

Lethal damage to endothelial cells by oxidized low density lipoprotein: role of selenoperoxidases in cytoprotection against lipid hydroperoxide- and iron-mediated reactions

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Abstract Oxidized low density lipoprotein (LDL_{ox}) is believed to be an important contributor to endothelial cytodamage and atherogenesis. The purpose of this study was to examine the role of glutathione (GSH) and GSH-dependent selenoperoxidases in cytoprotection against the damaging effects of LDL_{ox}. When irradiated in the presence of a phthalocyanine sensitizing dye, human LDL accumulated chromatographically detectable and iodometrically measurable lipid hydroperoxides (LOOHs). Photogenerated LDL_{ox} caused lethal damage to bovine aortic endothelial (BAE) cells in vitro, as determined by lactate dehydrogenase release and inhibition of thiazolyl blue reduction. When depleted of GSH by buthionine sulfoximine treatment, BAE cells became more sensitive to LDL_{ox}. Cells grown in 2% serum/DME-HAM's F-12 medium without added selenium [Se(-) cells] exhibited far lower GSH-peroxidase and phospholipid hydroperoxide GSH-peroxidase activities than selenium-supplemented controls [Se(+) cells], and were much more sensitive to oxidative injury induced by t-butyl hydroperoxide, liposomal cholesterol hydroperoxides, and LDL_{ox}. Preincubation of LDL_{ox} with GSH and Ebselen (a selenoperoxidase mimetic) resulted in a dramatic reduction in both LOOH content and cytotoxicity. Moreover, treating Se(-) cells themselves with Ebselen substantially restored their resistance to LDL_{ox}-induced damage. LDL_{ox} toxicity to Se(-) cells was strongly inhibited by desferrioxamine and stimulated by ferric-8-hydroxyquinoline (a lipophilic chelate), indicating that iron is an active participant in oxidative damage. These results demonstrate that the GSH-dependent selenoperoxidase(s) play(s) an important role in cellular defense against oxidized low density lipoprotein, presumably by detoxifying lipid hydroperoxides and thereby preventing their iron-catalyzed decomposition to damaging free radical intermediates. — Thomas, J. P., P. G. Geiger, and A. W. Girotti. Lethal damage to endothelial cells by oxidized low density lipoprotein: role of selenoperoxidases in cytoprotection against lipid hydroperoxide- and iron-mediated reactions. *J. Lipid Res.* 1993. 34: 479–490.

Supplementary key words selenium • glutathione peroxidase • Ebselen • glutathione • lipid hydroperoxide • iron

Human LDL is a molecular composite containing a single apoB-100 protein (~25 wt %) and several different lipid classes, including cholesteryl esters, phospholipids,

free cholesterol, and triacylglycerols (collectively ~75 wt %). Unsaturated lipids in LDL are subject to peroxidative degradation, a process that can cause profound changes in LDL structure and biological activity (1). There is a growing body of evidence that oxidatively modified LDL (LDL_{ox}) plays an important role in the atherogenic process (2, 3). LDL_{ox} exhibits cytotoxic (4–7) and chemotactic (8) properties, both of which could be involved in its atherogenicity. In vitro oxidation of LDL mediated by redox metals (Cu²⁺, Fe²⁺) or by vascular cells (macrophages, smooth muscle, or endothelial cells) is characterized by free radical lipid peroxidation resulting in formation of lipid hydroperoxides (LOOHs) and thiobarbituric acid (TBA)-reactive by-products, modification of apoB-100, and an increase in overall negative charge (1). This, in turn, promotes LDL uptake via the so-called scavenger receptor on macrophages, leading to the formation of lipid-laden foam cells (9). If occurring in vivo, these events could play a key role in the development of atherosclerotic lesions (2). There is considerable interest in understanding the pathological conditions that give rise to LDL_{ox} and also how chemical antioxidants such as α -tocopherol, β -carotene, ascorbate, or probucol might be

Abbreviations: AlPcS, aluminum phthalocyanine tetrasulfonate; BAE, bovine arterial endothelial (cell); BSO, DL-buthionine-S,R-sulfoximine; Ch, cholesterol; ChOOH, cholesterol hydroperoxide; DCP, dicetylphosphate; DFO, desferrioxamine; DMPC, dimyristoyl phosphatidylcholine; FCS, fetal calf serum; Fe(HQ)₂, Fe³⁺/8-hydroxyquinoline (1:2); Fe(HQS)₂, Fe³⁺/8-hydroxyquinoline-5-sulfonate (1:2); GRD, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; GPX, GSH-peroxidase; HQ, 8-hydroxyquinoline; LDH, lactate dehydrogenase; LDL, low density lipoprotein; LDL_{ox}, oxidized LDL; LOOH, lipid hydroperoxide; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; PCOOH, phosphatidylcholine hydroperoxide; PHGPX, phospholipid hydroperoxide GSH-peroxidase; Se(-), Se-deficient; Se(+), Se-sufficient; t-BuOH, t-butanol; t-BuOOH, t-butyl hydroperoxide.

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used to suppress LDL oxidation and LDL_{ox}-mediated cell damage. Numerous studies have focused on these aspects (9–12). Much less attention has been directed to the chemical species responsible for LDL_{ox} cytotoxicity and the protective role of enzymatic antioxidant systems, e.g., GSH-based systems. GSH may serve as a direct scavenger of toxic oxidants originating from LDL_{ox} (13), or as the reducing substrate for selenium (Se)-independent transferases or Se-dependent peroxidases, which can detoxify a wide variety of peroxides, including lipid-derived species (LOOHs). Two selenoperoxidases are known to exist: *a*) “classical” GSH-peroxidase, which acts on relatively polar peroxides such as H₂O₂ and fatty acid hydroperoxides (14), and *b*) the more recently discovered phospholipid hydroperoxide GSH-peroxidase (PHGPX), which acts on a broad range of relatively low polarity substrates, including phospholipid and cholesterol hydroperoxides (15, 16). GPX is located in the cytosol and mitochondrial matrix, whereas PHGPX is found in both cytosolic and membrane-bound forms. Recent studies in this laboratory have shown that PHGPX can catalyze the direct reduction of all LOOH classes in LDL_{ox}, whereas GPX is unreactive with cholesterol hydroperoxides and reacts with phospholipid, cholesteryl ester, and triacylglycerol hydroperoxides only after hydrolytic release of the oxidized fatty acyl moieties (16–18). The major goals of the present work were *a*) to examine the roles of LOOH and iron in LDL_{ox}-induced endothelial cell damage, and *b*) to ascertain the importance of selenoperoxidase-based cytoprotection against LDL_{ox}.

