ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 315, No. 2, December, pp. 244–254, 1994

Involvement of Preexisting Lipid Hydroperoxides in Cu^{2+}-Stimulated Oxidation of Low-Density Lipoprotein[1]

James P. Thomas,*,† B. Kalyanaraman,‡ and Albert W. Girotti*,[2]

*Department of Biochemistry, †Department of Internal Medicine, and ‡Biophysics Research Institute, Medical College of Wisconsin, Milwaukee, Wisconsin, 53226

Received April 26, 1994, and in revised form August 5, 1994

Oxidative modification of human low-density lipoprotein (LDL) is thought to play an important role in the development of atherosclerosis. LDL oxidizability is believed to be strongly influenced by factors such as (a) content of preexisting lipid hydroperoxides (LOOHs) and (b) content of endogenous antioxidants such as α-tocopherol and β-carotene. The purpose of this study was to examine the prooxidant role of preexisting LDL–LOOHs, using a recently developed method for ultrasensitive and selective LOOH analysis: high-performance liquid chromatography with mercury drop electrochemical detection (HPLC-EC). Exceedingly low detection limits for LDL–LOOHs have been achieved by HPLC-EC, e.g., ~100 fmol for cholesteryl ester hydroperoxide (CEOOH). This sensitivity has allowed us to monitor LDL–LOOHs at levels that are undetectable by most other methods. Fresh LDL prepared with the utmost care to prevent autoxidation was found to contain small, yet significant amounts of CEOOH, 6–12 pmol/mg protein. Our data suggest that these peroxides could not have arisen during LDL isolation or sample work-up for HPLC-EC. Incubation with GSH and phospholipid hydroperoxide glutathione peroxidase resulted in nearly complete reduction of the CEOOH. This LDL was found to be much more resistant to Cu^{2+}-induced peroxidation than starting material, exhibiting a lag period that was at least six times greater. We have also determined that LDL becomes progressively more susceptible to Cu^{2+}-induced lipid peroxidation (as evidenced by a shortened lag) when it is preloaded with increasing amounts of photochemically generated LOOHs. Taken together, these results provide strong support for the idea that preexisting LOOHs in LDL are important determinants of its overall oxidizability.

Key Words: **low-density lipoprotein; atherogenesis; lipid peroxidation; lipid hydroperoxides; phospholipid hydroperoxide glutathione peroxidase.**

Various lines of evidence suggest that oxidatively modified low-density lipoprotein (LDL)[3] plays a causal role in atherogenesis. For example, autoantibodies against oxidized LDL have been identified in human plasma, supporting the notion that this material exists *in vivo* (1). LDL that has been oxidized by exposure to trace metals (Cu^{2+}, Fe^{2+}), lipoxygenase, or endothelial cells is rapidly taken up by macrophages through a scavenger receptor pathway (2–4). Not being feedback-inhibited, this process can lead to foam cell formation, with eventual accumulation of atherosclerotic plaques (5). Interventions that decrease LDL oxidizability *in vitro*, e.g., use of antioxidants such as ascorbate or probucol, exert a marked antiatherogenic effect in experimental animals (6).

LDL oxidation has been studied extensively *in vitro* over the past decade (2–4, 7). In many of these studies, oxidation was triggered by redox metal ions, most notably Cu^{2+} (7). Among other things, it was shown that (a) unsaturated lipids are peroxidized, (b) endogenous antioxidants are consumed by reacting with free radical intermediates, and (c) apoB-100 protein is irreversibly modified such that it migrates more anodically on agarose gel electrophoresis (7). These processes can be effectively inhibited by the addition of lipophilic antioxidants (8) or metal chelators (9). It has been proposed that the susceptibility of LDL to free radical-mediated lipid peroxidation is dependent on its content of antioxidants as well as LOOHs that can drive propagation reactions. Endogenous an-

[1] This work was supported by NIH Grant HL47250.

[2] To whom correspondence should be addressed. Fax: (414) 266-8497.

