THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 265, No. 1, Issue of January 5, pp. 454-461, 1990
Printed in U.S.A.

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

IN SITU REDUCTION OF PHOSPHOLIPID AND CHOLESTEROL HYDROPEROXIDES*

(Received for publication, July 25, 1989)

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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 5α (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]cholesterol-labeled ghosts. Kinetic analyses indicated that the rate of ChOOH decay was ~1/4 that of phospholipid hydroperoxide decay. Photooxidized 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).

ROOH + 2 GSH → ROH + GSSG + H2O

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 sn-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 acyl 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 photooxidized, 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 LOOHs.

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

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This work was supported in part by Grant DCB-8808227 from the National Science Foundation (to A. W. G.) and by a grant from the Ministry of Public Education (to F. U.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 catalyze the reduction of membrane phospholipid hydroperoxides in situ without the necessity for 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, dioloyl phosphatidylethanolamine, cholesterol, and 7-keto-cholesterol from Sigma, sodium ascorbate 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 AZ, and xanthine oxidase from butter-milk. Rose bengal was obtained from Allied Chemical Co. and purified according to Brand et al. (13).

Förnic 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, 2.5 mM 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 lipoxxygenase-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 any pre-existing oxidation products by TLC (see below), located by radioactivity, and recovered by scraping. Erythrocyte ghosts were exchanged radioactively by incubating with unlabelled [14C]cholesterol/egg PC liposomes (0.8:1, mol/mol), as described previously (15). After 45 h of incubation at 37 °C, the ghosts were washed extensively with PBS to remove the liposomes and resuspended to a final concentration of about 1.8 × 10⁸/ml (1 mg of protein/ml) for experimental use.

**Photoperoxidation 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 beaker, and irradiated at 10 °C with continuous stirring (16). Incident light from a mercury arc lamp was focused through a yellow filter (Corning CS-68) to select wavelengths maximally absorbed by membrane-bound rose bengal (λ max ~570 nm). Light intensity at the membrane suspension surface was ~100 W/m², measured as 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 at this point 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 GSSH-reductase. Samples of photoperoxidized ghosts in PBS were mixed with desferrioxamine (25-50 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 GSSH-reductase (1-2 units/ml), 0.1% Triton X-100, 5 mM EDTA, 3 mM GSH, 0.1 mM NADPH, and 0.1 mM Tris-HCl, pH 7.6, in a thermostatted spectrophotometer cell (37 °C) equipped with a magnetic stirrer, and the basal rate of A₄₅₀ 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 to the cuvette and the ensuing decrement in A₄₅₀ decay was monitored by the rate of formation of reactive LOOH (7). Absorbance readings were corrected for small contributions of the peroxides and for volume changes that occurred when the enzymes were added.

**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 GSSH/GPX or GSSH/PHGPX. After a given period of irradiation (typically 1 h), membrane samples (0.05-0.1 mg of total lipids) were mixed with 3 μl of 0.1 M 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 Quick-Vap 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 butane/ethyl acetate (9:1, 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 boronate reduction products of 7-ketocholesterol (7α- and 7β-OH) were chromatographed alongside as standards. Immediately after developing, plates were either sprayed with TMPO (20) to detect hydroperoxides (Rf 0.60), or scanned using an densitometer (Radiomatic Instruments, model RS) equipped with data processing accessories. Subsequent to TMPO treatment or radioscanning, the plates were sprayed with 50% H₂SO₄ and warmed briefly at 80 °C to visualize cholesterol itself (Rf -0.6), and the reduction products of cholesterol hydroperoxides (diols; Rf 0.19-0.26) (20). Although hydroperoxides could also be detected with H₂SO₄, the sensitivity was much lower than with TMPO. In some instances, plates were photographed immediately after spraying with TMPO or H₂SO₄.

General separation of membrane LOOHs, including those derived from phospholipids, was accomplished with the solvent system oehroform/methanol/water (75:25:4, v/v). Immediately after development, the plates were sprayed lightly with TMPO to visualize phospholipid hydroperoxides. Identification of individual product classes was based on comigration with known hydroperoxides obtained, e.g. by rose bengal-sensitized photooxidation of egg PC, dioleoyl PE, or cholesterol in liposomal form (8).  Thiobarbituric Acid Assay—The effect of GPX or PHGPX on ascorbate- or xanthine/xanthine oxidase-stimulated lipid peroxidation in photooxidized membranes was monitored by TBA assay (1, 2). This colorimetric assay detected malonaldehyde and other thio- barbituric acid-reactive substances (TBARS) generated by free radical-mediated peroxidation of unsaturated lipids (excepting cholesterol and monoenoic or dienoic phospholipids). Absorbance readings at 532 nm were converted to TBARS values (nmol/mg protein), using an extinction coefficient of 1.47 (mmol)⁻¹ cm⁻¹ (21).