MATERIALS AND METHODS

Chemicals and reagents

Sigma Chemical Co. (St. Louis, MO) provided the following: cholesterol, cholesteryl linoleate, 7 β -hydroxycholesterol (7 β -OH), dicetylphosphate, dimyristoyl phosphatidylcholine, egg phosphatidylcholine, egg phosphatidylethanolamine, probucol, GPX, GRD, GSH, NADH, NADPH, pyruvate, t-butyl hydroperoxide, t-butanol, and MTT. Tissue culture reagents, including fetal calf serum, DME-HAM's F-12 medium, insulin, transferrin, L-glutamine, 10x-trypsin-EDTA, and antibiotics (penicillin, streptomycin) were also obtained from Sigma. 2-Phenyl-1,2-benzisoxselenazol (Ebselen) was from Ciba-Geigy (Summit, NJ) and desferrioxamine was from Ciba-Geigy (Suffern, NY). Aluminum phthalocyanine tetrasulfonate was a gift from Dr. E. Ben-Hur (Beer Sheva, Israel). Peroxide-free Triton X-100 was from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were of the highest purity available and aqueous solutions were prepared with deionized, glass-distilled water.

Cultured cells

Endothelial cells from bovine thoracic aorta (No. AG08132) were obtained from N.I.A. Aging Cell Culture Repository, Cornell Institute for Medical Research (Camden, NJ). Cells were obtained at the third passage, transferred to 75-cm² filter vent flasks (CoStar, Cambridge, MA), and grown to confluency in DME-HAM's F-12 medium containing 10% FCS, insulin (10 μ g/ml), transferrin (5 μ g/ml), L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Incubation was carried out at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Cells were passaged as described by Balla et al. (12), and used experimentally between passages 6 and 10. For preparing selenium-deficient cells, the serum concentration was decreased from 10% to 5%, at which point one population of cells, designated Se(–), was grown without added selenium, and the other, designated Se(+), was grown in the presence of sodium selenite (10 ng/ml). At the next passage, serum content of both cell populations was further decreased to 2%, and eventually to 1% in some experiments. Experiments were carried out within 48 h after growth to confluency in either 12-well (4 cm²) or 24-well (2 cm²) tissue culture plates. The protein content of confluent cells was measured by the method of Lowry et al. (19), using serum albumin as the standard. Se(–) and Se(+) cells were found to contain approximately the same amount of total protein (mg/10⁸ cells): 16.3 \pm 1.4 (n=3) and 14.7 \pm 3.9 (n=3), respectively.

Cell viability

Most viability determinations were based on retention of LDH as an indicator of cell integrity. Enzyme activity was measured by tracking the oxidation of NADH (A₃₄₀ decay) in the presence of pyruvate (20). Cells were washed immediately before the onset of an experiment to remove any extracellular LDH; total enzyme was measured on washed cells that had been lysed in the presence of 0.5% Triton X-100. A second assay, based on mitochondrial dehydrogenase-mediated reduction of MTT to a colored formazan (21), was introduced in the latter stages of the work. The formazan crystals were solubilized in isopropanol and absorbance at 570 nm was recorded. Viability of peroxide-treated cells is expressed as a percentage of the LDH retained or formazan generated by non-peroxide-treated controls.

Enzyme determinations

Enzyme activities were measured as soon as possible (no longer than 2 days) after cells reached confluency. The cells were removed by trypsinization, washed, and resuspended in 5% Triton X-100. GPX activity was measured by coupled enzymatic assay (16, 17), using 3 mM GSH, 0.2 mM NADPH, 1–2 units/ml GSSG-reductase, 0.2 mM t-BuOOH and cell sample (0.1–0.2 mg/ml pro-

tein) in 0.1 M phosphate buffer (pH 7.4) at 37°C. The rate of NADPH oxidation (A_{340} decay) was monitored. Assay conditions for PHGPX were identical except for a 4-fold increase in the amount of sample protein, and substitution of a phospholipid hydroperoxide (PCOOH, 0.15 mM) for t-BuOOH. (GPX does not react directly with phospholipid hydroperoxides (16, 17)). The PCOOH was prepared by AlPcS-sensitized photooxidation of liposomal egg phosphatidylcholine (22). Catalase was determined by monitoring the rate of decomposition of H_2O_2 , as measured by A_{240} decay (23). Assay mixtures contained 19 mM H_2O_2 and cell sample (20–40 μ g/ml protein) in 50 mM phosphate buffer (pH 7.0) at 25°C. Glutathione-S-transferase activity was measured as described (24), using 1.5 mM GSH, 0.5 mM 1-chloro-2,4-dinitrobenzene, and cell sample (0.3–0.4 mg/ml protein) in 0.1 M phosphate buffer (pH 6.5); determinations were based on the rate of increase of A_{340} .

Glutathione determination

Total GSH equivalents (GSH plus 2 GSSG) in BAE cells were measured by GSH recycling assay (25). Specific details have been reported elsewhere for another cell system (26).

Preparation and photooxidation of liposomes

Unilamellar DMPC/Ch/DCP liposomes (10:8:1 mol/mol; average diameter 100 nm) in which cholesterol was the only oxidizable lipid, were prepared by an extrusion procedure (27). Liposomal ChOOH was generated by AlPcS-sensitized photooxidation, as described for LDL (see below). The product consisted mainly of 7 α - and 7 β -hydroperoxide, which arose via the allylic rearrangement of 5 α -hydroperoxide (26, 28). The photooxidized liposomes were filter-sterilized (pore size 0.45 μ m), after which total peroxide content was determined by iodometric analysis (16).

Preparation of LDL and LDL_{ox}

Plasma was obtained from fasted human donors; EDTA (1 mM) was present from the outset. LDL was isolated by ultracentrifugal flotation in KBr (density range: 1.019–1.063 g/ml) (29). All steps were carried out with Chelex-treated, argon-sparged solutions. Before being used experimentally, the LDL was dialyzed against Chelexed PBS to remove EDTA and stored under argon at 4°C. Protein was determined by the method of Lowry et al. (19). Our preparations of LDL typically contained less than 1 nmol LOOH/mg protein. LDL (free of metals) was subjected to AlPcS-sensitized photoperoxidation as follows. A solution containing LDL (~1.0 mg protein/ml) and 20 μ M AlPcS was irradiated at 10°C in a 5-ml vial placed within a larger (45 mm inner diameter) thermostatted beaker (22). The light source was a 90-W quartz-halogen lamp positioned ~15 cm above the reac-

tion vial. Incident light intensity (fluence rate) was ~30 mW/cm², as measured with a YSI radiometer (Yellow Springs, OH). Solutions were stirred magnetically and left open to the air during irradiation. After being filter-sterilized, stock preparations of LDL_{ox} were analyzed iodometrically for LOOH content (16). Thin-layer chromatography of LDL-LOOHs was carried out as described (16, 18), using N,N,N',N'-tetramethyl-*p*-phenylenediamine for visualization.

Experimental conditions

For examining the cytotoxicity of t-BuOOH, liposomal ChOOH, or LDL-LOOH, confluent Se(+) and Se(–) cells (typically in 12-well plates) were overlaid with fresh media and then exposed to increasing amounts of oxidant (expressed as peroxide concentration). In experiments involving liposomes or LDL, total lipid content (unoxidized plus oxidized) was held constant to correct for any background effects. After 20 h of incubation at 37°C in 5% CO₂/95% air, cell viability was determined by LDH or MTT assay. For studying metabolic detoxification of t-BuOOH, Se(–) and Se(+) cells were grown in 25-cm² flasks. During incubation, samples of media were removed periodically for determination of residual peroxide, using a coupled GPX/GRD assay (16, 17). The volume of each reaction medium decreased by < 5% overall due to sampling.