[3] Abbreviations used: LDL, low-density lipoprotein; $AlPcS_4$, chloroaluminum phthalocyanine tetrasulfonate; CEOOH, cholesteryl ester hydroperoxide; CLOOH, cholesteryl linoleate hydroperoxide; HPLC-EC, high-performance liquid chromatography with electrochemical detection; LOOH, lipid hydroperoxide; 7α/7β-OOH, mixture of 3β-hydroxycholest-5-ene-7α-hydroperoxide and 3β-hydroxycholest-5-ene-7β-hydroperoxide; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLPC-OOH, hydroperoxide derived from 1-palmitoyl-2-linoleoyl phosphatidylcholine; TBARS, thiobarabituric acid-reactive substances; TLOOH, trilinolein hydroperoxide; BSP, bromosulfophthalein; PBS, phosphate-buffered saline.

0003-9861/94 $6.00

tioxidants such as α-tocopherol and ubiquinol-10 can scavenge peroxyl radical intermediates, thus prolonging the lag period that occurs before the onset of rapid LDL oxidation (10).

Whether preexisting LOOHs can sensitize LDL to further oxidative modification has been a subject of great interest. Thomas and Jackson (11) showed that pretreatment of LDL with GSH and the selenoperoxidase mimetic ebselen substantially inhibits Cu^{2+}-induced peroxidation, presumably by removing low levels of starting LOOHs, although such levels were not measured. Progress in this area has been limited by the inability of many conventional methodologies to detect trace levels of LOOH in LDL. However, many of these limitations have been eliminated with the advent of high-performance techniques for LOOH analysis. For example, using HPLC with chemiluminescence detection, Miyazawa (12) was able to measure at least 10 μM phosphatidylcholine hydroperoxide in freshly isolated plasma from healthy humans. On the other hand, Stocker *et al.* (10), using the same general approach, were unable to detect LOOHs in freshly prepared LDL, but could track their formation after antioxidant depletion during incubation with an azo initiator (peroxyl radical generator).

We have used several new strategies for determining LDL–LOOHs and for assessing the role of preexisting LOOHs in Cu^{2+}-stimulated LDL oxidation. Experiments were carried out on (a) freshly isolated LDL and (b) material that had been subjected to dye-sensitized (singlet oxygen-mediated) photooxidation to elevate its LOOH content. The availability of a new technique for ultrasensitive LOOH analysis, HPLC with mercury drop electrochemical detection (13, 14), was especially important in this work. Of additional importance was the recognition that LDL–LOOHs can be reductively inactivated in highly specific fashion by treating with GSH and phospholipid hydroperoxide glutathione peroxidase (PHGPX). We reported previously that all major classes of photochemically generated LOOH in LDL are susceptible to PHGPX-catalyzed reduction (15, 16). Our results indicate that freshly prepared LDL isolated under the most scrupulous conditions possible to prevent autoxidation contains trace amounts of peroxidized lipid (<0.01 mol CEOOH/mol protein), which are sufficient to initiate chain peroxidation reactions that profoundly alter overall structure.

MATERIALS AND METHODS

General materials. Cholesterol, cholesteryl linoleate, egg phosphatidylcholine, 1-palmitoyl-2-linoleoyl phosphatidylcholine, trilinolein, ubiquinone-10 (coenzyme Q_{10}), Chelex-100 (50–100 mesh), NADPH, GSSG reductase, GSH, and Sepharose–bromosulfophthalein (BSP)–glutathione affinity resin were supplied by Sigma Chemical Co. (St. Louis, MO). *Other chemicals were obtained from the following sources:* peroxide-free Triton X-100 from Boehringer-Mannheim Biochemicals (Indianapolis, IN), 2-thiobarbituric acid from Aldrich Chemical Co. (Milwaukee, WI), desferrioxamine from Ciba-Geigy Corp. (Suffern, NY), and $AlPcS_4$ from Porphyrin Products (Logan, UT). A cholesterol hydroperoxide standard (7α-OOH and 7β-OOH mixture) was prepared and characterized as described previously (14). HPLC grade chromatographic solvents were from Burdick and Jackson Corp. (Muskegon, MI). All other reagents and chemicals were of the highest purity available and all aqueous solutions were prepared with deionized, glass-distilled water.