**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 18O2-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 peroxidized ghosts in an enzymatic assay mixture (Fig. 1, trace A). (In this experiment, the small A340 decrement observed after the introduction of GPX is attributed primarily to dilution of the reaction mixture, with trace amounts of H2O2 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 A340 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 A340, indicating that the PHGPX was still active (data not shown). A second portion of the peroxidized membranes was treated with CaCl2/PLA2 before being analyzed. With this preparation, GPX produced a sizeable A340 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 ½ of that produced by GPX. This was seen consistently in all replicate determinations. It is important to note that the A340 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 PLA2; 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 μmol/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 μM rose bengal. After the addition of 25 μM desferrioxamine, the membranes were incubated for 15 min with 1 mM CaCl2 (A) or 1 mM CaCl2 plus PLA2 (20 units/ml) (B), and then reacted 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 μM rose bengal, mixed with 50 μM desferrioxamine, and then incubated in the dark at 37 °C with 3 mM GSH (●); GSH plus GPX, 1.1 unit/ml (△); or GSH plus PHGPX, 0.15 unit/ml (○). 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 detected with TMPD. A second portion of the peroxidized membranes was treated with CaCl2/PLA2 before being analyzed. With this preparation, GPX produced a sizeable A340 decrement (Fig. 1A) 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 PLA2; but even under these conditions, only ~60% of the LOOH population was removed by GPX.

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30 min of incubation, the LOOH had decayed to <5% of its starting value.\(^2\) 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 c). 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. 3A), 30 min incubation with GSH alone or GSH plus GPX caused little perceptible change in the spot intensity of each photoproducT. 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\(_2\) 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\(_2\) or GPX, or permeability barriers against the enzymes. Examination of TLC chromatograms clearly indicated that PLA\(_2\) 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\(_2\) 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 membrane 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) just 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.

FIG. 3. Enzymatic reduction of cholesterol hydroperoxides in erythrocyte membranes. A suspension of ghosts (1 mg of protein/ml) containing 5 \(\mu\)M rose bengal and 25 \(\mu\)M butylated hydroxytoluene was irradiated for 1 h. The peroxidized membranes were then mixed with 50 \(\mu\)M 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 \(H_2SO_4\) for overall detection of cholesterol and its reaction products (B). Solvent front (F); origin (O). Sample load (as starting cholesterol): ~40 \(\mu\)g/lane (lanes b-g).

\(^2\) 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.
ghost lipids converted cholesterol hydroperoxide to 5α-cholesten-6-one-3β,5-diol (5α-OH), which appeared as a blue spot (RF = 0.26) on the H2SO4-treated plate (Fig. 3B, cf. lanes c and d). As can be seen, 5α-OH migrated more slowly than parent cholesterol (RF ~ 0.62) or its hydroperoxide (RF ~ 0.37), but more rapidly than the reduction products of 7-ketocolesterol (lane h), cholest-5-en-3β,7α-diol (7α-OH, RF ~ 0.15) and cholest-5-en-3β,7α-diol (7β-OH; RF ~ 0.20). The immediate precursor of 5α-OH, 3β-hydroxy-5α-cholesten-6-ene-5-hydroperoxide (5α-OOH), is a characteristic product of singlet oxygen (1O2) attack on cholesterol that can be used as an unequivocal indicator of 1O2 intermediacy (24, 25). In addition to rose bengal, several other photosensitizing agents have been shown to be good 1O2 generators in ghost membranes by virtue of 5α-OOH formation (15, 26, 27). It should be pointed out that the photoperoxidation reaction described in Fig. 3 was carried out in the presence of an antioxidant (butylated hydroxytoluene) to prevent (or at least minimize) any formation of radical-derived products, e.g. 7α-7β-OOH.

