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REACTIVITY OF PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE WITH MEMBRANE AND LIPOPROTEIN LIPID HYDROPEROXIDES

MATILDE MAIORINO, JAMES P. THOMAS[§], ALBERT W. GIROTTI[§] and FULVIO URSINI

Department of Biological Chemistry, Univesity of Padova, Italy and
[§] Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA

A comparative study has been carried out on the general reactivity of lipid hydroperoxides in liposomes, biological membranes and lipoproteins with two Se-dependent peroxidases: Glutathione Peroxidase (GPX) and Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPX). While PHGPX reduces all hydroperoxides derived from phospholipids, cholesterol and cholesterol esters, GPX reduces only fatty acid hydroperoxides released after treatment of phospholipid hydroperoxides with phospholipase A_2. These findings highlight the role of PHGPX in protecting biomembranes from peroxidative damage and add new insight into how cholesterol hydroperoxides are detossified in cells.

KEY WORDS: Lipid hydroperoxides, glutathione, Se-peroxidases.

INTRODUCTION

Aerobic cells protect themselves from peroxidative damage by: i) primary antioxidants that prevent the formation of the initiating species – iron chelating agents, superoxide dismutase, catalase, glutathione peroxidase (GPX) – and ii) chain breaking or secondary antioxidants (e.g. vitamin E), that trap the chain propagating lipid hydroperoxyl radicals ($LOO^{\bullet}$) and thereby reduce the length of the peroxidative chain. Microsomal lipid peroxidation induced by NADPH or by ascorbate/Fe^{3+} ADP has shown to be inhibited very efficiently by vitamin E and the selenoenzyme Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPX).[1-3] PHGPX is a membrane bound, approx. 20 kDa enzyme that has been purified from different rat and pig organs.[2,4] It exhibits a peroxidase activity on phospholipid hydroperoxides (PLOOH) which are totally resistant to the 85 kDA cytosolic GPX.[5]

To learn whether also cholesterol and cholesterol ester hydroperoxides might also be substrate for PHGPX and/or GPX we measured the reactivities of both enzymes on photooxidized dimyristoylphosphatidylcholine-cholesterol (DMPC-CHOL) liposomes, photooxidized erytrocyte ghosts and peroxidized low density lipoproteins (LDL).

Please send correspondence and proofs to: Matilde Maiorino, Department of Biological Chemistry, University of Padova, Italy.

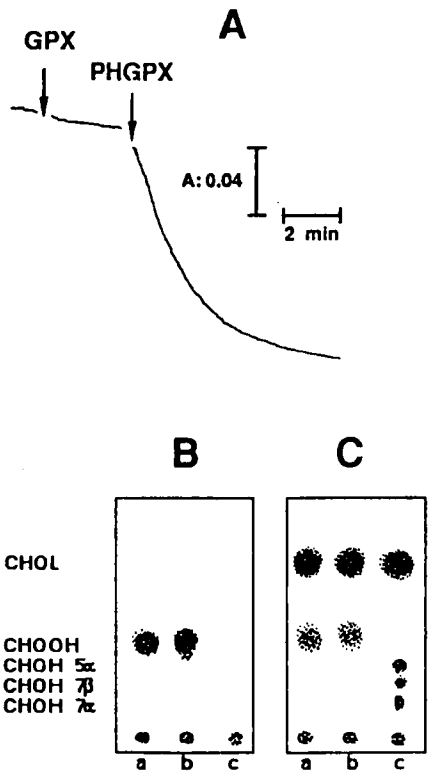

FIGURE 1 GPX and PHGPX activities on dimyreistoylphosphatidylcholine-cholesterol liposomes (DMPC-CHOL) photooxidized with rose bengal. Panel A: Liposomes (DMPC-CHOL 1–0.8 mol-mol, 1 mg/ml total lipids), were irradiated for 1 hr in the presence fo 5 μM rose bengal. After the addition of 50 μM desferrioxamine, sample of photooxidized liposomes (0.05–0.1 mg total lipids/ml) were transferred to a spectrophotometer cell at 37°C, containing in 2.5 ml: 0.1 M Tris-HCl pH 7.4, 5 mM EDTA, 3 mM GSH, 0.1 mM NADPH 2 U/ml GSSG reductase, 0.1% Triton X-100. Absorbance at 340 nm was recorded. GPX (0.5 U/ml) and PHGPX (0.2 U/ml) were added where indicated. Panels B and C: after 30 min. incubation with GSH (3 mM), or GSH (3 mM) + PHGPX (0.2 U/ml), liposomes, photooxidized as above, were extracted with chloroform/methanol (2/1) for TLC analysis on Silica-Gel 60. TLC was performed using a single irrigation of heptane/ethyl acetate (1/1). The plate was sprayed with TMPD (Panel B) to detect hydroperoxides, followed by 50% H_2SO_4 (Panel C) to detect cholesterol and its reduction products. In this system, phospholipids remain at the origin and cholesterol (CHOL, Rf 0.6) is separated from cholesterol peroxides (CHOOH, Rf 0.34–0.37) and the related reduction products (CHOH, Rf 0.19–0.26). Sample are as follows: lane A: photooxidized liposomes; Lane B: photooxidized liposomes + GSH; lane C: photooxidized liposomes + GSH + PHGPX.