Preparation of low density lipoprotein. LDL was prepared from freshly drawn human blood (in 1.0 mM EDTA) obtained from male donors (age 25–40) that had fasted overnight. Plasma from two to three individuals was pooled and the LDL was isolated by flotation ultracentrifugation in KBr (17). All preparative solutions were saturated with argon and dialyzed against Chelex-100. The isolated LDL fraction (density 1.019–1.063 g/ml) was dialyzed against deaerated PBS containing 1.0 mM EDTA and stored under argon at 4°C. Protein content was determined according to Lowry *et al.* (18), using bovine serum albumin as the standard. LDL ran as a single band on agarose gel electrophoresis (5). Immediately before experimental use, stock solutions were freed of EDTA by dialyzing exhaustively against Chelex-treated PBS under argon.

Preparation of phospholipid hydroperoxide glutathione peroxidase. The selenoenzyme PHGPX was isolated from rat testes and purified according to Maiorino *et al.* (19), with minor modifications. Approximately 10 g of tissue was homogenized for 5 min in 0.1 M Tris–HCl (pH 7.4) containing 5 mM β-mercaptoethanol. The homogenate was centrifuged for 15 min at 15,000*g*; the recovered supernatant fluid was passed through cheesecloth and then recentrifuged for 45 min at 100,000*g*. This supernatant was dialyzed exhaustively against 10 mM potassium phosphate (pH 7.4) containing 5 mM β-mercaptoethanol and then loaded onto a DEAE-Sepharose 6B (1 × 10-cm) column. The column was washed with loading buffer (19), after which PHGPX was eluted with 0.2 M potassium phosphate/5.0 mM β-mercaptoethanol (pH 7.0). Glycerol (10%) was added to the pooled active fractions (see below), and the material was loaded onto a 4 ml Sepharose–BSP–glutathione affinity column that had been equilibrated with 10 mM potassium phosphate (pH 7.0)/5 mM β-mercaptoethanol/10% glycerol. PHGPX was then eluted with 25 mM Tris–HCl (pH 7.6)/0.5 M KSCN/5 mM β-mercaptoethanol/10% glycerol. The enzyme solution was concentrated to <2 ml by ultrafiltration, loaded onto a Sephadex G-75 column, and eluted with the same buffer mixture used on the affinity column. The active fractions were pooled and concentrated to <5 ml. PHGPX purified in this fashion migrated as a single band of molecular weight ~20 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme was stored at −80°C; immediately before experimental use, its activity was determined by coupled enzymatic assay, using NADPH, GSH, GSSG reductase, and photoperoxidized egg phosphatidylcholine (20).

Oxidation of low density lipoprotein. Photoperoxidation was carried out as follows. LDL (1.0 mg protein/ml in Chelex-treated PBS) was sensitized with $AlPcS_4$ (10 μM) and irradiated in a thermostatted beaker at 10°C (21). The light source was a 90-W quartz–halogen lamp positioned above the reaction vessel. Light intensity (fluence rate) at the solution surface was measured with a Yellow Springs Radiometer (Yellow Springs, OH) and maintained at ~30 mW/cm^2 for all reactions. Solutions were open to the air and stirred magnetically during irradiation for increasing periods up to 30 min. After photooxidation, LDL lipids were recovered by extracting with chloroform/methanol (2/1, v/v). The $AlPcS_4$ remained entirely in the aqueous fraction during extraction. The organic fraction containing LDL–LOOHs was dried under a stream of argon and stored at −20°C for iodometric or HPLC-EC analysis. Samples were typically analyzed within a week after preparation.

Cupric ion-induced oxidation of LDL was carried out at 37°C in a Yellow Springs Stirrer Bath (Yellow Springs, OH). The typical reaction mixture contained 1.0 μM $CuCl_2$ and 0.1 mg LDL protein/ml in Chelex-treated PBS. At various intervals, samples were removed for determination of TBARS (see below).