RESULTS

Biochemical characteristics of selenium-deficient cells

When grown to confluency in 10% FCS/DME HAM's F-12 medium, BAE cells were found to be selenium-deficient by virtue of the fact that their GPX and PHGPX activities were 20% and 68%, respectively, of those expressed by selenium-supplemented counterparts grown in the presence of 10 ng/ml Na₂SeO₃ (Table 1). A dose-response experiment indicated that this concentration was sufficient for maximal expression of GPX and PHGPX activities (data not shown). Cells grown in the presence of 5% and 2% serum expressed progressively lower GPX and PHGPX activities than those grown in 10% serum (Table 1). In each case, supplemental Se increased these activities to their maximal levels, viz. ~155 and ~9 units/mg cell protein for GPX and PHGPX, respectively. At 2% FCS, Se-deficient [Se(–)] cells produced about 9% of the GPX activity and ~19% of the PHGPX activity measured in Se-satisfied [Se(+)] controls. Decreasing the serum concentration to 1% without added Se resulted in a small additional decrease in GPX and PHGPX. Most of our experiments on Se-based cytoprotection (see below) were carried out on cells grown in 2% serum, as confluency was achieved more rapidly and dependably at 2% than at 1% serum. At confluency in 2% FCS/DME

TABLE 1. Selenoperoxidase activities of BAE cells: effect of serum concentration and supplemental selenium

Enzyme	Specific Activity ^a			
	10% FCS	5% FCS	2% FCS	1% FCS
<i>units/mg cell protein</i>				
1. GPX				
Se(+)	142 ± 16	142 ± 36	168 ± 44	164 ± 37
Se(-)	29 ± 5	32 ± 1	15 ± 3	9 ± 6
2. PHGPX				
Se(+)	8.1 ± 1.5	7.3 ± 3.3	11.9 ± 0.8	8.5 ^b
Se(-)	5.5 ± 0.6	3.1 ± 1.0	2.3 ± 0.5	2.1 ^b

Bovine arterial endothelial cells (10^6 per flask) were grown to confluency in 75-cm² flasks, using DME-HAM's F-12 media plus the indicated concentrations of fetal calf serum with (+) or without (-) supplemental selenium in the form of sodium selenite (59 nM).

^aOne unit represents 1 nmol NADPH oxidized per min in coupled assay with GSSG-reductase. The peroxide substrate for GPX was t-BuOOH and for PHGPX was photooxidized egg phosphatidylcholine.

^bSingle determination. All other values are means ± SD of at least four determinations, using different cell inoculates.

HAM's F-12 medium, Se(-) cells were found to contain nearly twice as much total glutathione (GSH + 2 GSSG) as Se(+) controls, i.e., 21.9 ± 0.1 nmol/mg protein versus 13.9 ± 2.1 nmol/mg protein (mean ± SD, $n = 4$). An elevated glutathione content has been also observed in Se-deficient L1210 leukemia cells (26), suggesting that this may be an adaptive response to increased oxidative stress. Unlike L1210 cells, which expressed more catalase in response to selenium deprivation (21), BAE cells showed essentially no change in catalase: 32.3 ± 2.0 units/mg protein for Se(-) cells versus 36.7 ± 2.2 units/mg protein

for Se(+) cells (mean ± SD, $n = 9$). In addition, the GSH-S-transferase activity, some of which may reflect Se-independent GSH-peroxidases (30), remained virtually unchanged: 144 ± 1 units/mg protein for Se(-) cells versus 135 ± 1 units/mg protein for Se(+) cells (mean ± deviation, $n = 2$).

Effects of selenium deficiency on metabolism and cytotoxicity of t-BuOOH

BAE cells grown under Se-deficient conditions were found to be much less efficient in catabolizing t-BuOOH

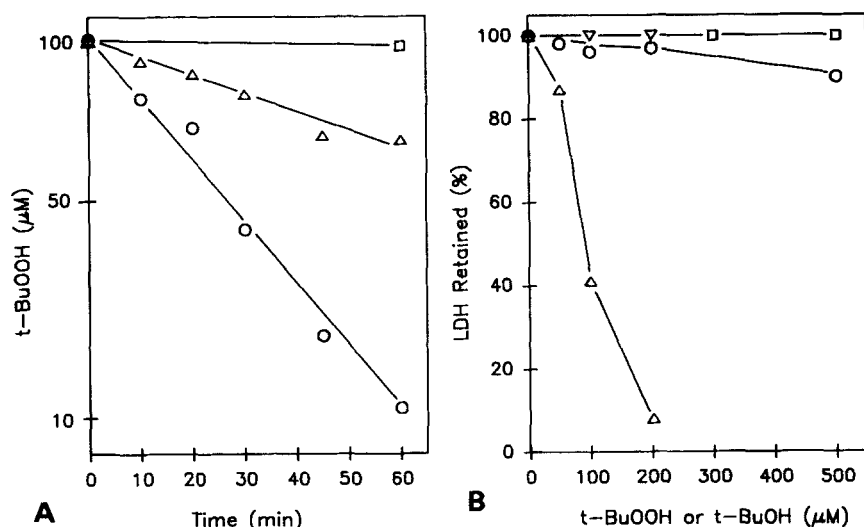


Fig. 1. Effect of selenium deficiency on (A) Ability of BAE cells to catabolize t-BuOOH, and (B) sensitivity of BAE cells to t-BuOOH-induced damage. (A) In 25-cm² flasks, confluent layers of Se(+) cells (○) and Se(-) cells (△) under 1% FCS/DME-HAM's F-12 medium plus and minus 60 nM Na₂SeO₃, respectively, were exposed to t-BuOOH (starting concentration ~100 μM). At the indicated times during incubation at 37°C, media samples were removed, centrifuged to remove any detached cells, and residual peroxide was determined by coupled GPX/GRD assay. No significant cell lysis (based on LDH) release) was observed during the 1 h incubation. A control without cells was analyzed alongside (□). (B) In 12-well plates, confluent layers of Se(+) cells (○, □) and Se(-) cells (△, ▽) cells were incubated with increasing concentrations of t-BuOOH (○, △) or t-BuOH (□, ▽) at 37°C. After 20 h, cytodamage was assessed by determining LDH activity in the medium. Values in each group are plotted as percentages of LDH retained relative to control cells incubated in the absence of t-BuOOH or t-BuOH.

than Se-satisfied counterparts. As shown in **Fig. 1A**, *t*-BuOOH underwent exponential decay during incubation with cells, whereas in the absence of cells (serum and medium alone), no significant loss was observed. The apparent first order rate constants for *t*-BuOOH decay were found to be as follows: 2.3 h^{-1} for Se(+) cells versus 0.6 h^{-1} for Se(-) cells. We have observed similar trends for Se(+) versus Se(-) leukemia L1210 cells (J. P. Thomas, P. G. Geiger, and A. W. Girotti, unpublished data). For L1210 cells, we have demonstrated that *t*-BuOOH loss is accounted for by stoichiometric formation of *t*-BuOH. Selenium-dependent metabolism of *t*-BuOOH is attributed primarily to GPX rather than PHGPX, as the latter is less active toward this peroxide, and is expressed in much lower relative amounts (31).

