Metabolism of hydroperoxy-phospholipids in human hepatoma HepG2 cells

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Abstract Two enzymatic mechanisms have been proposed for the metabolism of hydroperoxy-phospholipids: *i*) the combined action of phospholipase A_2 and glutathione peroxidase, and/or *ii*) direct enzymatic reduction. The latter reaction may be catalyzed by selenium-dependent phospholipid hydroperoxide glutathione peroxidase and/or by glutathione S-transferase α. To study the pathway of this reaction, we used human hepatoma HepG2 cells into which was incorporated labeled, hydroperoxy-phospholipids. The major product of incorporated 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine was the corresponding hydroxy-phospholipid with no hydroxy- or hydroperoxy-fatty acids. The contributions to reduction of hydroperoxy-phospholipids in HepG2 cells from glutathione S-transferase A1 and phospholipid hydroperoxide glutathione peroxidase were calculated to be 0.5% and 99.5%, respectively. Increasing selenium in the cell culture medium led to increases in selenium-dependent phospholipid hydroperoxide glutathione peroxidase activity but not in glutathione S-transferase α. This increase in the selenium-dependent enzyme was paralleled by a concomitant increase in the extent of reduction of the incorporated hydroperoxy-phospholipid. We conclude that the main metabolic fate of hydroperoxy-phospholipids in HepG2 cells is by direct reduction to hydroxy-phospholipids by phospholipid hydroperoxide glutathione peroxidase but also by glutathione S-transferase α, and that phospholipase A_2/selenium-dependent glutathione peroxidase does not play a significant role in the reduction.—**Bao, Y., and G. Williamson.** Metabolism of hydroperoxy-phospholipids in human hepatoma HepG2 cells. *J. Lipid Res.* 1996. **37:** 2351–2360.

Supplementary key words phospholipid hydroperoxide glutathione peroxidase • glutathione S-transferase • lipid peroxidation • selenium • HepG2 cells • phospholipase A_2 • phospholipid • membrane

Oxidized phospholipids are generated in cell membranes by specific iron-dependent intracellular lipoxygenases and cyclooxygenases (1), by extracellular signals such as phorbol esters (2), and by toxins, hypoxia or ischaemia (3, 4). The resulting hydroperoxy-phospholipids contribute to cellular peroxide levels that control synthesis of platelet thromboxane and arterial wall prostacyclin, and also further activate lipoxygenases (5). Fatty acid hydroperoxides, especially of arachidonic acid, are released from phospholipids and form the initial metabolites in pathways of leukotriene, prostaglandin, and thromboxane synthesis from arachidonic acid, as part of the inflammatory response to various cytokines (6). Estimates of the steady state concentration of oxidized phospholipid (mainly as hydroperoxy-phosphatidylcholine) in cells are not known, but in human plasma the levels show inter-individual variation, with values in the range of 4–24 nM (7); these are increased on oxidative stress (8). This concentration is sufficient for activation of prostaglandin synthase (9). Further, the decomposition products of hydroperoxy-phospholipids, malondialdehyde and 4-hydroxynonenal, modify the structure and correct functioning of DNA and protein (10, 11).

Fatty acid hydroperoxides are metabolized by selenium-dependent glutathione peroxidase (Se-GPx, glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) (12), by glutathione S-transferase (GST, RX: glutathione R-transferase EC 2.5.1.18) (13) α (primarily A1 and A2 in humans), by the hydroperoxidase component of prostaglandin synthase (9), and by a hemoprotein fatty acid lyase (14). These enzymes may therefore provide some protection against propagation of lipid peroxidation generated by oxidative stress. However, the fate of hydroperoxy-phospholipids is not clear, and two possible mechanisms have been proposed. The first is the release of oxidized fatty acids from membrane hydroperoxy-phospholipids by phospholipase A_2, fol-

Abbreviations: PHGPx, phospholipid hydroperoxide glutathione peroxidase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; PLA_2, phospholipase A_2; PLPC, 1-palmitoyl-2-linoleoyl-L-3-phosphatidylcholine; PLPC-OOH, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; PLPC-OH, 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; La-OOH, linoleic acid hydroperoxide; La-OH, linoleic acid hydroxide.

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lowed by reduction of the liberated hydroperoxy fatty acid to hydroxy fatty acid by either Se-GPx or by GST α (13, 15, 16). The second is a direct reduction of phospholipid hydroperoxides to their corresponding hydroxy-derivatives without the action of phospholipase A_2 (PLA_2).