Preparation of CLOOH, TLOOH, and PLPC-OOH standards. CLOOH, TLOOH, and PLPC-OOH were generated photochemically by irradiating each of the parent lipids (1.0 mg/ml in chlo-

roform/methanol (1/1, v/v)) for 30 min in the presence of 10 μM $AlPcS_4$. Following the addition of an equal volume of metal-free PBS, the peroxides were extracted and prepared for analysis as described for LDL–LOOHs.

Iodometric assay. The absolute LOOH content of standards and oxidized LDL samples was determined by iodometric assay (22). LOOHs were reduced anaerobically in the presence of excess iodide, with stoichiometric formation of triiodide. The latter was measured spectrophotometrically at 353 nm, quantitation being based on an extinction coefficient of 22.5 mM^{-1} cm^{-1}. Other details were as described (22).

High-performance liquid chromatography. Analytical reverse-phase HPLC was carried out at 25°C, using an Isco integrated system equipped with a C_{18} Ultrasphere column (4.6 × 150 mm; 5-μm particles) from Beckman Instruments (San Ramon, CA) and a C_{18} guard column (4.6 × 45 mm) from Alltech Associates (Deerfield, IL). The premixed mobile phase consisted of 54% isopropanol, 23% acetonitrile, 14% methanol, and 9% aqueous solution (pH 6.2) containing 10 mM ammonium acetate and 0.25 mM sodium perchlorate (13). Before and during use, this mixture was sparged with high-purity (99.998%) argon (Airco, West Allis, WI) that was passed sequentially through a Supelco OM-1 oxygen scrubber (Bellefonte, PA) and a mobile phase scrubber. The deoxygenated mobile phase was delivered isocratically at a flow rate of 1.0 ml/min. Column effluent was passed through two detectors in series: (a) an Isco V^4 variable wavelength detector set at 205 nm for cholesterol or 234 nm for conjugated dienes, and (b) an EG&G-Princeton Model 420 electrochemical detector with renewable mercury drop electrode (Princeton, NJ). The electrode potential was set at −150 mV vs a Ag/AgCl reference; this was sufficient to effect the complete reduction of all hydroperoxides examined. A fresh mercury drop was dispensed and equilibrated before each sample injection. An equilibration time of 3–4 min was sufficient to reduce background noise to optimally low levels. Immediately before injection, samples were dissolved in isopropanol and sparged for 1–2 min with helium. The injection volume was 10 μl for all samples. Output signals were collected and analyzed using Isco/ChemResearch software. Other details were as described elsewhere (14).

Thiobarbituric acid assay. TBARS in oxidized LDL samples were determined spectrophotometrically as described (23). An extinction coefficient of 157 mM^{-1} cm^{-1} (obtained with a malonaldehyde standard) was used for converting absorbance readings at 532 nm to TBARS values.

Other procedures. α-Tocopherol content of LDL samples was determined by HPLC with fluorometric detection (24). Total glutathione equivalents (GSH + 2 GSSG) were determined by recycling assay (25).

RESULTS

HPLC-EC analysis of lipid hydroperoxides. Different preparations of human LDL, each with no detectable LOOH by conventional iodometric analysis, were found to vary greatly in their susceptibility to Cu^{2+}-mediated oxidation, as indicated by duration of the initial lag period (J. P. Thomas and A. W. Girotti, unpublished observation). This prompted us to attempt measurement of trace LOOH levels in freshly prepared LDL by an ultrasensitive technique recently developed in this laboratory, HPLC-EC. Initial separations and determinations were performed with LOOH standards that are representative of some of the major lipid classes found in LDL, viz. cholesterol, cholesteryl ester, phosphatidylcholine, and triacylglycerol. In Fig. 1, HPLC profiles with EC detection and uv detection are compared for (A) a cholesterol hydroperoxide (epimeric mixture of 7α- and 7β-OOH); (B) trilinolein hydroperoxide (TLOOH); (C) 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (PLPC-OOH); and (D) CLOOH. Under the HPLC conditions used, the 7α/7β-OOH and CLOOH samples each exhibited a single EC peak, the former at 2.8 min (Fig. 1A) and the latter at 9.8 min (Fig. 1D). By contrast, TLOOH and PLPC-OOH showed multiple EC peaks, the former at least three (2.2, 3.0, and 5.7 min) (Fig. 1B), and the latter at least two (~3.5 and ~5.5 min) (Fig. 1C). In the case of TLOOH, PLPC-OOH, and CLOOH, the EC peaks overlapped with uv (234 nm) peaks representing conjugated dienes. However, compared with EC detection, absorbance at 234 nm is nonspecific, since hydroperoxides cannot be distinguished from coeluting hydroxide analogues (14). In accordance with this, treating CLOOH with triphenylphosphine, a deoxygenating agent which converts hydroperoxides to hydroxides (26), abolished its EC signal (Fig. 2A) without significantly affecting the uv (234 nm) signal (Fig. 2B). Similar results were obtained with 7α/7β-OOH, PLPC-OOH, and TLOOH (data not shown). In addition to being nonspecific, uv (234 nm) detection is relatively insensitive (the detection limit for CLOOH being estimated to be ~20 pmol) and does not allow measurement of nonconjugated species such as cholesterol hydroperoxides.