Cellular resistance to *t*-BuOOH was found to be strongly correlated with ability to metabolize and detoxify this compound. As shown in **Fig. 1B**, Se(-) cells were much more sensitive to *t*-BuOOH (as indicated by LDH release) than Se(+) controls. The peroxide concentration producing 50% release of LDH in 24 h (LC_{50}) was approximately $75 \mu\text{M}$ for Se(-) cells, but greater than $500 \mu\text{M}$ for Se(+) cells. Significantly, the alcohol reduction product of *t*-BuOOH, *t*-BuOH, was found to be nontoxic

at all concentrations up to at least $500 \mu\text{M}$, confirming that the peroxide moiety is required for cytotoxicity.

Cytotoxicity of cholesterol hydroperoxides

In order to more closely approximate oxidatively modified LDL, we challenged BAE cells with cholesterol hydroperoxides (ChOOHs) in liposomal form (**Fig. 2**). The ChOOHs were generated via AIPcS-sensitized photooxidation, the most prominent initial product being $5\alpha\text{-OOH}$, a singlet oxygen adduct (22). In membrane systems, $5\alpha\text{-OOH}$ can undergo allylic rearrangement to $7\alpha\text{-OOH}$, which in turn epimerizes to $7\beta\text{-OOH}$ (28). The 7-OOH epimers can also be generated via free radical reactions (28). For the experiment shown in **Fig. 2**, thin-layer chromatography revealed that the photooxidized liposomes contained mainly $7\alpha\text{-}$ and $7\beta\text{-OOH}$, with a trace of $5\alpha\text{-OOH}$ (data not shown). As shown in **Fig. 2**, Se(-) cells were more sensitive to ChOOH challenge than Se(+), the former releasing $\sim 75\%$ of their LDH after 24 h exposure to 0.3 mM ChOOH, and the latter releasing only $\sim 10\%$. It is important to note that $7\beta\text{-hydroxycholesterol}$ ($7\beta\text{-OH}$) in liposomes proved to be nontoxic to both Se(+) and Se(-) BAE cells when added in concentrations up to 1 mM (data not shown); thus, as noted for *t*-BuOOH (**Fig. 1**), cytotoxicity is ascribed specifically to the peroxide moiety. Similar evidence for Se-dependent protection against ChOOHs has been reported for L1210 cells (21).

Photooxidation of LDL

Dye-sensitized photooxidation was used for generating LDL_{ox} with discrete levels of LOOH. A major advantage of this approach over those involving free radical oxidation (e.g., Cu^{2+} -induced) is that the initiator (photosensitizer) is usually innocuous to cells in the dark. **Fig. 3A** shows a typical time course of LOOH formation during photooxidation of LDL in the presence of AIPcS. There is an apparent linear increase in LOOH content out to 1 h; the increasing curvature beyond this point was probably due to substrate depletion, since O_2 was not limiting and AIPcS did not undergo photobleaching. No LOOH formation was observed when irradiation was carried out in the absence of AIPcS. A thin-layer chromatogram of the LOOH profile in photooxidized LDL is shown in **Fig. 3B**. No peroxides can be detected at the outset, whereas during dye-sensitized photooxidation, there is a progressive accumulation of cholesteryl ester, triacylglycerol, and cholesterol hydroperoxides near the solvent front, along with lower mobility phospholipid hydroperoxides (mainly PCOOHs). Based on this qualitative evidence, the distribution of photochemically generated LOOHs among the different lipid groups appears to be fairly uniform, i.e., no large difference in reactivity of one class versus another is apparent.

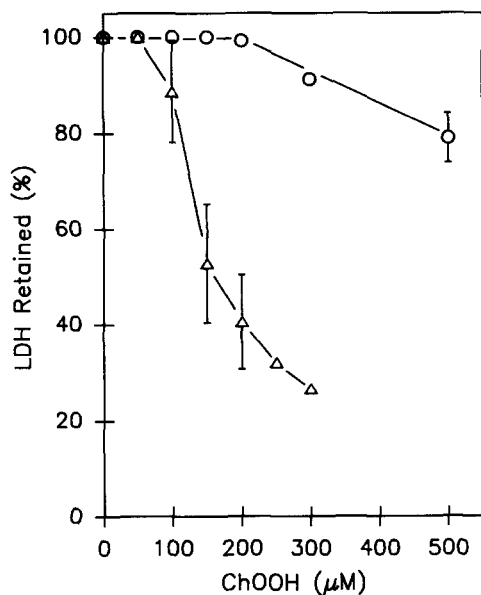


Fig. 2. Cytotoxicity of cholesterol hydroperoxides (ChOOH) toward selenium-deficient versus selenium-sufficient BAE cells. In 12-well plates, confluent Se(-) cells (Δ) and Se(+) cells (\circ) under nonsupplemented and Se-supplemented 1% FCS/DME-HAM's F-12 media, respectively, were incubated at 37°C with increasing concentrations of ChOOH in unilamellar DMPC/Ch/DCP (10:8:1) liposomes. (Liposomal ChOOH was generated by AIPcS-sensitized photoperoxidation.) Incubation mixtures were normalized to the same concentration of total liposomal lipid, $\sim 2.8 \text{ mM}$, which was nontoxic by itself. Cytotoxicity was based on the amount of LDH released after 20 h. Values shown are percentages relative to controls incubated with non-photooxidized liposomes. Points with error bars are means \pm deviation of values from duplicate experiments.