RESULTS AND DISCUSSION

Figure 1 shows that photochemically oxidized DMPC-CHOL liposomes were reactive with PHGPX, but not with GPX. In Panel A the activities of the two enzymes are compared in coupled spectrophotometric assay with GSH, NADPH and GSSG reductase. That the activity of PHGPX shown in Panel A (A_{340} decrement), is due to the presence of cholesterol hydroperoxides (CHOOH) was confirmed by thin layer chromatographic (TLC) analysis (Panels B and C). TLC of lipids extracted after

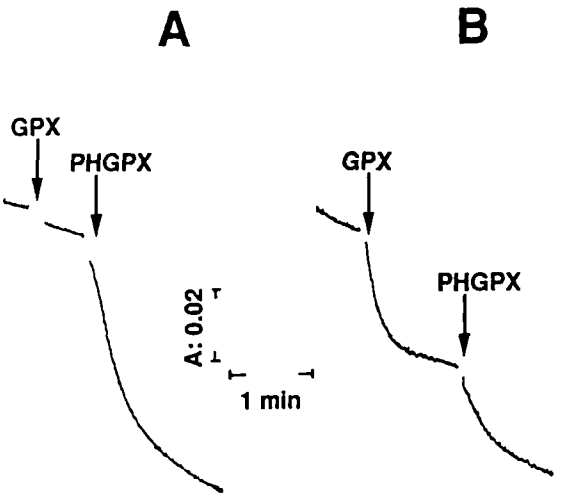

FIGURE 2 Reduction of eritrocyte ghost membrane lipid hydroperoxides. Human erithrocyte ghosts (2 mg protein/ml) were photooxidized with 5 μM rose bengal and tested in spectrophotometric assay under the conditions described in Figure 1 except that 25 μM butylated hydroxytoluene was added during photooxidation to prevent free radical mediated peroxide degradation. Prior to being analyzed, the membranes were incubated for 15 min. without (Trace A) or with (Trace B) PLA_2 (20 U/ml). Both samples contained 1 mM $CaCl_2$.

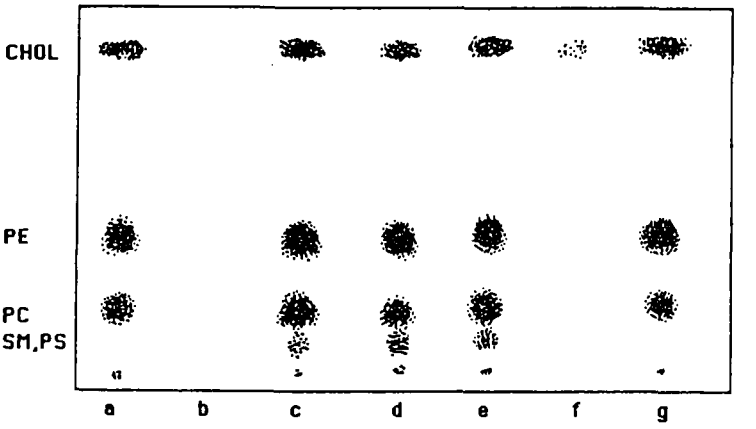

FIGURE 3 TLC visualization of lipid hydroperoxide loss in eritrocyte ghost membranes after enzymatic reduction. Erithrocyte ghosts were photooxidized as described in Figure 2. The membranes were mixed with 50 μM desferrioxamine and aliquots were incubated with GSH, GSH + GPX, or GSH + PHGPX. GSH was 3 mM, GPX 1.9 U/ml and PHGPX 0.4 U/ml. After 30 min at 37°C lipids were extracted and separated by TLC, using cholorform/methanol/water (75/25/4) as solvent system. Visualization of hydroperoxides was accomplished with TMPD. Photooxidized sample are shown immediately after irradiation (lane c); after 30 min of dark incubation in the presence of GSH (lane d); GSH + GPX (lane e); and GSH + PHGPX (lane f); lane b represents a non irradiated control. Lanes a and g show a mixture of photooxidized standards. Sample loads (as total lipid): 0.15 mg (lanes b–f) 0.45 mg (lanes a and g).