$$\text{PC-OOH} \xrightarrow[Ca^{2+}]{PLA_2} \text{La-OOH} + \text{lyso-PC} \quad 1)$$

$$\text{La-OOH} + 2\ \text{GSH} \xrightarrow[\text{or GST } \alpha]{\text{Se-GPx}} \text{La-OH} + \text{GSSG} + H_2O$$

$$\text{PC-OOH} + 2\ \text{GSH} \xrightarrow[\text{and GST } \alpha]{\text{PHGPx}} \text{PC-OH} + \text{GSSG} + H_2O \quad 2)$$

where PC is a phospholipid, and La is a fatty acid. The phospholipase A_2/GPx mechanism is supported by studies on oxidized phospholipid membranes in vitro. The specific activity of phospholipase A_2 on such membranes is increased relative to the action on unoxidized phospholipids (17). However, it is not clear whether the concentration of hydroperoxy-phospholipid in a cellular membrane would be sufficient to activate phospholipase A_2, as the effects appear to be dependent on membrane molecular order. On the other hand, agents that mediate the inflammatory response such as tumor necrosis factor and interleukin-1 (18), oxidative stress such as vitamin E deficiency, and agents that increase intracellular calcium (19) also activate phospholipase A_2, whereas ascorbate decreases it (20). Mechanism 2 could be catalyzed by either selenium-dependent phospholipid hydroperoxide glutathione peroxidase (Se-PHGPx, gene product of *GPX4*) (21–23) or by certain isoforms of GST, especially α in the liver but also "5.8" in other tissues (24).

Detailed studies have been reported on the comparison between human hepatoma HepG2 cells and normal liver parenchymal cells. Both cell types secrete similar levels of at least 17 major human plasma proteins (25), have similar levels and inducibilities of many xenobiotic-metabolizing enzymes (26), are morphologically comparable (27), are very similar in membrane lipid composition (28), and exhibit similar fatty acid transport characteristics (29). Therefore, this cell line serves as an appropriate and reliable model to study the metabolism of fatty acids and phospholipids (25, 28). The levels of two selenium-dependent glutathione peroxidases in HepG2 cells, Se-GPx and Se-PHGPx, can be manipulated by selenium supplementation (30), and we have used this as a tool to test the involvement of these enzymes, and of GST, in metabolism of oxidized phospholipids. In this study, we used a ^{14}C-labeled hydroperoxy-phospholipid incorporated into HepG2 cells in selenium-supplemented and normal medium to investigate the metabolism of hydroperoxy-phospholipids, and we conclude that the main product is hydroxy-phospholipid, and that the reaction is catalyzed mainly by Se-PHGPx.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-linoleoyl-L-3-phosphatidylcholine (PLPC), soybean lipoxidase (EC 1.13.11.12, type IV), dithiothreitol, phenylmethylsufonyl fluoride, choline chloride, sodium selenite, reduced GSH and Triton X-100 (peroxide and carbonyl free), insulin, and transferrin were purchased from Sigma Chemical, Poole, Dorset, UK. 1-Palmitoyl-2-[1-^{14}C]linoleoyl-L-3-phosphatidylcholine was from Amersham International plc, Bucks., UK. Methanol and acetonitrile, used for HPLC, were filtered and degassed. Monoflow 4 scintillant was from National Diagnostics, Manville, NJ. Human HepG2 hepatoma cell line was from European Collection of Animal Cell Cultures (UK). All other chemicals were analytical grade.

Preparation of PLPC-OOH and PLPC-OH

PLPC-OOH was prepared from PLPC using lipoxidase as described by Maiorino, Gregolin, and Ursini (31). The reaction mixture contained 0.2 M sodium borate (pH 9), 3 mM sodium deoxycholate, and 0.3 mM PLPC. The reaction was started by the addition of 0.05 mg/ml soybean lipoxidase and stirred at room temperature for 20 min. The resulting PLPC-OOH was separated from oxidized phospholipid by a PepRPC H5/5 column with a gradient of water (100%) to methanol (100%) in 10 min. PLPC-OOH was eluted with 100% methanol, and the central area of the peak was collected. The concentration of the hydroperoxide solution was determined by absorbance at 232 nm (ε = 25,000 $M^{-1}cm^{-1}$). The ^{14}C-labeled PLPC was diluted with unlabeled PLPC to a radioactivity of 4 μCi/μmol and the ^{14}C-labeled PLPC-OOH was prepared by the same method for unlabeled PLPC-OOH as described above. The efficiency of ^{14}C counting was 80% and was determined relative to a standard curve. PLPC-OH was prepared by $NaBH_4$ reduction of PLPC-OOH (32) and subsequent purification by Sep-Pak C18 cartridge (Waters Associates, MA). The identities of PLPC-OOH and PLPC-OH were confirmed by fast atom bombardment mass spectrometry (33). No molecular ions for PLPC-OH were seen in the preparation of PLPC-OOH. As determined by HPLC, the purity of PLPC-OH was >99.2%. This was based on injections of 500 pmol of PLPC-OH with a detection limit of <4 pmol under the described conditions.

Human HepG2 hepatoma cell culture

HepG2 cells were routinely grown in Eagle's minimal essential medium with L-glutamine, nonessential amino acids, and 10% fetal calf serum (FCS) at 37°C, 5% CO_2 in air. The medium was changed twice a week and confluent cells were sub-cultured weekly at a split ratio of 1:6 by treatment with 0.25% trypsin-EDTA. Normal HepG2 cells were grown in 10% FCS medium that contained endogenous selenium at ≤12.8 nM. One population of cells were made selenium-deficient (Se(−) cells) by gradual weaning from 10% FCS to 2% FCS in Eagle's minimal essential medium containing insulin (10 μg/ml) and transferrin (5 μg/ml). Se(+) cells were grown in 10% FCS with added selenium in the form of sodium selenite (100 nM) for 24 h. The cells were harvested using trypsin-EDTA; cell extracts for measurement of enzymatic activities were obtained by sonication in 0.1% digitonin in 0.1 M Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

