from either peroxide was cell-associated (see Figure 7). Under the conditions used (12-fold greater cell concentration and shorter contact time than in the Figure 6A experiment), the majority of cells appeared to be intact (Trypan Blue excluding) after incubation with either peroxide. In cells presented with 5α-OOH (experiment 1, Table 2), approximately 40% of the cell-associated radioactivity after 13 h was accounted for as 5α-OOH and 14% as 5α-OH. The existence of 7α-OOH (14%) and 7α-OH (~32%) is attributed to 5α-OOH rearrangement during incubation, followed by relatively favorable enzymatic reduction in the cell compartment. The low levels of 5α-OH and 7α-OH in the liposomal fraction of experiment 1 (~2% of the radioactivity for each) are ascribed to non-enzymatic reduction plus some efflux of the diols from cells. In experiment 2 (Table 2), where 6β-OOH was used, only 11% of the cell-associated radioactivity was found in 6β-OOH, the remaining 89% being found in 6β-OH. The relatively small fraction of 6β-OH observed in the liposomal compartment is attributed to nonspecific reduction and/or efflux, as indicated for 5α-OH and 7α-OH. These findings, consistent with those made on model systems (Figures 2−4) and cell extracts (Figure 5), confirm that ChOOHs differ in their susceptibility to reductive detoxification and that 5α-OOH is metabolized more slowly than 6β-OOH.

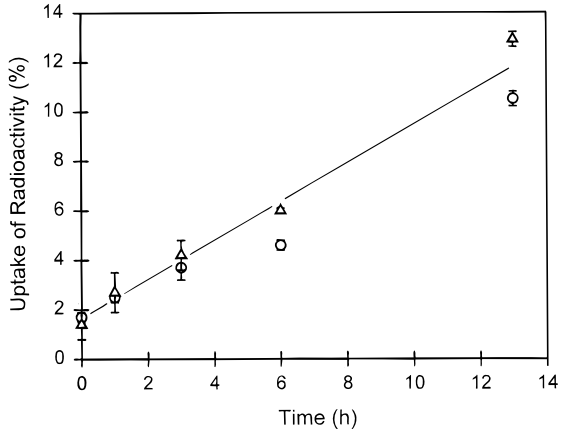

FIGURE 7: Uptake of radioactivity by L1210 cells during exposure to [^{14}C]5α-OOH or [^{14}C]6β-OOH in liposomal form. A stock suspension of L·Se(+) cells in 1% serum/RPMI medium (0.88 mL) was mixed with 0.62 mL of [^{14}C]5α-OOH/Ch/POPC or [^{14}C]6β-OOH/Ch/POPC liposomes in PBS. The resulting mixtures containing 1.0×10^7 cells/mL, 50 μM 5α-OOH or 6β-OOH (~0.75 μCi/mL) in bulk suspension, and 0.59 mM total liposomal lipid were incubated at 37 °C. HPLC-EC(Hg) analysis indicated that the 5α-OOH system contained ~3% 7α,7β-OOH at the outset, whereas the 6β-OOH system contained no other detectable peroxides. After various periods of incubation up to 13 h, samples were recovered and centrifuged. Cell fractions were washed twice with PBS to remove residual liposomes, mixed with EDTA (0.1 mM), and extracted with chloroform/methanol (2:1). After being dried under a stream of argon, the extracts were dissolved in a counting cocktail and subjected to scintillation counting. Designations are as follows: (○) [^{14}C]5α-OOH; (△) [^{14}C]6β-OOH. Data points represent means ± deviation of values from duplicate experiments.

