Protective Action of Phospholipid Hydroperoxide Glutathione Peroxidase against Membrane-damaging Lipid Peroxidation

IN SITU REDUCTION OF PHOSPHOLIPID AND CHOLESTEROL HYDROPEROXIDES*

James P. Thomas‡, Matilde Maiorino‡, Fulvio Ursini‡, and Albert W. Girotti†‡

From the ‡Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the †Institute of Biological Chemistry, University of Padova, 35131 Padova, Italy

The general reactivity of membrane lipid hydroperoxides (LOOHs) with the selenoenzyme phospholipid hydroperoxide glutathione peroxidase (PHGPX) has been investigated. When human erythrocyte ghosts (lipid content: 60 wt % phospholipid; 25 wt % cholesterol) were treated with GSH/PHGPX subsequent to rose bengal sensitized photoperoxidation, iodometrically measured LOOHs were totally reduced to alcohols. Similar treatment with the classic glutathione peroxidase (GPX) produced no effect unless the peroxidized membranes were preincubated with phospholipase A2 (PLA2). However, under these conditions, no more than ~60% of the LOOH was reduced; introduction of PHGPX brought the reaction to completion. Thin layer chromatographic analyses revealed that the GPX-resistant (but PHGPX-reactive) LOOH was cholesterol hydroperoxide (ChOOH) consisting mainly of the 5a (singlet oxygen-derived) product. Membrane ChOOHs were reduced by GSH/PHGPX to species that comigrated with borohydride reduction products (diols). Sensitive quantitation of PHGPX-catalyzed ChOOH reduction was accomplished by using [14C]cholesteralabeled ghosts. Kinetic analyses indicated that the rate of ChOOH decay was ~1/4 that of phospholipid hydroperoxide decay. Photoxidized ghosts underwent a large burst of free radical-mediated lipid peroxidation when incubated with ascorbate/iron or xanthine/xanthine oxidase/iron. These reactions were only partially inhibited by PLA2/GSH/GPX treatment, but totally inhibited by GSH/PHGPX treatment, consistent with complete elimination of LOOHs in the latter case. These findings provide important clues as to how ChOOHs are detoxified in cells and add new insights into PHGPX's protective role.

Aerobic cells are constantly exposed to the possibility of oxidative damage mediated by activated oxygen species such as superoxide (O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH·), or singlet oxygen (¹O₂). One of the most widely studied examples of such damage is lipid peroxidation, a process involving the oxidative degradation of unsaturated lipids, with corresponding formation and breakdown of lipid hydroperoxides (LOOHs). Lipid peroxidation is highly detrimental to membrane structure and function and has been linked to numerous cytopathological effects (1–3). Cytoprotection against lipid peroxidation and other types of oxidative damage is accomplished by diverse enzymatic and non-enzymatic means. An important participant in the former category is the classic selenoenzyme glutathione peroxidase (GPX), which can reduce and detoxify H2O2 and various organic hydroperoxides at the expense of GSH (Equation 1, where ROOH denotes a general hydroperoxide).

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\text{ROOH} + 2 \text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O} \quad (1)
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Natural regeneration of GSH from GSSG is catalyzed by GSSG reductase (Equation 2). Previous studies clearly established that phospholipid hydroperoxides (whether membrane-bound or detergent-dispersed) are not susceptible to direct reduction by GPX (4–6). Instead, the oxidized en-2 fatty acyl groups must first be hydrolyzed by phospholipase A2 (PLA2); GPX then acts on the liberated fatty acid hydroperoxides. On the basis of these findings, a mechanism for detoxification and repair of phospholipid hydroperoxide lesions has been proposed which involves consecutive action of PLA2 and GPX on membrane phospholipid hydroperoxides, followed by reinsertion of new fatty acid groups. Most of the early experiments leading to these conclusions were carried out with phospholipid vesicles (liposomes) that lacked cholesterol (4–6). Recent studies by Thomas and Girotti (7, 8) have indicated that photochemically generated phospholipid hydroperoxides in a well-characterized plasma membrane, the erythrocyte ghost (phospholipid ~50 mol %; cholesterol ~43 mol %), must also be hydrolyzed by PLA2 before GPX will act. On the other hand, cholesterol hydroperoxides were found to be resistant to GPX, even after extraction from the membrane. The photoxidized, PLA2/GPX-treated membranes were only partially protected against ascorbate/iron-stimulated lipid peroxidation, evidently because initiation reactions by cholesterol hydroperoxide-derived radicals persisted. These findings raised the important question of how cells might detoxify cholesterol hydroperoxides (CHOOH, cholesterol hydroperoxide; GPX, glutathione peroxidase; 5α-OOH, 5α-cholest-5-en-7α-hydroperoxide; 7α-OOH, 7α-hydroxycholest-5-en-3β-diol; 7β-OOH, 7β-hydroxycholest-5-en-3β-diol; 5α-OOH, 5α-hydroxycholest-5-en-3β-diol; 5α-OOH, 5α-hydroxycholest-5-en-7α-hydroperoxide; 5α-cholest-5-en-3β-diol; 7α-OOH, 7α-hydroxycholest-5-en-3β-diol; 7β-OOH, 7β-hydroxycholest-5-en-3β-diol; PBS, phosphate-buffered saline; PC, phosphatidylcholine;PE, phosphatidylethanolamine; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLA2, phospholipase A2; TLC, thin layer chromatography; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; TBA, thiobarbituric acid.}