Incorporation of PLPC-OOH to membranes

There is one report about the incorporation of hydroperoxy-phospholipids into cells (34). When we examined the stability of hydroperoxy-phospholipids in the cell culture medium reported in that paper (10% FCS in Eagle's minimum essential medium), the hydroperoxy-phospholipid was unstable and was broken down very rapidly. In our experiments using the conditions described (in ref. 34), only 3.0 ± 0.2% (n = 3) hydroperoxy-phospholipid was left after a 2 h incubation in 37°C with 5% CO_2. Further, no PLPC-OH was detected in the medium. A mixture of PLPC-OOH with 10% FCS in Eagle's minimum essential medium in the absence of cells led to breakdown of PLPC-OOH at a comparable rate as in the presence of cells. Fetal calf serum was responsible for the observed breakdown of PLPC-OOH, so that a modified serum-free medium containing 0.3% bovine serum albumin was used for the incorporation of hydroperoxy-phospholipids for a short period of time. PLPC-OOH (50 μM) was relatively stable in the modified medium: 89.7 ± 2.1% (n = 3) PLPC-OOH remained after 2 h, and 10.3 ± 2.1% (n = 3) was reduced to PLPC-OH. After 4 h, 80.3 ± 4.1% (n = 3) of the initial PLPC-OOH remained. When the cells were almost confluent, they were incubated with the serum-free Eagle's minimum essential medium containing 0.3% (w/v) bovine serum albumin and PLPC-OOH (5–100 μM in methanol) at 37°C with 5% CO_2 for the desired time. The final methanol concentration was less than 0.4%, and cells added with methanol were cultured as a control. Total cellular lipids were extracted using chloroform–methanol 2:1 (v/v), dried with nitrogen, redissolved in methanol, and then applied to HPLC. The values quoted here and throughout the text are the mean and standard deviations of n determinations.

Preparation of glutathione S-transferase A1-1

The plasmid ptacGST2 was expressed in *E. coli* (35) and glutathione S-transferase A1-1 was purified using affinity chromatography on S-hexyl-glutathione-agarose (36). The enzyme was >99% pure as judged by SDS-PAGE.

Protein and enzyme assays

Protein contents of the samples were determined by the dye-binding assay (Bio-Rad) of Bradford (37) using bovine serum albumin as a standard. Se-GPx activity was measured by a coupled spectrophotometric assay using hydrogen peroxide as substrate (38). GST activity was measured as described (39) using 1-chloro-2,4-dinitrobenzene as substrate. The rate of conversion of PLPC-OOH to PLPC-OH was measured by HPLC (33).

HPLC separation and detection of PLPC-OOH and PLPC-OH

PLPC-OOH and PLPC-OH contents in the cells after treatment with phospholipid hydroperoxide was determined by HPLC. Cells were rinsed twice with phosphate-buffered saline and treated with trypsin-EDTA, and the resulting cell suspension was centrifuged at 176 *g* for 5 min. The packed cells obtained were washed twice with phosphate-buffered saline, and extracted with 0.5 ml chloroform–methanol 2:1 containing 0.005% butylated hydroxytoluene. The mixture was then shaken for 3 min and centrifuged at 16,000 *g* for 5 min. The lower phase was dried under N_2, and the residue redissolved in methanol. Aliquots (20 μl) of the solution were analyzed by HPLC using an Ultracarb 5 ODS (20) column (250 × 4.6 mm). The mobile phase was an acetonitrile–methanol–water 50:49.5:0.5 (v/v/v) containing 10 mM choline chloride at 0.5 ml/min with detection at 232 nm. The peaks of PLPC-OOH and PLPC-OH were quantified from the peak heights using external standards, with a detection limit of 4 pmol. When using ^{14}C-labeled PLPC-OOH, the flow rate of scintillant was 1.5 ml/min with direct on line detection of radioactivity. The peaks were quantified by collecting fractions and scintillation counting.

RESULTS

Uptake of hydroperoxy-phospholipids in HepG2 cells

Phospholipids constitute about 3% of the wet weight of hepatocytes (40), which is equivalent to an apparent intracellular concentration of ~40 mM. HepG2 cells have a similar phospholipid content (28). HepG2 cells