DISCUSSION

Hydroperoxide species are among the most prominent intermediates generated when unsaturated lipids in biological

Table 2: Peroxide and Alcohol Levels in L1210 Cells after Incubation with 5α-OOH- or 6β-OOH-Containing Liposomes[a]

peroxide presented	species determined	concentration (μM)[b]	
		liposomal fraction	cellular fraction
1. 5α-OOH	5α-OOH	18.2 ± 1.2 (40.5%)	2.3 ± 0.3 (40.4%)
	7α-OOH	24.9 ± 2.5 (55.5%)	0.8 ± 0.4 (14.0%)
	5α-OH	0.7 ± 0.9 (1.6%)	0.8 ± 0.3 (14.0%)
	7α-OH	1.1 ± 1.2 (2.4%)	1.8 ± 0.2 (31.6%)
2. 6β-OOH	6β-OOH	36.7 ± 2.0 (82.5%)	0.6 ± 0.5 (10.9%)
	6β-OH	7.8 ± 2.0 (17.5%)	4.9 ± 0.5 (89.1%

[a] Data are from the same experiments as described in Figure 7. After a 13-h incubation of L·Se(+) cells with [^{14}C]5α-OOH- or [^{14}C]6β-OOH-containing liposomes, samples were collected and centrifuged. The recovered supernatant fractions containing liposomes and cellular fractions (washed free of liposomes) were extracted, and recovered lipids were subjected to TLC. ^{14}C-labeled ChOOHs and ChOHs were detected by Ambis radioimaging. Radioactivity not associated with the indicated compounds was negligible. Determination of ChOOH and ChOH concentrations in the different compartments was based on the distribution of radioactivity after 13 h (see Figure 7). Means ± deviation of values from duplicate experiments are shown; numbers in parentheses represent percent amounts within a given fraction. [b] Pertains to bulk suspension.

membranes, lipoproteins, and other organized assemblies undergo oxidative modification. Reactions that give rise to LOOHs include (i) free radical-mediated lipid peroxidation, triggered by strong oxidants such as ferryl or hydroxyl radical (Kappus, 1985; Halliwell & Gutteridge, 1989), and (ii) non-radical-mediated, ene addition of singlet oxygen ($^1O_2$), typically generated by photodynamic action (Girotti, 1985, 1990). In the former case, LOOHs arise during the secondary (propagative) phase of lipid oxidation, whereas in the latter case they arise as primary products. Since LOOHs are more polar than parent lipids, their presence can produce structural/functional perturbations in membranes and lipoproteins. Moreover, LOOHs are susceptible to iron-mediated reduction to oxyl radical (LO$^\bullet$) intermediates (eq 1). LO$^\bullet$

$$\mathrm{LOOH} + \mathrm{Fe}^{2+} \rightarrow \mathrm{LO}^\bullet + \mathrm{OH}^- + \mathrm{Fe}^{3+} \quad (1)$$

$$\mathrm{LO}^\bullet + \mathrm{O}_2 \rightarrow \mathrm{OLOO}^\bullet \quad (2)$$

$$\mathrm{OLOO}^\bullet + \mathrm{LH} \rightarrow \mathrm{OLOOH} + \mathrm{L}^\bullet \quad (3)$$

$$\mathrm{L}^\bullet + \mathrm{O}_2 \rightarrow \mathrm{LOO}^\bullet \quad (4)$$

$$\mathrm{LOO}^\bullet + \mathrm{LH} \rightarrow \mathrm{LOOH} + \mathrm{L}^\bullet \quad (5)$$

itself, or more likely, the epoxyallylic peroxyl radical (OLOO$^\bullet$) arising via rearrangement of LO$^\bullet$ and O_2 addition (eq 2; Gardner, 1989), can trigger chain peroxidation reactions, which exacerbate the damaging effects of oxidative processes (i) and (ii) described above. These reactions are depicted in eqs 3−5, where LH, L$^\bullet$, LOO$^\bullet$, and OLOOH denote an unsaturated lipid, a lipid radical, a peroxyl radical, and an epoxyallylic hydroperoxide, respectively. Eucaryotic cells are equipped with a rich variety of primary and secondary antioxidant defenses against the damaging and potentially lethal effects of these reactions (Halliwell & Gutteridge, 1989). Enzymatic detoxification of membrane-associated LOOHs is an important example of a secondary-level defense against peroxidative injury. These reactions are typically catalyzed by GSH-requiring enzymes, which fall into two classes: (i) the selenoperoxidases and (ii) certain (less important) GSH-*S*-transferases with peroxidatic activity, e.g., GST-α (Mannervik & Danielson, 1988). The general reaction catalyzed by these enzymes is depicted in eq 6, where ROOH denotes a general hydroperoxide and ROH is its alcohol reduction product. In metabolically competent