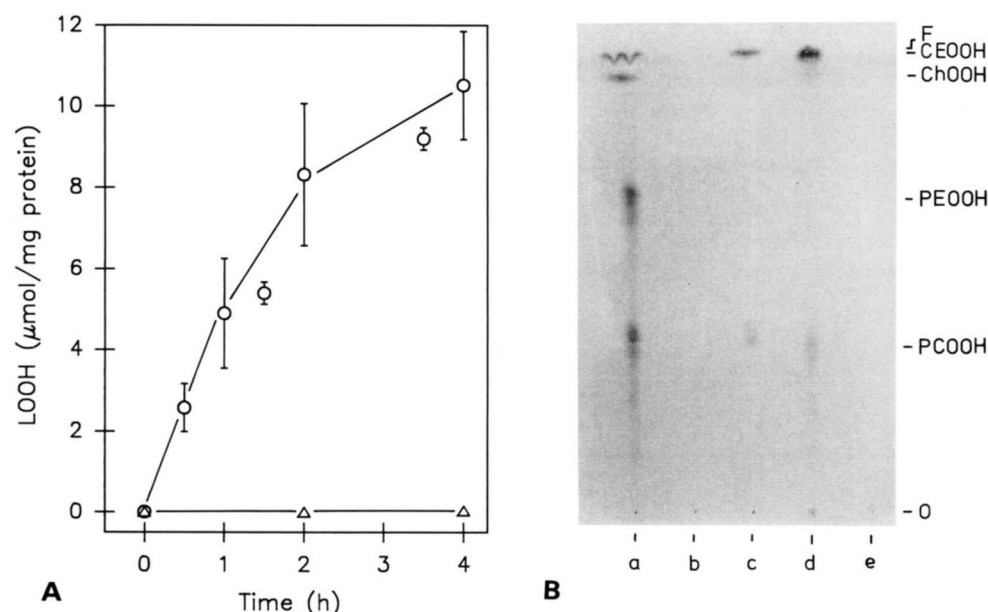


Fig. 3. Photogeneration of lipid hydroperoxides in LDL. (A) Iodometrically detectable lipid hydroperoxides (LOOHs). LDL (1.0 mg protein/ml) in Chelex-treated PBS was irradiated at 10°C in the absence (Δ) and presence (○) of 20 μM AIPcS. At the indicated intervals, samples containing 0.75 mg total lipid were extracted and the recovered lipids were analyzed iodometrically. Means ± deviation of values from duplicate experiments are shown. (B) Thin-layer chromatography of LOOHs. LDL from the same experiment as shown in panel (A) was analyzed for LOOHs before irradiation (lane b) and after irradiation for 0.5 h (lane c) and 4 h (lane d) in the presence of AIPcS. Lane e shows a nonsensitized control after 4 h irradiation. Lane a shows a mixture of photoperoxide standards derived from cholesteryl ester (linoleate) (CE), cholesterol (Ch), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). O, origin; F, solvent front. Sample load as total lipid: ~40 μg/lane (b-e).

Cytotoxicity of LDL_{ox}: GSH-dependent cytoprotection

When Se(+) BAE cells were incubated with photoperoxidized LDL, a dose-dependent cytotoxicity (LDH release) was observed (Fig. 4). (AIPcS present in the LDL_{ox} preparation has been ruled out as a possible contributing factor in this toxicity). In this experiment (as well as all others involving LDL_{ox}), iodometrically measured LOOH was increased while total LDL lipid was held constant. Preincubation of Se(+) cells with BSO, an inhibitor of γ-glutamylcysteine synthetase (32), reduced total glutathione content to < 10% of that measured in non-BSO-treated controls. Although BSO treatment was non-toxic by itself (at least in terms of LDH release), it made Se(+) cells much more sensitive to LDL_{ox} (LC₅₀ ~0.17 mM LOOH) than nontreated cells (LC₅₀ > 0.5 mM LOOH) (Fig. 4).

Cytotoxicity of LDL_{ox}: selenoperoxidase-dependent cytoprotection

Several possible explanations exist for the observed protective effect of GSH against LDL_{ox}-mediated cell killing. These include *a*) direct scavenging of free radical or carbonyl products derived from LDL-LOOHs (13); *b*) involvement as a substrate for certain peroxidatic GSH-S-transferases (30); and *c*) involvement as a substrate for GPX or PHGPX (16-18). In an attempt to narrow these

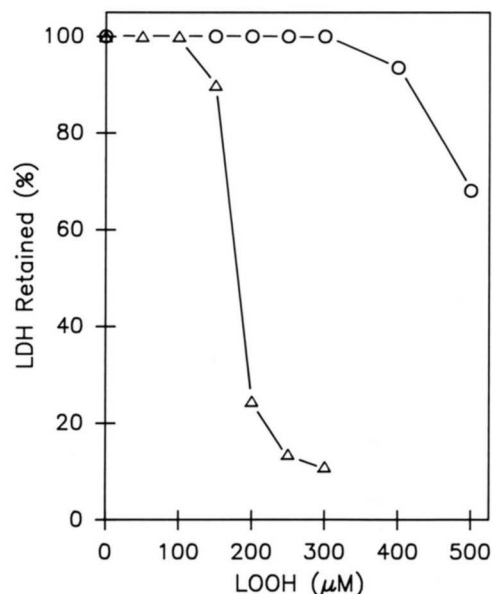


Fig. 4. Effect of buthionine sulfoximine (BSO) treatment on sensitivity of BAE cells to peroxidized LDL. In 12-well plates, confluent Se(+) cells under 1% FCS/DME-HAM's F-12 medium were preincubated with 2 mM BSO for 18 h, resulting in >90% decrease in glutathione content. The BSO-treated cells (Δ), along with a nontreated control (○), were washed and overlaid with fresh medium containing BSO plus increasing concentrations of LOOH in photooxidized LDL, as indicated. All incubation mixtures were standardized to the same concentration of total LDL, ~0.35 mg lipid/ml, which, if unoxidized, produced no detectable cytodamage on its own. Cell integrity was based on the amount of LDH retained after 20 h. Plotted values are percentages based on controls incubated with non-photooxidized LDL.

possibilities, we examined the effects of Se deficiency. As shown in Fig. 5, Se(-) cells in 2% FCS/DME-HAM's F-12 medium were markedly less resistant to photooxidized LDL than Se(+) counterparts. For example, 20 h exposure to 0.4 mM LDL-LOOH resulted in nearly 100% LDH release in Se(-) cells compared with only ~10% release in Se(+). As in the case of t-BuOOH and ChOOH (Figs. 1 and 2), therefore, cytoprotection against LDL_{ox}-induced cell injury was found to be strongly dependent on selenoperoxidase status. However, it is not clear from these results alone whether both PHGPX and GPX were involved in cytoprotection.

Involvement of lipid hydroperoxides in LDL_{ox}-mediated cytotoxicity

It was of interest to determine whether LOOHs per se were responsible for the oxidative cytotoxicity of LDL_{ox}, or whether other species (e.g., aldehydes derived from LOOHs) might be involved. To examine this question, we treated LDL_{ox} with GSH and Ebselen, a synthetic organoselenium compound with selenoperoxidase-like activity (33). As shown in Fig. 6, 10 mM GSH alone or 50

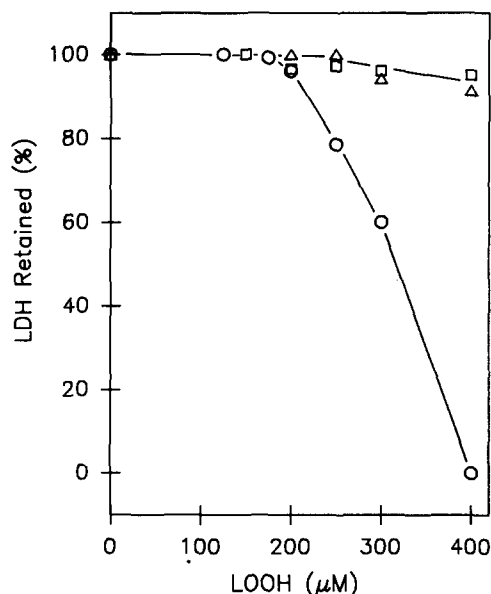


Fig. 5. Sensitivity of selenium-deficient BAE cells to peroxidized LDL and to Ebselen-treated peroxidized LDL. Confluent layers of Se(-) cells (○) and Se(+) controls (□) in nonsupplemented and Se-supplemented 2% FCS/DME-HAM's F-12 medium, respectively, were incubated with increasing concentrations of LOOH in photooxidized LDL, as indicated. An additional group of Se(-) cells (△) was incubated with photooxidized LDL (total LOOH ~5.2 mM) that had been exposed to 10 μM Ebselen plus 15 mM GSH for 4 h, then dialyzed overnight against argon-sparged PBS at 4°C. LOOH content was reduced to ~2% of its starting value after Ebselen/GSH treatment; thus, 400 μM LOOH was in actuality 8 μM LOOH for the Ebselen experiment. Incubation mixtures were normalized to the same concentration of total LDL, ~0.30 mg lipid/ml. Cytotoxicity based on LDH release was evaluated 20 h after the addition of LDL. Values shown as percentages relative to controls incubated with nonoxidized LDL.