* The abbreviations used are: LOOH, lipid hydroperoxide; ChOOH, cholesterol hydroperoxide; GPX, glutathione peroxidase; 5α-OOH, 5α-cholest-5-en-7α-hydroperoxide; 5α-OOH, 5α-cholest-5-en-3β-diol; 5β-OOH, 5β-hydroxycholest-5-en-7α-hydroperoxide; 7α-OOH, 7α-hydroxycholest-5-en-3β-diol; 7β-OOH, 7β-hydroxycholest-5-en-3β-diol; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLA2, phospholipase A2; TLC, thin layer chromatography; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; TBA, thiobarbituric acid.

† To whom correspondence should be addressed.

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cholesterol hydroperoxides. We have begun to examine this question by using a recently discovered second selenoenzyme, provisionally termed "phospholipid hydroperoxide glutathione peroxidase" (PHGPX) (9, 10). Unlike GPX, PHGPX can reduce membrane phospholipid hydroperoxides in situ without the necessity of prior hydrolysis by PLA₂ (9). Thus, a more direct protective role of PHGPX is apparent. Previous studies (10) indicated that PHGPX is relatively nonselective in its action on phospholipid hydroperoxides. However, whether it would also act on cholesterol hydroperoxides was not directly assessed. We now provide the first evidence for PHGPX-catalyzed reduction and detoxification of cholesterol hydroperoxides in a natural cell membrane.

EXPERIMENTAL PROCEDURES

Materials—Freshly drawn human blood was obtained from the Blood Center of Southeastern Wisconsin. Erythrocyte membranes (unsealed white ghosts) were prepared by standard lysing and washing procedures (11), stored under argon at 4 °C to minimize autoxidation, and used within a fortnight. Total membrane protein was determined by the method of Lowry et al. (12), using serum albumin as the standard. Other materials were obtained from the following sources: GSH, NADPH, xanthine, egg phosphatidylcholine, diolym phosphatidylethanolamine, cholesterol, and 7-keto-cholesterol from Sigma, sodium acetate from BDH Chemicals, 2-thiobarbituric acid and N,N,N',N'-tetramethyl-p-phenylenediamine from Aldrich Chemical Co., desferrioxamine from Ciba-Geigy, and [14C]cholesterol (40-60 mCi/mmol) from Research Products International. Sigma provided the following enzymes: bovine erythrocyte glutathione peroxidase and superoxide dismutase, bovine liver catalase, yeast glutathione reductase, pancreatic phospholipase A₂, and xanthine oxidase from buttermilk. Rose bengal was obtained from Allied Chemical Co. and purified according to Brand et al. (13).

Purification of Porphyrin heart phospholipid hydroperoxide glutathione peroxidase was prepared as described by Uraini et al. (9). The enzyme used in this study was purified by affinity chromatography on a glutathione-bromosulfophthalein-Sepharose column, followed by molecular exclusion chromatography on Sephadex G-50 (14). The final preparation in 0.5 M KSCN, 5 mM 2-mercaptoethanol, 10% glycerol, 25 mM Tris-HCl, pH 7.2, was stored at -20 °C. Activity of PHGPX was determined by coupled enzymatic assay, using lipoyxigenase-treated soybean PC as the substrate (14). All aqueous solutions were prepared with deionized, glass-distilled water.

Preparation of [14C]Cholesterol labeled Ghosts. Immediately before use, [14C]cholesterol plus carrier (0.2–0.3 mg total) was separated from my pre-existing oxidation products by TLC (see below), located by radioautography, and recovered by scraping. Erythrocyte ghosts were exchanged, labeled by incubating with unilamellar [14C]cholesterol/egg PC liposomes (0.8:1, mol/mol), as described previously (15). After 48 h of incubation at 37 °C, the ghosts were washed extensively with PBS to remove the liposomes and resuspended to a final concentration of ~1.8 × 10⁹/mg (1 mg of protein/ml) for experimental use.