$$\mathrm{ROOH} + 2\mathrm{GSH} \rightarrow \mathrm{ROH} + \mathrm{H_2O} + \mathrm{GSSG} \quad (6)$$

cells that are not overwhelmed by peroxide pressure, two-electron detoxification (eq 6) far exceeds one-electron toxicity enhancement (eqs 1−5) (Lin & Girotti, 1993). GPX and PHGPX are the intracellular selenoperoxidases that have been studied most extensively with regard to antiperoxidative activity. Although both enzymes contain an active site selenocysteine and have similar catalytic cycles (Flohe, 1989), they differ significantly in physical characteristics, subcellular distribution, and amino acid sequence, showing only ∼30% homology (Brigelius-Flohe et al., 1994). PHGPX and GPX also exhibit striking functional differences; the former can act directly on PLOOHs in membranes and other organized systems, whereas the latter is unreactive unless *sn*-2 fatty acyl bonds are cleaved to liberate fatty acid hydroperoxides (Grossman & Wendel, 1983; Sevanian et al., 1983). For PLOOHs in cell membranes, removal/detoxification could involve (i) sequential action of phospholipase A_2 (PLA_2) and GPX (hydrolysis followed by reduction) (Van Kuijk et al., 1987) or (ii) sequential action of PHGPX and PLA_2 (direct reduction, followed by hydrolysis) (Ursini et al., 1991). Each of these pathways would require a follow-up repair step in which lysolipids resulting from release of hydroperoxy or hydroxy fatty acids are reacylated (Ursini et al., 1991). The relative importance of the mechanism involving GPX (excision/reduction/repair) versus that involving PHGPX (reduction/excision/repair) for any given biological membrane undergoing peroxidative modification is not yet clear. The nature of the incident oxidant(s) and the hydroperoxides arising therefrom could be important in determining whether GPX or PHGPX plays a dominant protective role. For example, if LOOH formation is mediated by H_2O_2, GPX might be more important, since it reduces this peroxide more efficiently than PHGPX (Ursini et al., 1985). On the other hand, if $^1O_2$ gives rise to PLOOHs, removal/repair is the only known enzymatic defense, and PHGPX may be more effective in this regard. Since hydrolysis of PLOOHs by PLA_2 is not a prerequisite for PHGPX action, this alternative (direct reduction) would appear to be the more logical antioxidant strategy. A recent study based on kinetic modeling has provided strong support for this notion (Antunes et al., 1995). It was estimated that the reductive flux of PLOOHs through PHGPX in rat liver mitochondria is at least 4 orders of magnitude greater than the hydrolytic flux through PLA_2. Since GPX can only act after fatty acid hydroperoxides have been released by PLA_2, this finding suggests that PHGPX is far more important than GPX in PLOOH detoxification. *In vivo* experiments carried out by Hughes et al. (1983) have provided strong supporting evidence for this notion.

Since phospholipids are relatively abundant in most biological membranes, detoxification of PLOOHs has been given considerable attention. However, there is a growing awareness that cells must also deal with other LOOHs in order to avert peroxidative damage. Cholesterol-derived hydroperoxides may contribute significantly to the non-