HPLC-EC analysis of photooxidized LDL. Initial HPLC-EC determinations of LDL–LOOH were carried out on lipoprotein that had been enriched in LOOH by dye-sensitized photooxidation. The LDL was irradiated in the presence of $AlPcS_4$, a polar phthalocyanine derivative that sensitizes photooxidation predominantly via singlet oxygen ($^1O_2$) intermediacy (27). Singlet oxygen adds stoichiometrically to unsaturated lipids, giving allylic hydroperoxides (28). Thus, $^1O_2$-mediated lipid peroxidation (exclusive of any redox metal interaction) differs from free radical-mediated (chain) peroxidation in being easier to control (by simply applying or removing light) and more predictable in terms of LOOH yield. Moreover, unlike free radical peroxidation of LDL, $^1O_2$-mediated peroxidation does not exhibit a lag phase during which α-tocopherol and other chain-breaking antioxidants are depleted (7). Thus, although α-tocopherol is an excellent $^1O_2$ acceptor (29), its oxidation during dye/light LDL treatment would not necessarily preempt that of unsaturated lipids, as believed to be the case for Cu^{2+}-mediated oxidation (7). Irradiation of $AlPcS_4$-sensitized LDL for 5 and 30 min (corresponding to light fluences of ~9 and ~54 J/cm^2, respectively) resulted in a progressive accumulation of species bearing conjugated diene groups, as detected by uv absorbance at 234 nm (Figs. 3B and 3C, upper traces). Corresponding EC peaks are observed for most of the uv peaks, indicating that at least some of the conjugated dienes represent hydroperoxides. At the indicated sensitivity levels, no distinct UV or EC peaks were observed for nonphotooxidized LDL (Fig. 3A). The most prominent uv- or EC-detectable species in photooxidized LDL was identified as cholesteryl ester hydroperoxide