Photoradiolysis Reactions—In a typical experiment, ghost membranes (1 mg of protein/ml in PBS) were sensitized with rose bengal (5 μM), transferred to a thermostatted reaction vessel, and irradiated at 10 °C with continuous stirring (16). Incident light from a mercury arc lamp was passed through a yellow filter (Corning CS-68) to select wavelengths maximally absorbed by membrane-bound rose bengal (λ₅₄₀ ≈ 570 nm). Light intensity at the membrane suspension surface was ~100 W/m², as measured by a Yellow Springs thermopile. After a given period of irradiation (typically 1 h), samples were recovered for the various reactions and analyses described below. All manipulations were carried out under minimal illumination. Dark controls (with dye) and light controls (without dye) were prepared alongside.

Enzymatic Assays—The relative abilities of GPX and PHGPX to catalyze the reduction of membrane LOOHs were examined by coupled enzymatic assay with GSSG-reductase. Samples of photoradiolysed ghosts in PBS were mixed with desferrioxamine (25-60 μM) and incubated at 37 °C for 15 min in the presence of 1 mM CaCl₂ (control) or 1 mM CaCl₂ plus PLₐ₂ (15–20 units/ml). (Calcium was used to activate PLₐ₂ and desferrioxamine to prevent iron-catalyzed LOOH decomposition (17).) Membrane samples (0.05–0.1 mg of protein/ml) were mixed with GSSG-reductase (1–2 units/ml), 0.1% Triton X-100, 5 mM EDTA, 3 mM GSH, 0.1 mM NADPH, and 0.1 mL Tris-HCl, pH 7.6, in a thermostatted spectrophotometer cell (37 °C) equipped with a magnetic stirrer, and the basal rate of A₅₄₀ decay was recorded. The volume of the reaction mixture at this point was typically 2.5 ml. (The detergent was used primarily to minimize light scattering.) After ~1 min, GPX (0.3–0.5 units/ml) or PHGPX (0.1–0.2 units/ml) was added, based on the catalytic activity of the enzyme in the presence of reactive LOOH (7). Absorbance readings were corrected for small contributions of the peroxidases themselves and for volume changes that occurred when the enzymes were added.

Iodometric Assays—Iodometric determination of total LOOH in photooxidized membranes was carried out before and after treatment with GPX/GSH or PHGPX/GSH. At various intervals during peroxide treatment, 0.5-ml samples of the membrane suspension (~0.45 mg of lipid) were mixed with 1 mM EDTA and extracted with 0.8 ml of chloroform/methanol (2:1, v/v). Aliquots of 0.4 ml from the organic phases were evaporated under argon, and the recovered LOOHs were analyzed iodometrically as described previously (18). Quantitation was based on an extinction coefficient of 21.9 (mM)-¹ cm⁻¹, which was obtained by using enzymatically standardized cumene hydroperoxide in the assay.

Chromatographic Methods—The procedure for TLC separation of cholesterol hydroperoxides and their reduction products was adapted from published methods (19). Photooxidized membranes were examined before and after reaction with GSH/GPX or GSH/PHGPX. After 0.25 (typical) or 0.25 (mg of total) of membrane mixtures were mixed with 3 μl of 0.1 mM EDTA and extracted with 0.4 ml of chloroform/methanol (2:1, v/v) in polypropylene microcentrifuge tubes. After centrifugation, 0.2 ml of the organic phase was transferred to a second microcentrifuge tube, and solvent was removed at 50 °C under a stream of argon. Simultaneous treatment of several samples was accomplished with a 9-port manifold (Pierce Vacuum Evaporator). In some instances, solvent evaporation was preceded by 5 min of incubation with 1-2 mM borohydride (added as 50 mM sodium borohydride in methanol, 10 mM NaOH). Each lipid residue was dissolved in 10 μl of cold chloroform/methanol, applied to a Silica Gel-60 TLC plate (EM Science), and chromatographed, using a single irrigation of hexane/ethyl acetate (1:1, v/v). In this system, phospholipid hydroperoxides remain at the origin, and, therefore, do not interfere with the colorimetric detection of cholesterol hydroperoxides. Authentic cholesterol and the borohydride reduction products of 7-ketocholesterol (7α-OH and 7β-OH) were chromatographed alongside as standards. Immediately after developing, plates were sprayed with TMMD (20) to detect hydroperoxides (R₂ > 0.6), or scanned using a linear array detector (Radiomatic Instruments, model RP) equipped with data processing accessories. Subsequent to TMMD treatment or radioautography, the plates were sprayed with 50% H₂SO₄ and warmed briefly at 80 °C to visualize cholesterol itself (R₂ < 0.6), and the reduction products of cholesterol hydroperoxides (diols; R₂ 0.19–0.26) (20). Although hydroperoxides could also be detected with H₂SO₄, the sensitivity was much lower than with TMMD. In some instances, plates were photographed immediately after spraying with TMMD or H₂SO₄.