are able to efficiently take up a variety of forms of fatty acids from the culture medium (29), and there are no defects in the incorporation of phospholipids into the maturing lipoprotein by HepG2 cells (41). In order to study the metabolism of hydroperoxy-phospholipids, we specifically incorporated a low level of pure hydroperoxy-phospholipid, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH), into the cells. To facilitate this, a modified medium was used in which PLPC-OOH is relative stable (see Experimental section). At a concentration of 50 μM PLPC-OOH in the medium, 1.05 ± 0.04% (n = 4) of the total PLPC-OOH was incorporated into 10^7 cells in 2 h as determined by HPLC analysis of cell extracts. This demonstrates uptake and is equivalent to a total apparent concentration of 200 μM in the cell, although clearly the local intracellular concentrations in each organelle will vary widely. Thus the incorporated phospholipid derivatives constitute only ~0.5% of the total phospholipid. The concentration of peroxides in unstressed cells is unknown, but the total cellular peroxide is profoundly increased by various stimuli and this can lead to apoptosis (2, 6). However, after incorporation of PLPC-OOH (50 μM for 2 h) into the HepG2 cells, there was no effect on either cell viability as assessed by trypan blue exclusion (97.7 ± 1.6% (n = 4) after trypsin/EDTA treatment for control cells, 97.0 ± 1.2% (n = 4) for PLPC-OOH-treated) or on cellular morphology as judged by scanning electron microscopy. Uptake of PLPC-OOH was also demonstrated using labeled PLPC-OOH. ^{14}C-labeled 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine was added to the cell culture medium. After 2 h, the cells were washed twice and the incorporated label was measured in a membrane extract. Of the total ^{14}C-labeled PLPC-OOH, 1.06 ± 0.15% (n = 3) was taken up by the cells, which again shows uptake of PLPC-OOH. Uptake was also demonstrated indirectly by comparing the ratios of PLPC-OOH to PLPC-OH in the medium and in the cells. After incorporation into the cell membrane, metabolism results in the formation of PLPC-OH (see below). The distribution of PLPC-OOH and PLPC-OH in the cell culture medium and in cell extracts was tested by HPLC after addition of PLPC-OOH (50 μM, **Fig. 1**). In cell culture medium, 24.9 ± 3.7% (n = 3) PLPC-OOH was reduced to PLPC-OH and 75.1 ± 3.7% (n = 3) PLPC-OOH remained intact after 4 h. On the other hand, incorporation of phospholipid into HepG2 cells resulted in an intracellular ratio of PLPC-OH to PLPC-OOH of 97:3.

Conversion of hydroperoxy- to hydroxy-phospholipids

HepG2 cells were incubated with PLPC-OOH (50 μM) for 1–6 h. The PLPC-OH and PLPC-OOH contents

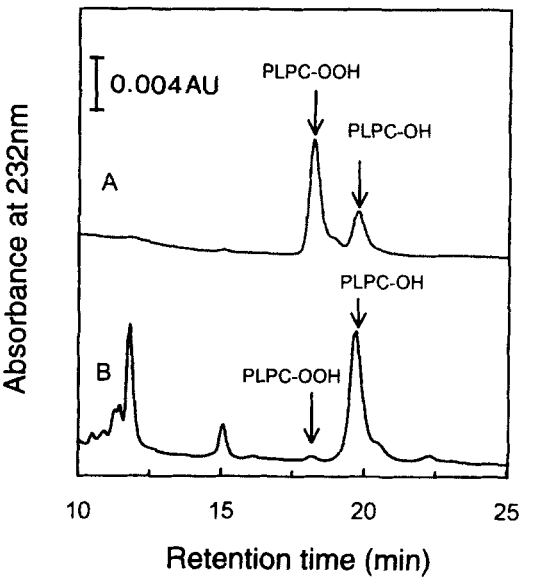

Fig. 1. Comparison of the ratio of PLPC-OOH to PLPC-OH after addition of PLPC-OOH to the HepG2 cell culture medium (50 μM) and incubation for 4 h. Chromatograms showing the ratio of PLPC-OOH to PLPC-OH in A: the cell culture medium and B: the cell extract. The arrows show the elution position of standards.

in the total lipid extracts from the cells were analyzed by HPLC (**Fig. 2**). The presence of the main product, intracellular PLPC-OH, increased with time. The amount of intracellular PLPC-OH also increased with increasing amount of PLPC-OOH (5–100 μM) added to the medium (**Fig. 3**). The concentration of unconverted intracellular PLPC-OOH was low under these conditions.

These results suggest that PLPC-OOH was converted into PLPC-OH in HepG2 cells. To confirm this, we used ^{14}C-labeled 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-

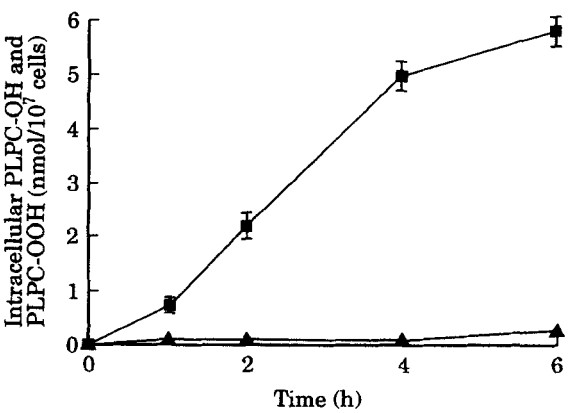

Fig. 2. Time course of PLPC-OOH incorporation into HepG2 cells. HepG2 cells were incubated with PLPC-OOH (50 μM) for 1–6 h. The PLPC-OH (■) and PLPC-OOH (▲) content in total lipid extracts from HepG2 cells were analyzed by HPLC. The error bars show the standard deviation (4 replicates).