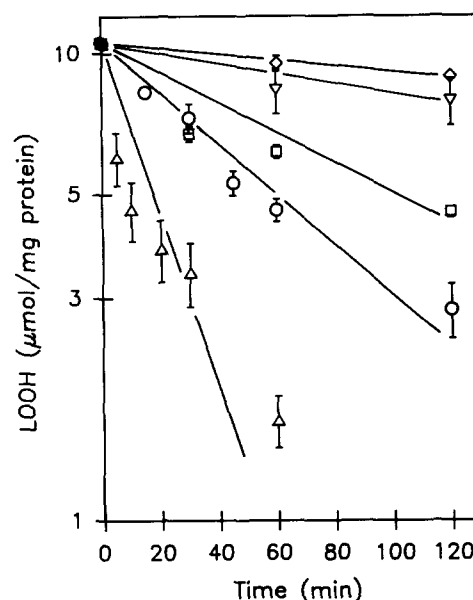


Fig. 6. Ebselen-catalyzed reduction of LDL lipid hydroperoxides. LDL (1.0 mg protein/ml) in Chelexed PBS was photoperoxidized to a level of ~10.5 mM LOOH and then incubated in the presence of 10 mM GSH alone (◇), 50 μM Ebselen alone (□), or 10 mM GSH plus Ebselen at the following concentrations: 2 μM (○); 10 μM (○); 50 μM (△). At the indicated times during incubation at 25°C, samples were removed for iodometric determination of residual LOOH. Data points are means ± deviation of values from duplicate experiments.

μM Ebselen alone produced only a small drop in LOOH content under the conditions used (< 10% in 1 h). However, incubation with GSH plus Ebselen in increasing concentrations resulted in a strong, dose-dependent decay of LOOH such that at 50 μM Ebselen, ~80% of the LOOH was lost in 1 h. Separate experiments indicated that hydroperoxide decay was accompanied by formation of hydroxy derivatives, e.g., TLC evidence showed that cholesterol hydroperoxides were replaced by diols, mainly 7α-OH and 7β-OH. Similar findings have been reported recently by Maiorino, Roveri, and Ursini (34). The toxicity of GSH/Ebselen-treated LDL_{ox} was tested on Se(-) cells. As shown in Fig. 5, a preparation that was at least 97% depleted in LOOH was completely nontoxic over a wide range of concentrations. Treating LDL_{ox} with GSH alone, followed by dialysis, did not diminish its cytotoxicity (data not shown). Taken together, these results provide strong evidence that LOOHs were the most important toxic species present in photooxidized LDL.

Protective effect of phenolic antioxidants

We were interested in learning whether interventions other than those that enhance the cell's ability to metabolize toxic peroxides might be beneficial. One mechanism by which LOOHs might produce lethal cell injury is via 1-electron reduction to oxyl radicals, which, either directly or indirectly (35), may magnify damage by triggering free radical chain reactions. Oxyl (LO•) and

peroxyl (LOO^\bullet) intermediates can be trapped and detoxified by lipophilic antioxidants such as α -tocopherol, butylated hydroxytoluene, or probucol (1). Probucol is an especially potent free radical interceptor in both LDL and cells (10, 11). Recent studies have shown that it can retard the development of atherosclerotic lesions in cholesterol-fed animals (36). As shown in Fig. 7, probucol provided substantial protection against LDL_{ox} -induced damage to $\text{Se}(-)$ cells, increasing LC_{50} from $\sim 170 \mu\text{M}$ LOOH to $\sim 250 \mu\text{M}$ LOOH. A similar effect has been observed with butylated hydroxytoluene. Thus, the cytotoxic effects of photoperoxidized LDL appear to be mediated by free radical processes.

Involvement of iron in LDL_{ox} cytotoxicity

Redox iron is known to play an important role in many different types of oxidative damage. We investigated the possible involvement of non-heme iron in LOOH-induced cell killing by exposing cells to desferrioxamine (DFO) prior to introducing photoperoxidized LDL. DFO binds Fe^{3+} with very high affinity and prevents it from undergoing redox cycling (37). As shown in Fig. 7, DFO had a large protective effect on $\text{Se}(-)$ BAE cells, increasing LC_{50} for LDL -LOOH from $\sim 170 \mu\text{M}$ to $\sim 350 \mu\text{M}$. Since the LDL_{ox} was effectively metal-free when introduced into the reaction system, we deduced that most of the iron sequestered and inactivated by DFO originated in the cells and/or medium. To further examine iron's involvement in LOOH-mediated cytotoxicity, we studied the effects of supplemental iron in the form of a lipophilic chelate, $\text{Fe}^{3+}/8$ -hydroxyquinoline (1:2), which has been shown to markedly enhance the sensitivity of endothelial cells to H_2O_2 (38). As indicated in Table 2, $1.5 \mu\text{M}$ $\text{Fe}(\text{HQ})_2$ enhanced LDL_{ox} cytotoxicity at all three of the indicated LOOH levels (cf. expts. 6 and 1). A small effect was even noticed at a 10-fold lower concentration of

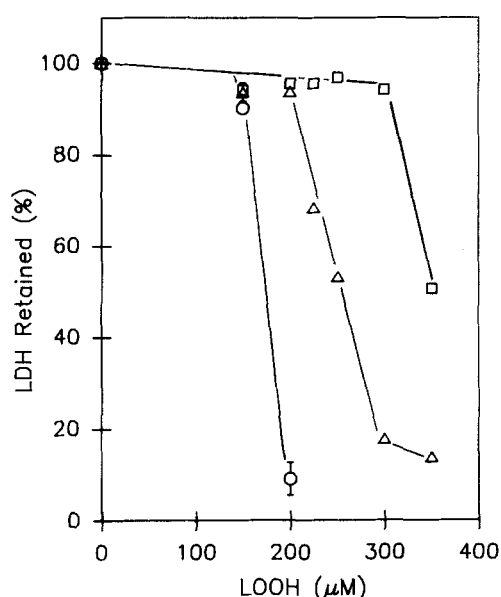


Fig. 7. Effect of desferrioxamine and probucol on cell sensitivity to peroxidized LDL. Confluent $\text{Se}(-)$ cells in 1% FCS/DME-HAM's F-12 medium were preincubated for 3 h in the presence of $50 \mu\text{M}$ probucol (\triangle), $200 \mu\text{M}$ DFO (\square), or no additions (\circ), and then exposed to increasing concentrations of photoperoxidized LDL. Cytotoxicity (based on LDH release) was assessed 20 h after the LDL was introduced. Points with error bars are means \pm deviation of values from duplicate experiments.