General separation of membrane LOOHs, including those derived from phospholipids, was accomplished with the solvent system chromatography (15, 20). Immediately after development, the plates were sprayed lightly with TMMD (20) to visualize phospholipid hydroperoxides and scanned using a linear array detector (Radiomatic Instruments, model RP) equipped with data processing accessories. Subsequent to TMMD treatment or radioautography, the plates were sprayed with 50% H₂SO₄ and warmed briefly at 80 °C to visualize cholesterol itself (R₂ < 0.6), and the reduction products of cholesterol hydroperoxides (diols; R₂ 0.19–0.26) (20). Although hydroperoxides could also be detected with H₂SO₄, the sensitivity was much lower than with TMMD. In some instances, plates were photographed immediately after spraying with TMMD or H₂SO₄.

RESULTS

Enzymatic Reduction of Lipid-derived Hydroperoxides—In initial experiments, the relative abilities of GPX and PHGPX to catalyze the GSH-dependent reduction of membrane
LOOHs in situ was examined by coupled enzymatic assay with GSSG-reductase. In these determinations, the rate and also extent of NADPH oxidation during the reductase-catalyzed regeneration of GSH (Equation 2) was used as a measure of peroxidatic action on LOOHs (Equation 1). The test system consisted of isolated membranes that were photoperoxidized in the presence of rose bengal, a $^{18}O_2$-generating dye (22). As expected from previous studies (7), GPX alone (0.4 unit/ml) caused little (if any) LOOH loss when added to the photoperoxidized ghosts in an enzymatic assay mixture (Fig. 1, trace A). (In this experiment, the small $A_{340}$ decrement observed after the introduction of GPX is attributed primarily to dilution of the reaction mixture, with trace amounts of H$_2$O$_2$ or fatty acid hydroperoxide making a possible contribution.) When added subsequently to GPX, PHGPX (0.1 unit/ml) caused an immediate and rapid increase in the rate of $A_{340}$ decay, which slowed to approximately the background rate after ~1 min. Introduction of more PHGPX (0.1 unit/ml) at this point caused no further change; alternatively, introduction of a known amount of cumene hydroperoxide produced the expected decrement in $A_{340}$, indicating that the PHGPX was still active (data not shown). A second portion of the peroxidized membranes was treated with CaCl$_2$/PLA$_2$ before being analyzed. With this preparation, GPX produced a sizeable $A_{340}$ decrement (Fig. 1, trace B). However, the reaction was clearly not complete, since subsequent addition of PHGPX resulted in another decrement, the magnitude of which was approximately 60% of that produced by GPX. This was seen consistently in all replicate determinations. It is important to note that the $A_{340}$ value generated by PHGPX (Fig. 1A) is nearly the same (<10% difference) as that generated by successive additions of GPX and PHGPX (Fig. 1B). Moreover, there was good agreement between LOOH values calculated from these measurements (116 and 105 nmol/mg of protein, respectively) and the absolute value of total LOOH determined independently by iodometric assay (108 nmol/mg of protein). These results suggested that PHGPX could react quantitatively with all LOOHs in the membrane. By contrast, and in agreement with earlier findings (7, 8), GPX reactivity was expressed only after the hydrolytic action of PLA$_2$; but even under these conditions, only ~60% of the LOOH population was removed by GPX.

Reducibility of membrane LOOHs was studied in other ways, viz. by iodometric determination of residual LOOH during peroxidase treatment and by TLC analysis of different LOOH classes. Results of a typical experiment are shown in Fig. 2. The starting level of photoperoxides in this experiment was 0.12 $\mu$mol/mg of protein, which represents ~10% of the membrane phospholipid plus cholesterol (23). When incubated with GSH plus PHGPX, the membranes underwent a rapid loss of total LOOH, with a half-time of 2–3 min. After

![Fig. 1. Susceptibility of membrane lipid hydroperoxides to enzymatic reduction. Erythrocyte ghosts were irradiated for 1 h in the presence of 5 $\mu$M rose bengal. After the addition of 25 $\mu$M desferrioxamine, the membranes were incubated for 15 min with 1 mM CaCl$_2$ (A), or 1 mM CaCl$_2$ plus PLA$_2$ (30 units/ml) (B), and then incubated with GPX and PHGPX in coupled assay with GSSG-reductase. Assay mixtures contained membranes (0.06 mg of protein/ml), GSSG-reductase (1 unit/ml), 3 mM GSH, 0.1 mM NADPH, 5 mM EDTA, and 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 7.6. Absorbance scans (340 nm) were started at the indicated points (0). Subsequent additions of GPX (0.4 unit/ml) and PHGPX (0.1 unit/ml) were made as shown. Total LOOH content measured enzymatically in assays A and B (116 and 105 nmol/mg of protein, respectively) agreed closely with the value obtained by iodometric assay (108 ± 5 nmol/mg of protein).