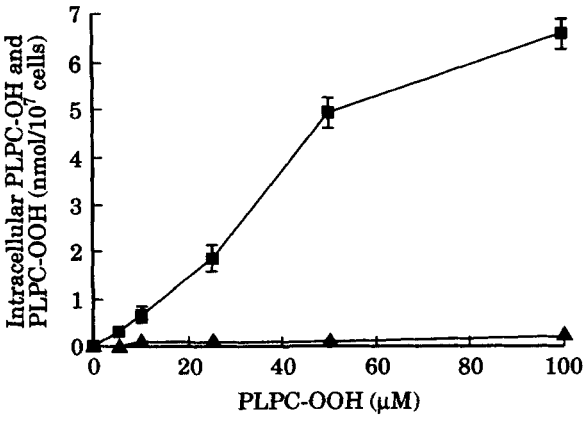

Fig. 3. Treatment of HepG2 cells with PLPC-OOH. PLPC-OH (■) and PLPC-OOH (▲) were measured after treatment of cells with PLPC-OOH for 4 h.

octadecadienoyl)-L-3-phosphatidylcholine containing only one ^{14}C label at C(1) of the *sn*-2 hydroperoxy-fatty acid. After incorporation into the cell, if a hydroperoxy- or hydroxy-fatty acid is subsequently released by the action of phospholipase A_2, then a radioactive peak would be detected at the retention time of the relevant fatty acid standards. In contrast, if phospholipase A_2 is not involved, then a peak corresponding to 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OH) would be expected. **Figure 4** shows that, in HepG2 cells, 81% of incorporated intracellular PLPC-OOH was metabolized to PLPC-OH, 5% of the total PLPC-OOH remained unchanged, and 10% was converted to a peak that eluted at about 15 min. This latter peak has the same retention time as heme-catalyzed breakdown products of PLPC-OOH, but has no absorbance at 277 nm. This suggests that the product is a lower molecular weight and/or higher polarity derivative containing the conjugated diene moiety (absorption maximum at 232 nm) as described by Maiorino, Ursini, and Cadenas (42). There were no detectable hydroperoxy- or hydroxy-fatty acids.

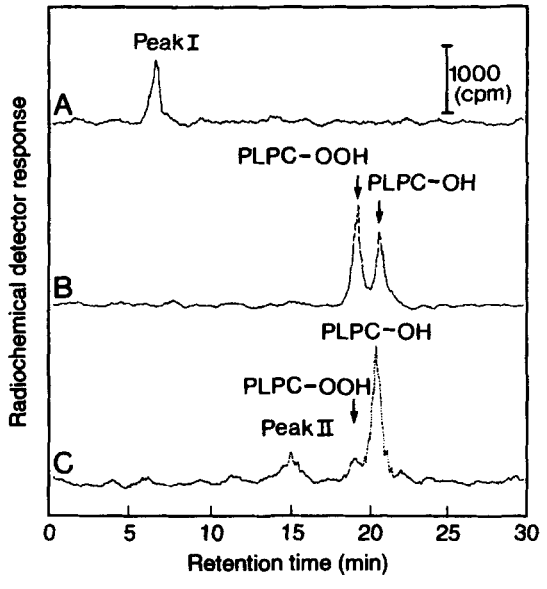

Fig. 4. HPLC-radiochemical detection of metabolites of incorporated PLPC-OOH in HepG2 cells. A: Linoleic acid hydroperoxide hydrolyzed from [^{14}C]-PLPC-OOH by the action of PLA_2 (peak I). B: Separation of the standards [^{14}C]-PLPC-OOH and -PLPC-OH. C: HepG2 cells were incubated in 50 μM ^{14}C-labeled PLPC-OOH for 2 h before harvest, and total lipid was extracted using chloroform–methanol 2:1 as described under Experimental Procedures.

A mathematical modelling study on the relative importance of PHGPx and PLA_2/GPx in the reduction of hydroperoxy-phospholipids, based on published data using a kinetic model of mitochondrial lipid peroxidation in rat liver, showed the PHGPx was theoretically more important in the reduction of hydroperoxy-phospholipids. This study also reported that the PLA_2 activity in HepG2 cells is several orders of magnitude lower than that of PHGPx (43). A pathway exists that enables phospholipids to be deacylated and reacylated with a different fatty acid (44). This pathway involves PLA_2, followed by reacylation by an acyltransferase and an acyl-CoA synthetase. However, PLA_2 is low in most unstressed cells, including HepG2 cells, so the reacylation of fatty acids would be limited by the deacylation step. If this reaction was occurring to a significant extent in HepG2 cells, then the chance for reacylation of ^{14}C-labeled fatty acids into exactly the same lysophospholipids is extremely low; reacylation of the ^{14}C-labeled hydroxy fatty acid to a different lysophospholipid (any species with no palmitic acid at the first position) would give a different retention time on HPLC. Taken together, this evidence and the results reported here show that the PLA_2/GPx pathway is unlikely to play a significant role, and that the predominant metabolism of PLPC-OOH reduction is via PHGPx or a PHGPx-like pathway.