PLOOH pool, particularly in the plasma membrane, where cholesterol comprises 40−45 mol % of the total lipid (Bloch, 1983). In keeping with this, we have shown that ChOOH accumulates at approximately one-third the rate of phosphatidylcholine hydroperoxide during lethal irradiation of L1210 cells in the presence of a plasma membrane-associated photosensitizer (Bachowski et al., 1994). Throughout irradiation, ChOOH accounted for 2−3 mol % of total LOOH, which approximates the mol % of cholesterol relative to total lipid in these cells. We showed earlier that PLOOHs in photooxidized erythrocyte ghosts are inert to GPX unless first hydrolyzed by Ca^{2+}-activated PLA_2, whereas ChOOHs (examined as a group) are completely unreactive with GPX, even after membrane solubilization with Triton X-100 (Thomas & Girotti, 1988). Significantly, pretreatment with PLA_2 and then with GSH/GPX only partially protected LOOH-containing membranes against free radical peroxidative damage induced by iron and ascorbate (see eqs 1−5). The residual activity correlated with the persistence of ChOOHs in the membranes after exposure to PLA_2 and GSH/GPX. This result prompted us to ask whether another SePX is involved in ChOOH detoxification and led to the discovery that porcine heart PHGPX can catalyze the reduction of ChOOHs in addition to PLOOHs in various membrane systems (Thomas et al., 1990a). Of special importance was our observation that, in contrast to PLA_2/GSH/GPX treatment, GSH/PHGPX treatment made LOOH-containing erythrocyte ghosts completely resistant to iron/ascorbate-induced lipid peroxidation. Other studies have shown that all measurable LOOHs in low-density lipoprotein, including cholesteryl ester hydroperoxides, can be disposed of by PHGPX (Thomas et al., 1990b), further demonstrating the great versatility of this enzyme as a proximal LOOH scavenger.

In the present work and recent preliminary work (Geiger et al., 1995), we have shown that several photochemically-generated ChOOH isomers are reduced at different first-order rates by the GSH/PHGPX system. The same general order of reactivity (6β-OOH > 6α-OOH ≈ 7α,7β-OOH > 5α-OOH) was observed for three different test systems, detergent micelles, liposomes, and red cell ghosts. Decay rates for individual peroxides matched those for formation of the corresponding alcohol products, indicating that the expected overall reactions were taking place (eq 6). Comparable kinetic patterns observed for the different test systems (Figure 2) suggest that reducibility depended more on structural properties of the substrates themselves than on environmental factors. It is important to note in this regard that 5α-OOH is unique among the substrates tested in being a tertiary hydroperoxide. As such it might not be recognized by PHGPX as well as the other substrates, which, like PLOOHs, are secondary hydroperoxides. Although the same trend in reactivity was observed in the different systems, there was considerable variability in the decay rate of one peroxide relative to another from system to system (Figure 2). Most obvious was the 6β-OOH/5α-OOH rate ratio, which was 8 times greater in Triton X-100 micelles than in erythrocyte ghosts. These differences are attributed mainly to effects on 5α-OOH reactivity, although the underlying reason is unknown. One possibility is that 5α-OOH's structural orientation or configuration in Triton micelles makes it less accessible to PHGPX than in membrane bilayers. Consistent with this is our preliminary observation (data not shown) that Triton solubilization of peroxidized liposomes (cf. Figure 2B) slows 5α-OOH reduction somewhat while strongly enhancing 6β-OOH and 7α,7β-OOH reduction. We have focused on ChOOH behavior, but it is important to realize that the photooxidized membranes used in this work also contained PLOOHs. Comparative studies on PHGPX-catalyzed reduction of PLOOHs and ChOOHs are underway and will be the subject of a future report.