![Fig. 2. Enzymatic reduction of liquid hydroperoxides in photoperoxidized erythrocyte ghosts. A, iodometric quantitation of LOOH loss. Membranes (2 mg of protein/ml in PBS) were photooxidized for 1 h in the presence of 5 $\mu$M rose bengal, mixed with 50 $\mu$M desferrioxamine, and then incubated in the dark at 37 °C with 3 mM GSH (O); GSH plus GPX, 1.1 unit/ml (A); or GSH plus PHGPX, 0.15 unit/ml (O). At the indicated time points, samples were removed for LOOH determination. Means ± deviation of values from duplicate experiments are shown. B, TLC visualization of LOOH loss in the same experiment. LOOHs were determined independently by iodometric assay (108 nmol/mg of protein). These results suggested that PHGPX could react quantitatively with all LOOHs in the membrane. By contrast, and in agreement with earlier findings (7, 8), GPX reactivity was expressed only after the hydrolytic action of PLA$_2$; but even under these conditions, only ~60% of the LOOH population was removed by GPX.

![Hydroperoxide classes are designated as follows: sphingomyelin and phosphatidylserine (I, 2), PC (3), PE (4), cholesterol (5). Solvent front (F); origin (O). Sample load (as total lipid): 0.15 mg (lanes b–f), 0.45 mg (lanes a and g).](http://www.jbc.org/)
30 min of incubation, the LOOH had decayed to <5% of its starting value. By contrast, incubation with GSH plus GPX produced no net effect on LOOH over that observed with GSH alone (~15% loss after 30 min). Lipid extracts from this experiment were also analyzed by TLC (Fig. 2B), which allowed hydroperoxides of cholesterol and different phospholipid classes to be scrutinized. Whereas no TMPD-reactive hydroperoxides were detected in a non-irradiated control (lane b), these products were clearly evident in the photooxidized sample (lane a). Based on the chromatographic migration of photooxidized standards, hydroperoxides of two major membrane phospholipids (PC and PE) could be identified, along with unresolved phosphatidylserine and sphingomyelin products. In agreement with the iodometric measurements (Fig. 2A), 30 min incubation with GSH alone or GSH plus GPX caused little perceptible change in the spot intensity of each photoprod. However, similar incubation with GSH plus PHGPX resulted in an almost total disappearance of the phospholipid-derived hydroperoxides and a sizeable, albeit incomplete, loss of cholesterol hydroperoxides. Although membrane hydroperoxides were not susceptible to direct reduction by the GSH/GPX system, they did react after PLA, treatment (Fig. 1; Ref. 7). Under these conditions, the peroxide content decreased rapidly to 35-40% of its starting value, but remained at this level after prolonged incubation with GSH/GPX (7). Trivial explanations for this incomplete reaction were ruled out, e.g., progressive inactivation of PLA, or GPX, or permeability barriers against the enzymes. Examination of TLC chromatograms clearly indicated that PLA, action had released fatty acid hydroperoxides, which were then susceptible to GPX attack. Significantly, cholesterol hydroperoxides were shown to be the only major LOOHs to resist enzymatic reduction subsequent to PLA, treatment. The poor reactivity of these peroxides with GPX could not be attributed to hindered accessibility, since solubilization of the membranes with Triton X-100 or extraction from the mem-

branes had no significant effect (7). It is apparent from these earlier results that the discrepancy between the iodometrically determined and the GPX-determined LOOH values for the experiment shown in Fig. 1B was due to cholesterol hydroperoxides. In contrast, to GPX, PHGPX appeared to react directly with these species in situ (within the membrane) as it reacted with phospholipid hydroperoxides. In subsequent experiments, we studied PHGPX-catalyzed reduction of cholesterol hydroperoxides in greater detail, focusing on (a) substrate-product relationships and (b) kinetics of substrate loss.