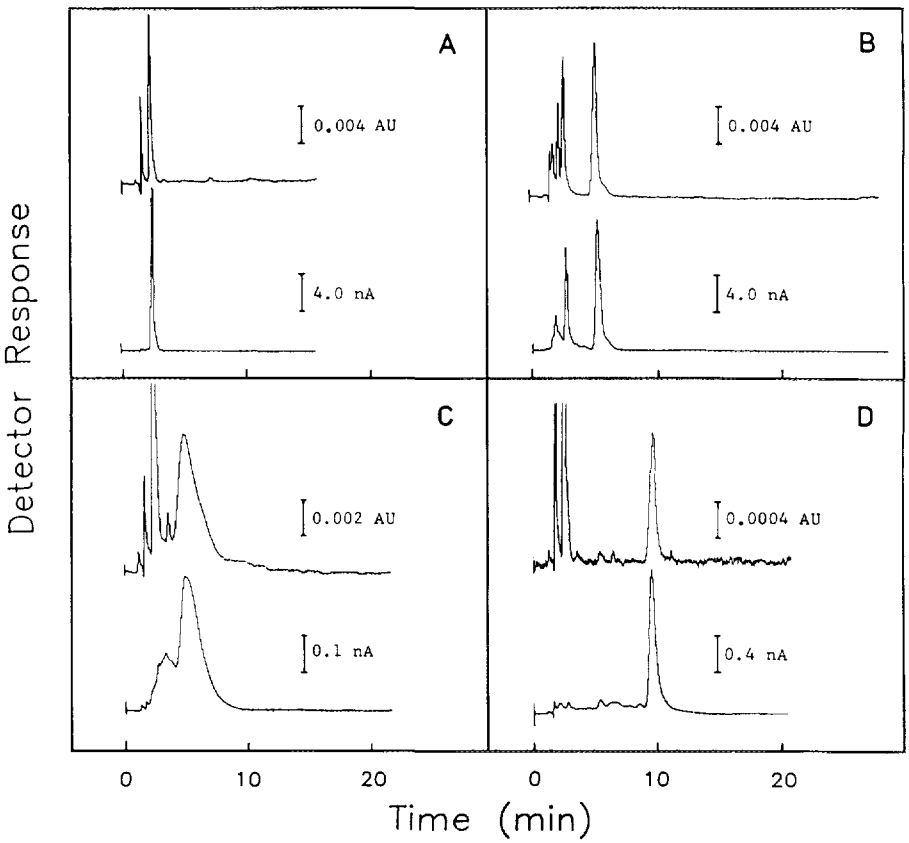

FIG. 1. HPLC of photochemically generated lipid hydroperoxide standards. (A) 7α/7β-OOH (0.3 nmol): top trace, uv detection (205 nm); bottom trace, EC detection (10-nA full-scale sensitivity). (B) TLOOH (2.0 nmol): top trace, uv detection (234 nm); bottom trace, EC detection (10-nA full-scale sensitivity). (C) PLPC-OOH (0.25 nmol): top trace, uv detection (234 nm); bottom trace, EC detection (5-nA full-scale sensitivity). (D) CLOOH (0.2 nmol): top trace, uv detection (234 nm); bottom trace, EC detection (10-nA full-scale sensitivity).

(CEOOH), based on matching retention time of a CLOOH standard (Fig. 3C). Two other peaks were also assigned, a sharp one at ~2.5 min attributed to cholesterol ring hydroperoxide (matching time of 7α/7β-OOH standard) and a broad one at ~5.5 min attributed to phospholipid hydroperoxide (matching time of PLPC-OOH standard). An unusual species eluting at ~11.0 min appears to be a hydroperoxide that lacks conjugated diene groups, since it exhibits an EC signal, but no uv (234 nm) signal (Fig. 3C). Although photooxidized LDL probably contained triacylglycerol hydroperoxide, this could not be distinguished under the conditions used because of low yield (triacylglycerol being the least abundant LDL lipid) and poor resolution from phospholipid hydroperoxide.

Enzymatic reduction of photoperoxidized LDL: Effect on Cu^{2+}-stimulated peroxidation. As shown in Fig. 4, a 30-min incubation of photooxidized LDL (29 μM in total LOOH) with GSH (5 mM) and PHGPX (10 mU/ml) resulted in almost complete disappearance of the CEOOH peak on HPLC-EC (compare traces a and b). Other peroxides were similarly reduced. (Desferrioxamine was included in the reaction mixtures to prevent iron-catalyzed decomposition of LOOHs.) Samples from control mixtures lacking GSH or PHGPX showed essentially the same HPLC-EC profiles as starting material (Fig. 4, traces c and d). Thus, all LOOHs in photoperoxidized LDL are susceptible to reduction and detoxification, as demonstrated previously by thin layer chromatographic analysis (15, 16).