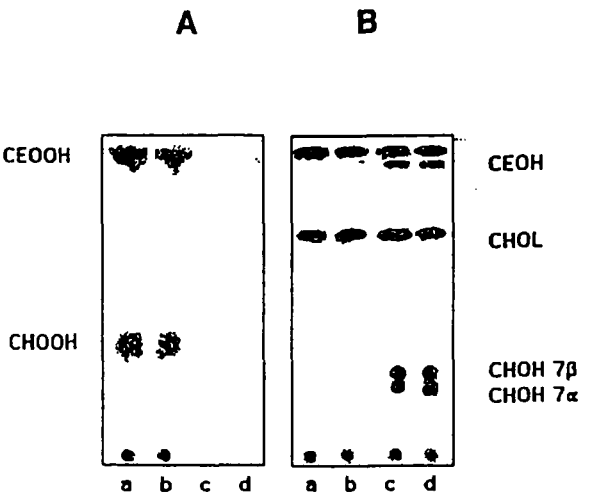

FIGURE 4 Susceptibility of peroxidized cholesterol and cholesterol esters in low density lipoproteins (LDL) to PHGPX catalyzed reduction. LDL (2 mg cholesterol/ml) were peroxidized in the presence of 10 mM 2'2' Azobis 2-amidinopropane (a diazo compund that generates free radicals at constant rate) at 40°C in PBS and oxygen consumption followed polarographically. when all oxygen was consumed, 5 mM EDTA was added and sample immediately incubated at 37°C for 30 min with 3 mM GSH or 3 mM GSH + 0.4 U/ml PHGPX. After lipid extraction TLC was carried out as indicated under Figure 1. 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). Sample load: approx. 0.15 mg total cholesterol per lane. Samples are as follows: lane a: peroxidized LDL; lane b: peroxidized LDL + GSH; lane c: peroxidized LDL + GSH + PHGPX; lane d: borohydride reduced, peroxidized LDL.

30 min incubation with GSH alone or GSH plus PHGPX allowed to distinguish among cholesterol (CHOL), its hydroperoxide(s) (CHOOH) and their reduction products (CHOH). Tetramethyl-p-phenylenediamine (TMPD)-reactive peroxides are not seen in lane C, where PHGPX/GSH were present during incubation (Panel B). The complete disappearance of peroxides under these conditions is accompanied by the appearance of three CHOH isomers spots (Panel C, lane C). In the direction of increasing Rf these represent 7α, 7β, and 5α-hydroxycholesterol.[6]

PHGPX also catalyzes the direct reduction of lipid hydroperoxides in physiological structures (membranes and lipoproteins) as shown by experiments with erythrocite ghosts (Figures 2 and 3) and LDL (Figure 4).

Coupled enzymatic assay indicated that peroxides in photooxidized ghosts were essentially non-reactive with GPX (Figure 2, trace A). By contrast, PHGPX produced a rapid decay of A_{340}. As anticipated,[7] after ghost phospholipids were hydrolyzed by calcium-activated phospholipase A_2, GPX also produced a decrement in A_{340}, albeit smaller than that produced by PHGPX (Figure 2, trace B). The reaction with GPX was apparently due to the release of fatty acids hydroperoxides, which were more accessible to the enzyme.[5,8] Subsequent addition of PHGPX resulted in a further decrement, the magnitude of which is approximately 2/3 of that produced by GPX. It was shown that the latter reaction represents CHOOH reduction. The complete reduction by PHGPX of all hydroperoxides in ghost membranes is confirmed: i) by

iodometric titration (not shown), ii) by TLC of extracted lipids that allows to scrutinize PLOOH and CHOOH (Figure 3).

Additional studies were carried out on peroxidized LDL. As shown in Figure 4 (Panel A), hydroperoxides of cholesterol (CHOOH) and cholesterol esters (CEOOH) disappeared upon incubation with GSH/PHGPX. The reaction products generated in this reaction (CHOH and CEOH) had R_f values identical to those obtained upon borohydride reduction of oxidized LDL (Figure 4, Panel B).

These findings provide additional strong support for the previous contention[1] that PHGPX is more important than GPX in protecting biomembranes against lipid peroxidation damage. The results are in agreement with the kinetic analysis of GPX and PHGPX, suggesting that each of these enzymes plays a unique physiological role, GPX acting on relatively polar hydroperoxides in the cytosol (e.g. H_2O_2), and PHGPX on lipid hydroperoxides in membrane compartment.[9] The fact that GPX reacts with PLOOH after hydrolysis leaves open the question as to whether this sequence is important physiologically, and, if so, how repair is accomplished. For GPX, the protection/repair mechanism would involve hydrolysis, reduction, and then reacylation, whereas with PHGPX direct in situ reduction takes place, followed by hydrolysis and reacylation. Further studies are needed to establish the true working mechanism *in vivo*, but since lipid hydroperoxides are easily converted to reactive radicals in the presence of redox metals, direct reduction by PHGPX would be a definite advantage. Also peroxidized membranes may contain CHOOH, the reduction of which can only be catalyzed by PHGPX.

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