We also considered the rates of uptake of PLPC-OOH and of PLPC-OH from the medium, and calculated whether very different rates of uptake for the two compounds could make it appear that there was intracellular conversion of PLPC-OOH to PLPC-OH. The reactions can be represented as follows:

$$\begin{array}{ccc} \text{PLPC-OOH}_{(\text{intracellular})} & \xrightarrow{\text{PHGPx } (k_1)} & \text{PLPC-OH}_{(\text{intracellular})} \\ k_2 \uparrow \downarrow k_3 & & k_4 \uparrow \downarrow k_5 \\ \text{PLPC-OOH}_{(\text{extracellular})} & \xrightarrow[\text{cell culture medium } (k_6)]{} & \text{PLPC-OH}_{(\text{extracellular})} \end{array}$$

Because of the dynamic nature of the system, it is not possible to measure directly the uptake of PLPC-OOH. However, we have estimated some of the rates above in order to determine whether the rates of uptake of PLPC-OOH and of PLPC-OH are comparable. The

PHGPx activity (k_1) in these cells is equivalent to 4550 pmol product per min per 10^7 cells (33). The uptake of PLPC-OH into cells (k_3) was measured by incubation of cells in PLPC-OH (5–50 μM), followed by a measurement of intracellular PLPC-OH. The rate of uptake was concentration-dependent. At 10 μM PLPC-OH, the rate of uptake was 6.8 ± 0.68 pmol per min per 10^7 cells. This demonstrates that the rate of uptake of PLPC-OH is 600- to 700-fold lower than the potential rate of intracellular reduction of incorporated PLPC-OOH. Because of this, PLPC-OOH added to the medium and incorporated into cells is almost entirely found as intracellular PLPC-OH. The apparent rate of uptake of PLPC-OOH, calculated from intracellular [PLPC-OH], was concentration-dependent between 5 and 50 μM initial PLPC-OOH, and at 10 μM was 3.1 ± 0.2 pmol per min per 10^7 cells (k_2). Clearly, in this calculation, intracellular enzymic reduction of PLPC-OOH is rapid compared to uptake, and so the rate-limiting step is the uptake. As stated in the Experimental section, there is also some reduction of PLPC-OOH in the culture medium which is represented above by k_6. This extracellular reduction was minimized in the experimental protocol, but cannot be completely abolished. The rate of this reduction was 33 pM per min, and was almost independent of the presence or absence of cells. The volume of cell culture medium containing 10^7 cells is 4 ml, and so this rate is much slower than intracellular reduction predicted from PHGPx content above. The loss of PLPC-OH from cells was also measured, using ^{14}C-labeled PLPC-OH. Cells were incubated with PLPC-OH and then transferred to a medium containing no added phospholipids. After 2 h, ~75% of the incorporated intracellular PLPC-OH was lost (**Fig. 5**), and this allowed the rate (k_5) to be calculated as 6.6 ± 0.3 pmol per min per 10^7 cells. This rate of loss of PLPC-OH from cells is identical within experimental error to the rate of uptake. We can also conclude that the rates of transfer of PLPC-OOH and of PLPC-OH between culture medium and the cell are similar, but are slow compared to the rate of intracellular reduction of PLPC-OOH. This is also supported by the observation above that the intracellular ratio of PLPC-OOH to PLPC-OH is very different from that of the cell culture medium, which is further evidence that the rate of reduction of PLPC-OOH is much faster in cells than in the cell culture medium. The results demonstrate that the observed intracellular reduction of PLPC-OOH is primarily due to enzymic reduction and not due to preferential uptake of PLPC-OH compared to PLPC-OOH.

Pathway of reduction of hydroperoxy- to hydroxy-phospholipids

The reduction of hydroperoxy-phospholipids can be catalyzed by certain isoforms of GST (24) as well as by Se-PHGPx (21). In HepG2 cells, GSTA1-1 constitutes about 90% of the total GST (45–47). This isoform is active on hydroperoxy-phospholipids, but with a much lower specific activity compared to Se-PHGPx (24). The value reported by these authors, however, was using a substrate concentration equivalent to 200 μM peroxide, whereas the values in the literature for Se-PHGPx used a lower concentration. Further, some assay methods use detergents, whereas others do not. Because the relative contributions to the reduction of hydroperoxy-phospholipids is therefore controversial, we have measured the activity of recombinant GSTA1-1, which is completely free of Se-PHGPx, on PLPC-OOH under the same conditions as for Se-PHGPx. The method used was direct measurement by HPLC which is the most accurate and sensitive method. This experiment provides the first valid comparison between these two enzymes. The ratio of activities on 1-chloro-2,4-dinitrobenzene and PLPC-OOH for GSTA1-1 is shown in **Table 1,** and the level of glutathione S-transferase activity in HepG2 cells on 1-chloro-2,4-dinitrobenzene is shown in **Table 2.** Using this data, the activity of GSTA1-1 on PLPC-OOH can be estimated to be 0.04 nmol/min per mg protein in HepG2 cell extract. This compares to a total value for reduction of PLPC-OOH in HepG2 extract of 2.30 ± 0.31 (n = 4) nmol/min per mg protein, which shows that GSTA1-1 contributes 0.5% to the reductive reaction of PLPC-OOH.