**Enzymatic Reduction of Cholesterol Hydroperoxides**—Because of (a) adequate sterol resolution and (b) possible interference with phospholipid-derived products, the solvent system used for the TLC shown in Fig. 2B (chloroform/methanol/water, 75:25:4 (v/v/v)) was not suitable for examining reduction products of cholesterol hydroperoxides. For this reason, we selected a less polar system (heptane/ethyl acetate, 1:1 (v/v)) which affords good resolution of cholesterol products (A), followed by H2SO4, for overall detection of cholesterol and its reaction products (B). Solvent front (F); origin (O). Sample load (as starting cholesterol): ~40 μg/lane (lanes b-g).

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**Fig. 3. Enzymatic reduction of cholesterol hydroperoxides in erythrocyte membranes.** A suspension of ghosts (1 mg of protein/ml) containing 5 μM rose bengal and 25 μM butylated hydroxytoluene was irradiated for 1 h. The peroxidized membranes were then mixed with 50 μM desferrioxamine, and aliquots were incubated with GSH alone (3 mM), GSH + GPX (1.9 units/ml), or GSH + PHGPX (0.4 unit/ml). After 30 min at 37 °C, lipids were extracted and cholesterol products were analyzed by TLC. The samples were as follows: non-irradiated ghosts (lane b), photooxidized ghosts before (lane c) and after (lane d) borohydride reduction, photooxidized ghosts after incubation with GSH (lane e), GSH/GPX (lane f), or GSH/PHGPX (lane g). Standards were chromatographed alongside: lane a, cholesterol hydroperoxides from photooxidized egg PC/dioleoyl PE/cholesterol-containing liposomes; lane h, borohydride-reduced 7-ketocholesterol. The TLC plate was sprayed with TMPD to detect peroxides (A), followed by H2SO4, for overall detection of cholesterol and its reaction products (B). Solvent front (F); origin (O). Sample load (as starting cholesterol): ~40 μg/lane (lanes b-g).

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2 Unlike the experiments of Fig. 1, those of Fig. 2 were carried out in the absence of Triton X-100. Therefore, near quantitative reaction of PHGPX with LOOHs did not require dispersal of membrane lipids.
Enzymatic Detoxification of Membrane Lipid Hydroperoxides

Fig. 3. Radioscanning of residual cholesterol hydroperoxide at selected time points (A, TLC detection; B, determination by radioscanning). C, total lipid hydroperoxide (determined indirectly) was measured and compared with residual cholesterol hydroperoxide (determined by TLC/radioscanning). D, total lipid hydroperoxide (determined indirectly) was measured and compared with residual cholesterol hydroperoxide (determined by TLC/radioscanning). E, total lipid hydroperoxide (determined indirectly) was measured and compared with residual cholesterol hydroperoxide (determined by TLC/radioscanning). F, total lipid hydroperoxide (determined indirectly) was measured and compared with residual cholesterol hydroperoxide (determined by TLC/radioscanning).

Fig. 5. Kinetics of total lipid hydroperoxide loss versus cholesterol hydroperoxide loss during phospholipid hydroperoxide glutathione peroxidase treatment. Ghost membranes (1 mg of protein/ml) were photooxidized as described in Fig. 2, and then incubated at 37°C in the presence of PHGPX (0.15 unit/ml), 3 mM GSH, and 50 μM desferrioxamine. At various time points, samples were removed for iodometric determination of residual total LOOH and ChOOH. A, TLC profiles of cholesterol products after 1, 5, 15, and 30 min of incubation. Sample load (as starting cholesterol): ~40 μg (~2000 cpm). B, time course of total LOOH decay (Δ) and ChOOH decay (C). Also plotted is loss of total LOOH (V) and ChOOH (E) in reaction mixtures lacking PHGPX.
Enzymatic Detoxification of Membrane Lipid Hydroperoxides

Membrane lipid peroxidation is one of the most prominent forms of cellular damage induced by conditions of oxidative stress. Aerobic cells are equipped with a battery of defenses against the deleterious effects of lipid peroxidation. Primary defense is based on prevention of initiating reactions. This can be achieved by agents such as (a) enzyme scavengers of reactive oxygen species, e.g. superoxide dismutase, catalase, peroxidases, (b) chemical antioxidants, e.g., α-tocopherol, β-carotene, ascorbate, and (c) iron-sequestering proteins, e.g., apoferitin, apolactoferrin. A second mode of protection involves enzymatic removal of lipid-derived hydroperoxide intermediates. These reactions are typically catalysed by GSH requiring enzymes which fall into two classes: Se-dependent GSH-peroxidases and certain Se-independent enzymes, e.g. GSH-S-transferase B (28). This latter pathway can be considered as a back-up to the various primary lines of defense that involve iron inactivation or oxid/Fe/H2O2 scavenging.