We found that the kinetic profiles observed with purified PHGPX in micellar or membrane systems could be mimicked by using lysates of Se-replete L1210 or K562 cells (Figure 5A,C). This, coupled with the fact that Se(+) lysates without GSH or Se(−) lysates with GSH were ineffective, leaves little doubt that the active cellular factor was PHGPX. We have shown that making L1210 or K562 cells Se deficient typically reduces their PHGPX activity by only 60%−70%, as measured by coupled enzymatic assay (see Experimental Procedures). It is curious, therefore, that residual activity in L·Se(−) or K·Se(−) lysates was barely detectable when measured in terms of ChOOH reduction by means of HPLC-EC(Hg) (Figure 5B,D). This discrepancy is attributed to the relatively poor sensitivity of the coupled assay, which resulted in large errors at the low PHGPX activities observed. Therefore, the data acquired by HPLC-EC(Hg) are regarded as being much more accurate, suggesting that the extent of PHGPX depletion in Se(−) cells was probably closer to that of GPX, i.e., 90%−95%. Other workers have also pointed out the advantages of an HPLC-based assay for PHGPX (Bao et al., 1995). Although 6β-OOH and 7α,7β-OOH were readily reduced in the presence of Se(+) cell lysates, no net loss (relative to freeze-fractured controls) was observed after 1 h when intact cells at concentrations equal to or even greater than those represented in the lysate experiments were used (data not shown). The likely explanation for this is that ChOOH uptake was very slow, <1% per h for L·Se(+) cells (Figure 7), making it the rate-limiting step in intact systems.

Having demonstrated that 6β-OOH is reduced more rapidly than 5α-OOH in the presence of cell lysates, we predicted that the latter species would be more cytotoxic. Cell survival determinations indicated that this prediction was correct; thus, the LC_{50} value of 5α-OOH was $\sim^1/_5$ of that of 6β-OOH for both L·Se(+) and K·Se(+) cells. Faster uptake of 5α-OOH could have accounted for its greater lethality; however, the rate of incorporation of 5α-OOH and 6β-OOH into L·Se(+) cells was found to be essentially the same. Once taken up, the peroxides were converted to their respective diols, but the fractional amount of 5α-OOH so converted was substantially smaller than that of 6β-OOH, mimicking the results obtained with model systems and cell lysates. Although a detailed kinetic analysis of this metabolism has not been carried out with intact cells, it is not unreasonable to conclude from the results at hand that relatively inefficient reductive metabolism of 5α-OOH is the biochemical basis for its greater cytotoxicity. The longer metabolic lifetime of 5α-OOH would make it more available for inducing cell injury, e.g., via iron-mediated peroxidative pathways (eqs 1−5). That 5α-OOH can trigger such reactions was demonstrated in recent work involving erythrocyte ghosts to which it had been translocated from liposomal donors (Geiger et al., 1995). Such ghosts, containing 5α-OOH as the only major peroxide, underwent damaging free radical lipid peroxidation when exposed to a lipophilic Fe^{3+} chelate and

an electron donor, ascorbate. How 5α-OOH compares with other ChOOHs in this regard has not been determined. Thus, whether these peroxides might differ from one another in their susceptibility to one-electron reduction and, if so, whether this is a contributing factor in determining cytotoxicity are presently unknown.

Although it is well accepted that reductive metabolism of LOOHs can protect cells against peroxidative damage, little is known about the actual mechanisms by which cells dispose of endogenous or exogenous LOOHs. We have addressed this question in comprehensive fashion with an overall goal of attaining a better understanding of how cells cope with peroxidative stress. Several aspects of the work are novel. To our knowledge, this is the first study in which (i) the relationship between LOOH cytotoxicity and metabolic reducibility is systematically evaluated for mammalian cells; (ii) several LOOHs within a given lipid family are compared in terms of cytotoxic potency and susceptibility to metabolic detoxification; (iii) LOOH uptake by cells is measured; and (iv) detoxification via the GSH/PHGPX system is singled out. Our findings have specific bearing on singlet oxygen-mediated phototoxicity, since 5α-OOH, 6α-OOH, and 6β-OOH are generated exclusively by singlet oxygen attack on cholesterol (Smith, 1981). We have determined (Korytowski et al., 1992) that 5α-OOH is produced 2−3 times more rapidly than 6α- or 6β-OOH during dye-sensitized photooxidation of cholesterol in membrane systems. Faster generation on the one hand and slower reductive metabolism on the other could make 5α-OOH the most damaging ChOOH to arise in photodynamically stressed cells.

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