Se-PHGPx can be manipulated in HepG2 cells by the

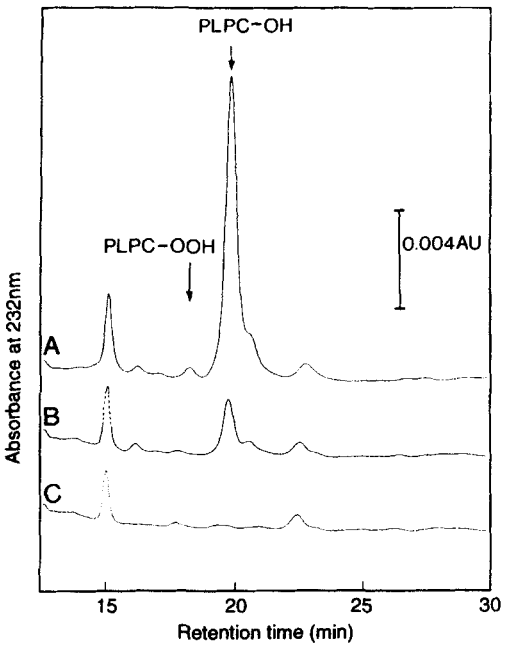

Fig. 5. The metabolic fate of PLPC-OH in HepG2 cells. A: Cells were incubated with 50 μM PLPC-OOH for 2 h, before replacing the medium. B: After replacement with fresh medium for 4 h. C: After 24 h.

TABLE 1. Activities of purified human liver phospholipid hydroperoxide glutathione peroxidase and of glutathione S-transferase A1

	Specific Activity			
	Triton X-100		No Triton X-100	
	CDNB	PLPC-OOH	CDNB	PLPC-OOH
	nmol/min/mg			
GST	20,100 ± 2600	8 ± 1	20,700 ± 2900	32 ± 3
Se-PHGPx	0	336,000 ± 8000	0	40,000 ± 6000

Specific activities were calculated using substrate concentrations of 1 mM for CDNB and 25 μM for PLPC-OOH. Triton X-100, were indicated, was 0.04% (v/v) for the CDNB assay and 0.1% (v/v) for the PLPC-OOH reduction; CDNB, 1-chloro-2,4-dinitrobenzene.

level of selenium (30). Table 2 shows the levels of PHGPx in HepG2 cells in selenium-supplemented, "normal," and selenium-deficient cells. The morphology of the cells in selenium supplementation was unchanged, as was the cell viability. However, the viability of the selenium-deficient cells was decreased (88.5 ± 4.3%, n = 4), and scanning electron microscopy showed that the cells were more fragile. Selenium supplementation increased the level of intracellular Se-PHGPx and Se-GPx activity on hydrogen peroxide, but did not affect the level of GST (see Table 2). PLPC-OOH was incorporated into the cells, and the levels of remaining PLPC-OOH and product PLPC-OH was measured (**Fig. 6**). The selenium-deficient cell extract contained the highest amount of PLPC-OOH (7-fold more than control cells) and the lowest amount of PLPC-OH. Selenium supplementation decreases the amount of residual PLPC-OOH (Fig. 6). If selenium-deficient cells are re-supplemented with 100 nM selenium for 20 h, and the cells are then treated with PLPC-OOH, they exhibit less remaining PLPC-OOH than selenium-deficient cells (706 ± 112 compared to 408 ± 56 pmol/10^7 cells). This demonstrates that selenium-deficient cells can partially recover their ability to metabolize hydroperoxy-phospholipids.

TABLE 2. Effects of selenium depletion and supplementation on enzyme activities in HepG2 cells

	Enzyme Activities		
Cell Type	GPx	PHGPx	GST
	nmol/min/mg protein		
Se(+)	18.6 ± 2.0	10.2 ± 1.6	26.4 ± 3.9
Normal	6.0 ± 0.52	2.3 ± 0.31	25.6 ± 2.7
Se(−)	1.2 ± 0.25	0.65 ± 0.2	24.3 ± 4.5

HepG2 cells were grown in Eagle's minimum essential medium containing 5% FCS for 3 days followed by a reduction to 2% FCS for 4 days, with no added selenium (Se(−)). Cells were also grown in 10% FCS medium with added selenium (100 nM) for 24 h (Se(+)). Normal cells were grown in 10% FCS medium that contained endogenous selenium at ≤12.8 nM. Values represent the mean and standard deviation of 4 replicates.