Early studies on a thermolabile cytosolic factor capable of inhibiting microsomal lipid peroxidation in the presence of GSH suggested that this factor might be GPX (29). Inasmuch as GPX was known to catalyze the reduction of a wide range of hydroperoxides, including fatty acid hydroperoxides, it was inferred that direct reduction of membrane lipid hydroperoxides in situ might also take place. However, subsequent work clearly indicated that this could not be the case. For example, Grossman and Wendel (4) and Sevanian et al. (5) reported that phospholipid hydroperoxides in micelles or unilamellar liposomes are poor substrates for GPX unless first acted upon by PLAs. Similar observations were made in connection with GSH-S-transferase action on phospholipid hydroperoxides (30). The proposed mechanism in each case involved (i) PLAs-catalyzed hydrolysis of sn-2 fatty acyl hydroperoxide groups, (ii) "release" of the fatty acyl hydroperoxide, with subsequent reincorporation of a new unsaturated fatty acyl group, and (iii) GPX- or transferase-catalyzed reduction
of the hydroperoxide. More recent studies by Thomas and Girotti (7,8), using photooxidized erythrocyte membranes, indicated that phospholipid-derived hydroperoxides were completely eliminated by Ca²⁺/PLA₂ treatment followed by GSH/GPX. Thus, phospholipid-derived hydroperoxides were unaffected. Extracted cholesterol products (mainly 5α-OOH in 50 mM Tris·HCl, pH 7.4, 20% ethanol) were resistant to GSH/GPX, thereby ruling out physical inaccessibility in the membrane as a possible reason for nonreactivity. The only other known investigation of GPX activity on cholesterol hydroperoxides was that of Littke (31), who showed that free radical-derived products, e.g., 7β-OOH and 25-OOH, are also poor substrates for GPX, the reaction rates being less than 5% of the rate observed with H₂O₂ or linoleic acid hydroperoxide.

These findings prompted us to carry out the present study with PHGPX, the second selenium-requiring peroxidase to be isolated and characterized (9,10). Although PHGPX also contains an active site selenocysteine group, it differs from classical GPX in several respects (28), e.g., (i) relatively high molecular weight (~20 kDa for monomeric PHGPX versus ~85 kDa for tetrameric GPX), (ii) lack of absolute specificity for GSH as the reducing substrate, (iv) a broad specificity for hydroperoxides, including phospholipid hydroperoxides. PHGPX was first isolated from rat and porcine liver by Ursini et al. (9) and characterized in terms of its ability to inhibit free radical-mediated lipid peroxidation in phosphatidylcholine liposomes and microsomes.

Chromatographically distinct from any known GPX or GSH-S-transferase, the enzyme was provisionally termed a "Peroxidation Inhibiting Protein" (PIP) and later given its present designation, PHGPX. It is likely that the previously described cytosolic factor with peroxidation inhibiting properties (29) was, in fact, PHGPX (28). Maiorino et al. (14) reported that the GSH/PHGPX system, coupled with NADPH/glutathione reductase, could be employed for accurate determinations of membrane LOOH content. Using peroxidized microsomal membranes as a test system, they observed agreement between enzymatically determined LOOH values and values obtained by iron/thiocyanate assay. It was deduced, therefore, that all classes of membrane LOOHs were accessible and were reacting quantitatively with GSH/PHGPX. Since microsomes contain only small amounts of cholesterol (typically <10% of the total lipid weight), it was not clear from these and related studies (10,14) whether cholesterol hydroperoxides (in addition to phospholipid hydroperoxides) were substrates for PHGPX. Consequently, the present work has provided the first direct evidence for PHGPX-catalyzed reduction of cholesterol hydroperoxides in a biological membrane. Relatively little else of related interest has been done in this area, other than the one study already mentioned (31), an earlier report on the peroxidatic action of cytochrome P-450 on steroid hydroperoxides (32), and a more recent study dealing with the metabolism of 5α-OOH and 7α-OOH by Staphylococcus typhimurium (33). In the latter case, evidence was presented for a slow isomerization and/or reduction of the hydroperoxides, but the putative enzymes involved were not identified.

While PHGPX was capable of reacting with both cholesterol hydroperoxides and phospholipid hydroperoxides in the membranes studid (erythrocyte ghosts), the rates of these reactions were found to be significantly different. Thus, under the conditions described (cf. Fig. 5), cholesterol species were reduced at a first order rate which was only ~15% of that ascribed to phospholipid species. It is not clear at this point whether this difference is an intrinsic one (i.e. based on structural properties of the hydroperoxides per se) or whether other factors (e.g., substrate arrangement in the bilayer, interaction with other membrane elements) are more important. It should be noted that both classes of hydroperoxides disappeared completely after sufficiently long periods of exposure to GSH/PHGPX (cf. Figs. 2 and 4), indicating that there were no absolute permeability barriers to PHGPX, i.e. that LOOHs in both membrane leaflets were accessible to externally added enzyme. This was not unexpected, however, since the ghost membranes were known to be unsealed, i.e. leaky to macromolecules at least as large as hemoglobin. Similar results were reported earlier for GPX-catalyzed reduction of phospholipid-derived hydroperoxides in photooxidized, PLA₂-treated ghosts (7).