To enhance the sensitivity of cholesterol product detection and to allow quantitation as well, we used membranes that were radiolabeled with [14C]cholesterol. Prior to dye-sensitized photooxidation, cholesterol itself was the only detectable radioactive species (Fig. 4, lane A). Immediately after irradiation, a peak corresponding to [14C]cholesterol hydroperoxide was observed (10% of the total radioactivity), with lesser amounts of 7α-OH and 5α-OH (~5% collectively). As in the experiment shown in Fig. 3 (cf. lanes c, e, and f), there was a relatively little change in this profile after 30 min of dark incubation in the presence of desferrioxamine plus GSH (lane B) or desferrioxamine plus GSH/PHGPX (lane E). An anticipated, however, GSH/PHGPX treatment (lane D) caused a large decrease in the peroxide peak similar to that observed with borohydride treatment (lane C). Correspondingly, the amounts of 7α-OH and 5α-OH increased substantially in each case. Relatively little 7β-OH was seen. In comparing the results of Fig. 3 with those of Fig. 4, one should note that butylated hydroxytoluene was added before irradiation in the former case, but not in the latter. Previous studies (27) have shown that butylated hydroxytoluene inhibits free radical-mediated formation of 7α-/7β-OOH from cholesterol and also the slow allylic rearrangement of 5α-OOH to 7α-OOH that may accompany 1O2-mediated photooxidation. The inferred presence of significant 7α-OOH in the product profile shown in Fig. 4 (cf. lanes B and C) is attributed primarily to allylic rearrangement of 5α-OOH. It is apparent from Fig. 4 that PHGPX can reduce not only membrane-bound 5α-OOH but also 7α-OOH. Any kinetic differences in these reactions remain to be determined.

Additional insights into LOOH removal by PHGPX were gained by comparing the time course of cholesterol hydroperoxide decay with that of the overall lipid population. Fig. 5 shows the results of such an experiment in which the reduction of [14C]cholesterol hydroperoxide (determined by TLC/radioscanning) and total lipid hydroperoxide (determined iodometrically) are tracked in a common membrane system. Radionuclides of residual cholesterol hydroperoxide at selected time points are shown in Fig. 6A. Note that in this experiment (similarly to the one described in Fig. 4), peroxide disappearance was accompanied by the formation of increasing amounts of 7α-OOH as well as 5α-OOH. As indicated above, partial rearrangement of 5α-OOH to 7α-OOH (during irradiation as well as enzyme treatment) probably accounts for the appearance of 7α-OOH. As shown in Fig. 5B, cholesterol hydroperoxide decayed in apparent first order fashion (k ~ 2.8 h⁻¹) over a 30-min period of incubation with GSH/PHGPX. By contrast, total LOOH decay was biphasic, with an initial rapid decay, with subsequent slow decay. The 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 for determination of ChOOH by TLC and radioscanning. 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.

Fig. 5. Kinetics of total lipid hydroperoxide loss versus cholesterol hydroperoxide loss during phospholipid hydroperoxide glutathione peroxidase treatment.

Fig. 4. Reactivity of cholesterol hydroperoxides with glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase. Ghost membranes (1 mg of protein/ml) containing [14C]cholesterol were photoperoxidized as described in Fig. 2, mixed with 50 μM desferrioxamine, and dark incubated with 2.7 mM GSH (B), GSH plus PHGPX, 0.4 unit/ml (D), GSH plus GPX, 1.9 unit/ml (E), or treated with borohydride (C). A non-irradiated control (A) was analyzed alongside. Sample load (as starting cholesterol): ~40 μg/lane (~2000 cpm).
**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 catalyzed 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 oxidative 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 PLAP. Similar observations were made in connection with GSH-S-transferase action on phospholipid hydroperoxides (30). The proposed mechanism in each case involved (i) PLAP-catalyzed hydrolysis of sn-2 fatty acyl hydroperoxide groups, (ii) "release" of the fatty acid hydroperoxide, with subsequent reincorporation of a new unsaturated fatty acyl group, and (iii) GPX- or transferase-catalyzed reduction

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**Fig. 6. Inhibitory action of phospholipid hydroperoxide glutathione peroxidase on post-irradiation lipid peroxidation.**

A, ascorbate/iron system. Photoperoxidized ghosts (cf. Fig. 2), 1 mg of protein/ml, were incubated in the dark at 37 °C with 0.5 mM ascorbate, 0.05 mM FeCl₃ after a 30 min preincubation in the presence of the following additives: none (C); 3 mM GSH (Δ); 3 mM GSH plus PHGPX, 0.3 unit/ml (C). A non-irradiated control was also treated with ascorbate/iron (●). B, xanthine/xanthine oxidase/iron system. Photoperoxidized ghosts were incubated for 30 min with 3 mM GSH alone (○) or 3 mM GSH plus PHGPX (0.3 unit/ml, •), washed to remove residual GSH, and then treated with xanthine oxidase (0.01 unit/ml), 1 mM xanthine, and 0.05 mM FeCl₃ at 37 °C. Other incubations with xanthine/xanthine oxidase/iron were as follows: photoperoxidized ghosts (non-PHGPX-treated) plus superoxide dismutase (25 µg/ml, ▲); non-photoperoxidized ghosts (●). At the indicated times, samples from each reaction mixture were removed for determination of TBARS (thiobarbituric acid reactive substances). Points with error bars are means ± deviation of values from duplicate determinations.