$\text{Fe}(\text{HQ})_2$ (cf. expts. 5 and 1 at $200 \mu\text{M}$ LOOH). Ferric iron alone ($1.5 \mu\text{M}$) had no effect. Interestingly, however, HQ alone ($3.0 \mu\text{M}$) produced as large a stimulation of cytotoxicity as did the iron complex (cf. expts. 3 and 6). This effect could be completely blocked by DFO, suggesting that HQ was binding endogenous Fe^{3+} (e.g., in the medium) to generate a complex that acted like "pre-formed" $\text{Fe}(\text{HQ})_2$. DME-HAM's F-12 medium is known to contain 5–10 μM iron in salt form (39). Relatively polar

TABLE 2. Effect of supplemental iron on cytotoxicity of peroxidized LDL

Additions	LDH Retained		
	100 μM LOOH	150 μM LOOH	200 μM LOOH
	%		
1. None	98.7 \pm 2.8	95.5 \pm 6.8	75.9 \pm 8.2
2. FeCl_3 (1.5 μM)	99.5	94.7	ND
3. HQ (3.0 μM)	91.4	43.9	ND
4. HQ (3.0 μM) + DFO (100 μM)	100.0	99.5	99.0
5. $\text{Fe}(\text{HQ})_2$ (0.15 μM)	98.4	93.0	71.7
6. $\text{Fe}(\text{HQ})_2$ (1.5 μM)	71.9 \pm 14.1	52.2 \pm 4.2	34.0 \pm 3.5
7. $\text{Fe}(\text{HQS})_2$ (1.5 μM)	100.0	99.5	75.8
8. $\text{Fe} \cdot \text{EDTA}$ (3.0 μM)	96.6	ND	79.1

Confluent layers of $\text{Se}(-)$ BAE cells in 2% FCS/DME-HAM's F-12 medium without (none) or with the indicated additions of iron and iron chelates/chelators were incubated with LDL_{ox} containing 100 μM , 150 μM , and 200 μM lipid hydroperoxide (LOOH). Oxidized LDL was introduced 30 min after additions 2–8 were made. Cytotoxicity based on LDH release was determined 20 h after adding LDL_{ox} . Values for additions 1 and 6 are means \pm SD (three experiments); other values are for single experiments. ND, not determined.

chelates such as $\text{Fe}(\text{HQS})_2$ or $\text{Fe} \cdot \text{EDTA}$ were completely inactive in our system (Table 2: expts. 7 and 8). These findings are consistent with the idea that $\text{Fe}(\text{HQS})_2$ exacerbates LDL-LOOH cytotoxicity by localizing in highly oxidizable lipid-rich domains, e.g., cell membranes. Localization on the LDL itself may also be an important factor.

Protective effect of Ebselen

Having demonstrated that Ebselen plus GSH can reduce and detoxify LDL_{ox} extracellularly, we asked whether Ebselen might also restore resistance in $\text{Se}(-)$ BAE cells. To examine this, we preincubated $\text{Se}(-)$ cells with increasing (nontoxic) concentrations of Ebselen up to $5 \mu\text{M}$ and then challenged them with LDL_{ox} . As shown in Fig. 8A, Ebselen over the concentration range $2\text{--}5 \mu\text{M}$ was able to completely protect cells against LDL-LOOH at two different toxic levels, $150 \mu\text{M}$ and $250 \mu\text{M}$. At a fixed concentration ($2 \mu\text{M}$), Ebselen increased the LC_{50} of LDL-LOOH from $\sim 75 \mu\text{M}$ to $\sim 250 \mu\text{M}$ (Fig. 8B). Cell viability in these experiments was assessed by MTT assay; similar trends were observed with the less sensitive LDH assay. We have recently shown that Ebselen also protects (albeit partially) Se -deficient L1210 cells against lethal injury induced by $t\text{-BuOOH}$; GPX activity remained low, thus ruling out the release of free Se as a possible reason for this effect (J. P. Thomas, P. G. Geiger, and A. W. Girotti, unpublished results). The Ebselen results indicate that the most important defect in $\text{Se}(-)$ cells was GPX/PHGPX deficiency, and not some other manifestation of selenium deprivation.

DISCUSSION

The involvement of LDL in atherosclerosis has been under intensive investigation for quite some time (2, 10). Recent interest has focused on the role of oxidatively modified LDL in the atherogenic process (2, 3). Oxidative modification of LDL renders it recognizable by scavenger receptors on macrophages, thereby giving rise to "foam" cells (9). If generated *in vivo*, these cells could accumulate as fatty deposits (plaques) in the arterial wall. Endothelial cell damage by oxidized LDL may also play an important role in both atherosclerosis and thrombotic disease. Denuded of endothelium, the arterial wall might serve as a nidus for repair processes, leading to plaque formation and also the appearance of thrombi (2). The physiological role of the scavenger receptor is uncertain. However, an intriguing possibility is that it protects vascular cells from large-scale oxidative damage by constantly removing minimally oxidized LDL from the circulation.

In this study, the GSH, selenium, and iron levels of arterial endothelial cells have been manipulated in order to test the hypothesis that lipid hydroperoxides play a role in LDL_{ox} -induced cytotoxicity. Initial experiments demonstrated that Se -deficient cells are markedly more sensitive to $t\text{-BuOOH}$ or ChOOH s than Se -satisfied counterparts. In the case of $t\text{-BuOOH}$, Se -dependent protection is attributed primarily to GPX, as PHGPX is present in much smaller amounts, and is less efficient in catalyzing $t\text{-BuOOH}$ reduction than GPX (31). Conversely, the Se -dependent detoxification of ChOOH s is ascribed to PHGPX, as our previous work with isolated membrane