The major cholesterol photoproduct generated by rose bengal-sensitized photooxidation of erythrocyte membranes is the 5α-OH adduct, 5α-OOH (26,27). When generated in the presence of a free radical trap which prevented (or at least minimized) its isomerization to 7α-OOH, 5α-OOH was the principal steroid-based substrate for PHGPX (Fig. 3). Under these conditions, a clean conversion of 5α-OOH to 5α-OOH was observed, with no evidence of other diol products. In other experiments (cf. Fig. 4), allylic rearrangement was allowed to occur during irradiation and subsequent incubation steps. In these instances, GSH/PHGPX treatment produced significant amounts of 7α-OH (and traces of 7β-OOH epimer) in addition to 5α-OOH, indicating that membrane-bound 7α-OOH and 7β-OOH are also substrates. However, any kinetic differences in the reduction of the three different hydroperoxides remain to be elucidated.

The present findings add further support to the proposal of Ursini et al. (29) that PHGPX plays a unique role in protecting cells against the damaging effects of lipid peroxidation. We have shown that erythrocyte ghosts primed with LOOHs by dye-sensitized photooxidation produce large amounts of TBARS when exposed to ascorbate or xanthine/xanthine oxidase as a source of O₂. Earlier work with resealed ghosts (16,18) indicated that because of their propagative nature, these reactions cause far more lytic damage than photooxidation alone. One of the most significant findings of the present work is that pretreatment of LOOH-containing ghosts with GSH/PHGPX prevented ascorbate- or O₂-stimulated lipid peroxidation from occurring. Under similar reaction conditions, GSH/GPX had no effect. Even after PLA₂ treatment, GSH/GPX afforded only partial protection against peroxidation, the residual reaction being ascribed to reductive decomposition of cholesterol hydroperoxides (7). The relative superiority of PHGPX as a direct inhibitor of lipid peroxidation is clearly evident from the present study. These results are consistent with previous ones (9), which showed that GSH/PHGPX can totally inhibit NADPH/iron-ADP-driven lipid peroxidation in mitoplasts and microsomes. Thus, PHGPX is seen to be highly effective in protecting plasma membranes as well as subcellular membranes against the damaging effects of LOOH-mediated lipid oxidation.

By comparison with GPX, PHGPX is a relatively lipophilic enzyme. In the large number of tissues from which PHGPX has been isolated (28), significant amounts of its activity have been shown to be associated with subcellular membranes. This could explain its ability to act directly on membrane LOOHs, whereas GPX, having limited ability to interact with membranes, may be more important in removing cytosolic.
Enzymatic Detoxification of Membran Lipid Hydroperoxides

Hydroperoxides, e.g. H$_2$O$_2$ and certain fatty acid hydroperoxides. According to this idea, the functional significance of PHGPX versus GPX in any given tissue would depend on the nature of the incident oxidative stress and the hydroperoxides arising therefrom (28).

Although hydrolysis of phospholipid hydroperoxides by PLA$_2$ is not a prerequisite for PHGPX action, such hydrolysis may occur secondarily. Thus, it is reasonable to expect that in cellular systems, fatty acyl alcohols generated by PHGPX will be cleaved by PLA$_2$ as part of the repair process. Subsequent insertion of a new fatty acyl group into the lysolipid sn-2 position would regenerate the glycerophospholipid. This has been proposed as a more logical mechanism for damage prevention and repair than one involving consecutive action of PLA$_2$ and GPX (28). As the physiological role of PHGPX unfolds, it will be important to understand how the processes of reduction, excision, and reacylation are coordinated in tissues. It will be equally important to understand how the enzymatic reduction products of cholesterol hydroperoxides (diols) are metabolized. Although we have been primarily concerned with cell membranes in this work, in certain tissues removal of LOOHs from other cellular structures could be equally important, e.g. internalized lipoproteins in the vascular wall (34). In this regard, we have recently shown that the GSH/PHGPX system can readily reduce hydroperoxides of cholesterol, cholesteryl esters, and phospholipids in low density lipoproteins.

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Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides.

J P Thomas, M Maiorino, F Ursini and A W Girotti


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