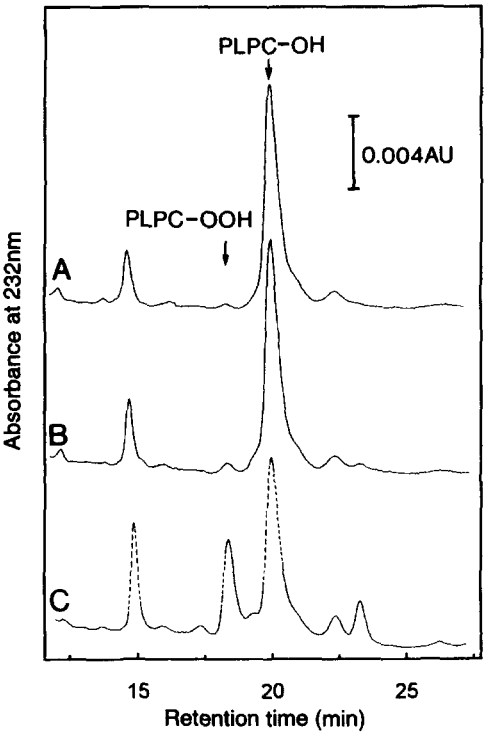

Fig. 6. Elution profiles of lipids extracted from HepG2 cells cultured with different levels of selenium. A: PLPC-OOH and PLPC-OH were 52 ± 5.4 and 2143 ± 176 pmol/10^7 cells (n = 4), respectively, in selenium-supplemented medium (100 nM sodium selenite, 24 h). B: PLPC-OOH and PLPC-OH were 98 ± 6.8 and 2102 ± 103 pmol/10^7 cells (n = 4), respectively, in cells cultured in control medium (Se ≤ 12.8 nM). C: In selenium-deficient cells, PLPC-OOH and PLPC-OH were 706 ± 112 and 1453 ± 91 pmol/10^7 cells (n = 4), respectively

DISCUSSION

There are few reports on the comparison of activities of GPx and PHGPx in tissues, and it is not easy to distinguish the contributions from these two enzymes as both are increased on selenium supplementation. However, a human leukemia cell line, K562, expresses PHGPx but not GPx. This line exhibited a 5- to 10-fold lower PHGPx activity under Se deficiency relative to Se-suffi-

cient conditions (48) and the Se-deficient cells were more susceptible to photoperoxidation and hydroperoxides than the controls. The fact that PHGPx, and not GPx, can act directly on hydroperoxy phospholipids suggests the importance of PHGPx. The expression of PHGPx in cultured cells has also been used to examine the role of PHGPx (49). cDNA expressing PHGPx was transfected into guinea pig 104C1 cells. The cells were incubated with PC-OOH (300 μM) for 24 h, and cell viability (assessed by the release of LDH) was significantly higher in transfectants (104C1/04C) containing the human PHGPx cDNA (49).

In this report, the role of PHGPx has been further probed. One of the central questions involves the metabolism of hydroperoxy-lipids by selenoenzymes. Here we show that the cellular metabolism of hydroperoxy-phospholipids is by direct reduction to hydroxy-phospholipids by both Se-PHGPx and GSTA1. The former plays the more important role in HepG2 cells, as the reduction of hydroperoxy- to hydroxy-phospholipid can be enhanced by selenium supplementation, whereas under selenium deficiency, the reaction of hydroperoxy- to hydroxy-phospholipid is decreased. There are no detectable hydroperoxy- or hydroxy-fatty acid products, and no products of a possible reacylation reaction, which shows that the action of phospholipase A_2/GPx on hydroperoxy-phospholipids is not significant. Even under conditions where the intracellular hydroperoxy-phospholipid concentration may be as high as 200 μM, which may activate phospholipase A_2, there are no detectable fatty acid products (17). In in vitro assays, phospholipase A_2 activity can be increased by 1–3 mM Ca^{2+}, and although the concentration of Ca^{2+} in human HepG2 cells is unknown, the free Ca^{2+} in hepatocytes is about one magnitude lower than this (50).

The activity of GST in human liver is subject to large inter-individual variations. GST μ (M1) exhibits a null allele in about 50% of the population (51), but this isoform has almost no peroxidase activity (13). GST π (P1) is present at low levels in human liver (52), and also has almost no peroxidase activity (13). GST α (GSTA1 and A2) is the predominant isoform in human liver but is subject to considerable variation. The total hepatic GST activity (on 1-chloro-2,4-dinitrobenzene) varies from 0.39 to 2.17 μmol/min per mg protein (53). These variations are reflected in the level of GSTA1 (7.0 to 47.0 μg A1 protein/mg cytosolic protein) and of GSTA2 (4.4 to 40.5 μg A2 protein/mg cytosolic protein). The range of inter-individual variation of Se-PHGPx in humans is not known, but the one estimate available shows that the total activity for reduction of hydroperoxy-phospholipids is 3 nmol/min per mg protein (54). On the basis of these data, it is clearly not yet possible to calculate the precise contribution of GST α and Se-PHGPx to reduction of cellular hydroperoxy-phospholipids in human individuals. However, for the ranges of GST activity on 1-chloro-2,4-dinitrobenzene shown above, and using the values in Table 1, the activity contributed by GST on hydroperoxy-phospholipids is between 0.15 and 0.86 nmol/min per mg protein; this compares to the total value for this activity, albeit measured in only one human liver sample, of 3 nmol/min per mg protein. Although accurate figures cannot yet be calculated, it is clear that reduction of hydroperoxy-phospholipids depends on both GST α levels (which vary between individuals) and on Se-PHGPx levels (which are affected by dietary selenium (30, 55)). Whether Se-PHGPx in humans shows inter-individual variation requires further investigation. Up to 13 selenium-containing proteins or protein subunits have been identified by in vivo labeling with [^{75}Se]selenate in rat tissues (56), but apart from glutathione peroxidases, the functions of most of these selenoproteins are not well characterized. The metabolism of hydroperoxy-phospholipids by Se-PHGPx demonstrates the role of the essential trace element selenium.

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