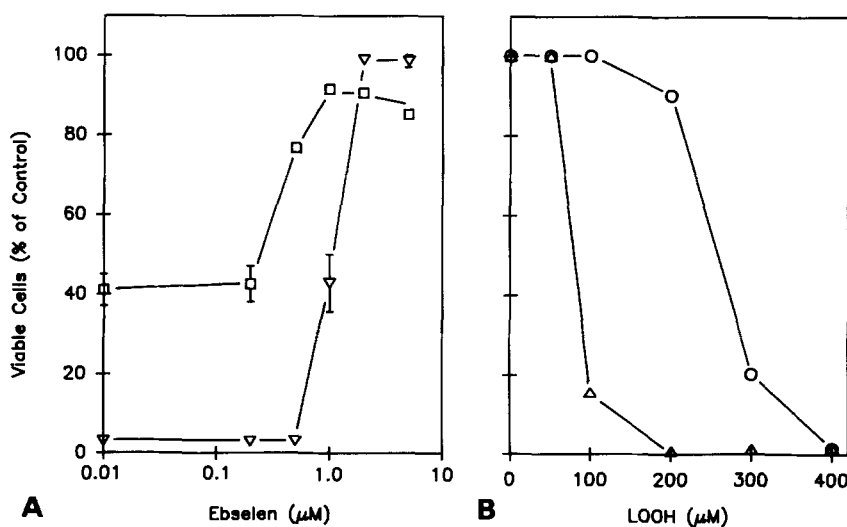


Fig. 8. Susceptibility of Ebselen-treated cells to the cytotoxic effects of peroxidized LDL. (A) Confluent $\text{Se}(-)$ cells in 2% FCS/DME-HAM's F-12 medium were pre-incubated with Ebselen ($0.01\text{--}5 \mu\text{M}$) for 30 min at 37°C , and then exposed to photooxidized LDL containing $150 \mu\text{M}$ LOOH (\square) and $250 \mu\text{M}$ LOOH (∇). (B) Confluent $\text{Se}(-)$ cells were pre-incubated in the absence (\triangle) and presence (\circ) of $2 \mu\text{M}$ Ebselen, and then incubated with increasing concentrations of LOOH in photooxidized LDL. After 24 h in (A) and (B), cell viability was assessed by MTT assay. Points with error bars are means \pm deviation of values from duplicate experiments.

systems indicated that PHGPX can reduce ChOOHs, whereas GPX cannot (16, 18). The present findings further illustrate the complementary role of these two enzymes, GPX acting on H₂O₂ and other relatively polar hydroperoxides such as fatty acid hydroperoxides, and PHGPX acting on these as well as membrane-associated lipid hydroperoxides.

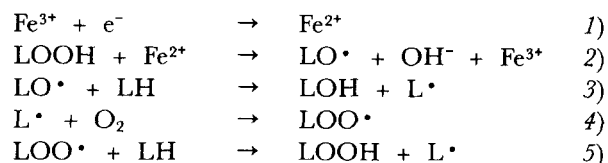
The oxidized LDL used in this study was generated by AlPcS-sensitized photooxidation. Like many other photosensitizing agents, AlPcS generates singlet oxygen (¹O₂), which reacts with unsaturated lipids via the so-called "ene" mechanism (40). This approach for preparing LDL_{ox} has certain advantages over conventional autooxidation approaches, e.g., treatment with Cu²⁺ or azo-initiators (1). For example, LOOHs typically accumulate linearly with time during photooxidation, but not necessarily during free radical-mediated autooxidation. Also, photoperoxidation can be terminated by simply removing light; the sensitizing agent can be left in the reaction mixture, since, at the concentrations used, it rarely shows any "dark" toxicity. By contrast, stopping LDL autooxidation typically requires chelating agents and/or lengthy dialysis to remove toxic initiators. Cells containing a full complement of GSH and Se were found to be relatively resistant to LDL_{ox}. If, however, cellular defenses were compromised by either depleting GSH or Se, a dose-dependent cytotoxicity of LDL-LOOH was observed. Selenoperoxidase-dependent cytoprotection is consistent with the involvement of LOOHs in LDL_{ox}-mediated cell killing. This view was further strengthened by showing that lethal damage could be prevented by pretreating LDL_{ox} with GSH/Ebselen, which reduced virtually all of the LOOH. How toxic LOOHs contacted and entered cells in our system is not yet clear. However, two viable possibilities are *a*) endocytosis of the LDL_{ox} and *b*) some type of lipid transfer or exchange process.

The relative importance of GPX and PHGPX in cellular defense against LDL_{ox} is uncertain at this point. Previous studies carried out on photooxidized erythrocyte membranes (16) and photooxidized LDL (18) have shown that GPX is unreactive with cholesterol ring hydroperoxides, and reacts with phospholipid hydroperoxides only after hydrolytic release of the fatty acyl hydroperoxide groups. By contrast, PHGPX can react directly (but not necessarily at the same rate) with all LOOHs in membranes or LDL (18). It is conceivable that upon interacting with cells, LDL-LOOHs give rise to hydroperoxide species (self-derived or from cellular lipids) that are directly metabolizable by GSH/GPX. How this pathway compares in importance with one involving direct reduction of LOOHs by GSH/PHGPX remains to be seen.

Of special interest vis-a-vis Se-based defense against cytotoxic LDL_{ox} is our finding that the selenoperoxidase mimetic Ebselen exerts a strong protective effect on Se(-) BAE cells. Currently being evaluated as an antioxidant

and antiinflammatory agent, Ebselen has been shown to be very effective in inhibiting free radical lipid peroxidation in isolated hepatocytes (41). Recent studies have indicated that Ebselen can react with a wide variety of hydroperoxides, including phospholipid, cholesterol, and cholesteryl ester hydroperoxides (34). Thus, the compound acts more like PHGPX than GPX. Indeed, in studies to be reported elsewhere, we have shown that Ebselen is ineffective in protecting Se(-) BAE cells against t-BuOOH, but highly effective against LDL_{ox}, ChOOH, or photoperoxidative insult.

Desferrioxamine and probucol were found to be potent inhibitors of photooxidized LDL's cytotoxic effects, suggesting that iron and free radical intermediates are involved in the cytotoxic process. In addition, we found that oxidative cell damage could be exacerbated by supplementing the system with ferric-8-hydroxyquinoline, a lipophilic (membrane-directed) chelate. A high degree of site selectivity was demonstrated by showing that relatively polar chelates such as ferric-8-hydroxyquinoline-5-sulfonate or ferric-EDTA have no effect. Based on these observations, a plausible reaction pathway is as follows:



According to this scheme, LOOHs in LDL_{ox} undergo iron-catalyzed reduction to LO radicals (Eqs. 1 and 2). The latter trigger chain peroxidation reactions (Eqs. 3–5), in which damage may be "broadcasted" far from its site of origin, the LOOH delivered by LDL_{ox}. The electron donor that might initiate this series of reactions is unknown, but could conceivably be superoxide (O₂⁻) generated by the host cell. There is good evidence that certain cells (e.g., activated monocytes/macrophages and smooth muscle cells) produce O₂⁻ at relatively high rates (3, 42) and that their ability to promote LDL oxidative damage in vitro is mediated at least in part by O₂⁻ (43, 44). By reducing LOOHs as they enter cells on LDL_{ox} or after translocation from LDL_{ox} onto cell membranes, PHGPX (or GPX) would attenuate the cytodamaging effects of chain peroxidation. It is reasonable to expect that PHGPX plays a major role in this process, since *a*) the enzyme can reduce and detoxify membrane- or LDL-LOOHs directly (18), and *b*) some copies of the enzyme reside on cell membranes (31).

Up to now, relatively little attention has been given to the question of how target cells might cope with LDL_{ox}-mediated damage by utilizing enzymatic defenses associated with the glutathione cycle. The present findings suggest approaches by which selenium-based defenses might be augmented (e.g., administration of Se or Ebselen).

len) so as to minimize both the formation of LDL_{ox} and the damage it produces in target cells. The rationale for considering these approaches appears well founded, as a correlation has been observed between selenium deficiency in humans and increased incidence of atherosclerotic heart disease (45, 46). ■■

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