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**DISCUSSION**

The rapid loss of total LOOH depicted in Fig. 2B is ascribed primarily to phospholipid hydroperoxides and the slow reaction approximated that of [¹⁴C]cholesterol hydroperoxide disappearance (see above).

Enzymatic Protection against Free Radical-mediated Lipid Peroxidation—Earlier studies have shown that photoperoxidized ghosts undergo a large burst of free radical-mediated lipid peroxidation (as detected by TBARS formation) when exposed to ascorbate and ferric iron (15, 16, 18, 27). A similar effect was observed when the membranes were treated with xanthine and xanthine oxidase in the presence of iron. Non-photooxidized membranes showed essentially no reaction with ascorbate/iron and relatively little reaction with xanthine/xanthine oxidase/iron. In either system, stimulation of lipid peroxidation was attributed to LOOH-dependent initiation, i.e., ferrous iron-induced reduction of LOOHs to lipid oxyl radicals (LO·), which trigger chain reactions via H abstraction (1). Classical GPX had no effect on these reactions unless the membranes were first treated with Ca²⁺/PLA₂. While this treatment alone had a sizeable inhibitory effect, the combination of Ca²⁺/PLA₂ and GSH/GPX was even more (but not completely) inhibitory. TLC analyses indicated that liberated fatty acid hydroperoxides were totally reduced by GPX, leaving cholesterol hydroperoxides as the only probable initiating species. With this information at hand, it was of obvious interest to determine how PHGPX would affect these reactions.

As shown in Fig. 6A, incubation of photoperoxidized ghosts with 0.5 mM ascorbate and 0.05 mM FeCl₃ resulted in a strong surge of lipid peroxidation (TBARS formation) which leveled off at ~11 nmol/mg protein after 15 min. Little peroxidation (if any) was seen with non-photooxidized ghosts. Low concentrations of butylated hydroxytoluene (e.g., 25 µM) inhibited the ascorbate/iron-stimulated reaction (data not shown), indicating that it was free radical-mediated. The small zero-time level of TBARS (~1 nmol/mg protein) represents partial degradation of starting photoperoxides that occurred during the TBA assay (18). Significantly, preincubation of the photooxidized membranes with GSH/PHGPX resulted in essentially total inhibition of the ascorbate/iron-stimulated lipid peroxidation, i.e., TBARS formation was little different from that seen in the dark control. GSH alone had relatively little effect, if any. Prevention of peroxidation by GSH/PHGPX is consistent with the fact that all measurable initiating LOOIs, including cholesterol hydroperoxides, were removed by GSH/PHGPX treatment (Fig. 2B). Similar results were obtained in the case of xanthine/xanthine oxidase-driven lipid peroxidation (Fig. 6B). Thus, when photoperoxidized ghosts were incubated with xanthine, xanthine oxidase, and FeCl₃, they underwent a burst of O₂⁻-dependent (superoxide dismutase-inhibitable) lipid peroxidation, which was almost totally nullified by prior treatment with GSH/PHGPX (Fig. 6B). The small residual reaction after such treatment was identical in magnitude to the reaction of the non-irradiated control.

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**FIG. 6.** Inhibitory action of phospholipid hydroperoxide glutathione peroxidase on post-irradiation lipid peroxidation.
of the hydroperoxide. More recent studies by Thomas and Girotti (7, 8), using photoperoxidized erythrocyte membranes, indicated that phospholipid-derived hydroperoxides were completely eliminated by Ca\(^{2+}\)/PLA\(_2\) treatment followed by GSH/GPX. Phospholipid hydroperoxides were unaffected. Extracted cholesterol products (mainly 5α-OOH in 50 mM Tris-HCl, pH 7.4, 30% ethanol) were resistant to GSH/GPX, thereby ruling out physical inaccessibility in the membrane as a possible reason for nonreactivity. The only other known involvement of GPX activity on cholesterol hydroperoxides was that of Little (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 H\(_2\)O 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, (iii) 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 excellent 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 to 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 studied (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 unssealed, 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, PLA\(_2\)-treated ghosts (7).

The major cholesterol photoprodut generated by rose bengal-sensitized photooxidation of erythrocyte membranes is the 5α OO 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 diole 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-OOH (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. (28) 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 O\(_2\). 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 O\(_2\)-stimulated lipid peroxidation from occurring. Under similar reaction conditions, GSH/GPX had no effect. Even after PLA\(_2\) 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 Membrane 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 PLAr 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.

Acknowledgment—The technical assistance of Peter Geiger is appreciated.

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

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J. Biol. Chem. 1990, 265:454-461.

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