Photoperoxidized LDL was found to be more susceptible to Cu^{2+}-induced free radical peroxidation. This was observed as a more rapid onset of TBARS formation relative to a nonirradiated control (Fig. 5). At the relatively low Cu^{2+} concentration used in this experiment, 0.2 μM, there was no increase in the TBARS level of the control over an incubation period of 6 h. A 30-min exposure of photooxidized LDL to GSH/PHGPX as described in the legend to Fig. 4 resulted in a large decrease in its ability to undergo free radical peroxidation, as indicated by a lag period at least as long as that of the control (Fig. 5). This is consistent with the almost complete disappearance of CLOOH and other peroxides after GSH/PHGPX treatment (Fig. 4). Incubation of photooxidized LDL with GSH alone or PHGPX alone had virtually no effect on the time

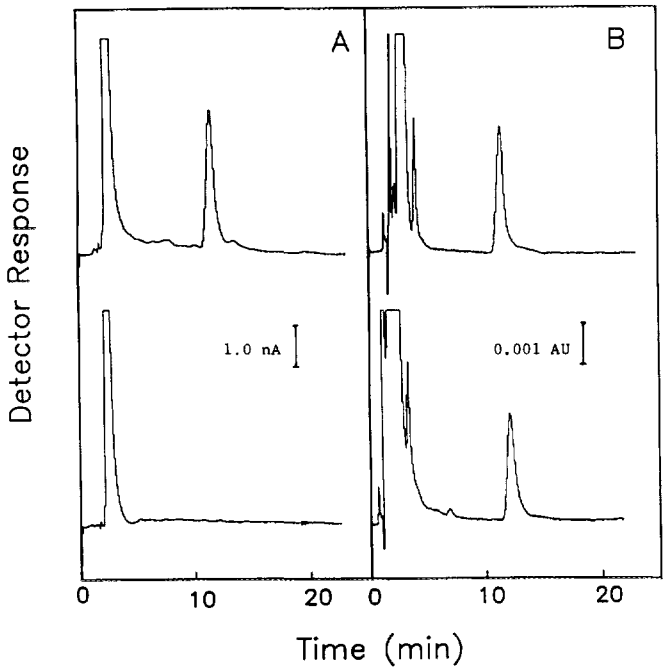

FIG. 2. Comparison of electrochemical detection and ultraviolet absorbance detection of cholesteryl linoleate hydroperoxides. A sample containing 1.1 nmol of photochemically generated CLOOH was analyzed by HPLC before (upper traces) and after (lower traces) treatment with triphenylphosphine. Treatment consisted of incubating the peroxide for 5 min at room temperature with a 58-fold molar excess of triphenylphosphine in hexane. (A) EC detection (5 nA full scale); (B) uv (234 nm) detection (0.005 AU full scale).

course of Cu^{2+}-stimulated TBARS formation, in agreement with our observation that LOOHs were not affected in these controls (Fig. 4).

Cholesteryl ester hydroperoxide content of freshly prepared LDL. Cholesteryl ester is the most abundant lipid class found in LDL (30). The fact that CEOOH appears as a distinct, well-resolved peak on HPLC-EC (Figs. 1–4) prompted us to look for the presence of this peroxide family in freshly prepared LDL. On a constant lipid basis, these determinations required much higher EC sensitivities than were necessary for photooxidized LDL. For accurate quantitation of LDL-CEOOH, we first examined the EC response characteristics of a CLOOH standard. The use of CLOOH as a reference standard is appropriate, since cholesteryl linoleate is the major cholesteryl ester found in LDL (30). As shown in Fig. 6, the EC response (peak area) is linear with increasing amount of injected CLOOH over a 0.1–4.0 pmol range. Additional data (not shown) have indicated that linearity is maintained out to at least 10 nmol of peroxide. Under the chromatographic conditions used, the detection limit for CLOOH was found to be ~100 fmol, based on a signal-to-noise ratio of 3:1 at a full scale sensitivity of 0.1 nA. This exceeds the sensitivity limits reported for other detection methods, e.g., 2–5 pmol with chemiluminescence detection (10). When analyzed by HPLC-EC at a full-scale sensitivity setting of 0.1 nA, a preparation of "virgin" LDL (examined within 24 h after isolation) exhibited a well-defined CEOOH peak at 9.9 min and a larger peak at 14.1 min representing ubiquinone-10 (Fig. 7A, top trace). The other peaks observed in this chromatogram have not yet been assigned. Integration of the CEOOH peak and quantitation based on standardized CLOOH (Fig. 6) indicated that this particular LDL preparation contained 11.2 pmol CEOOH/

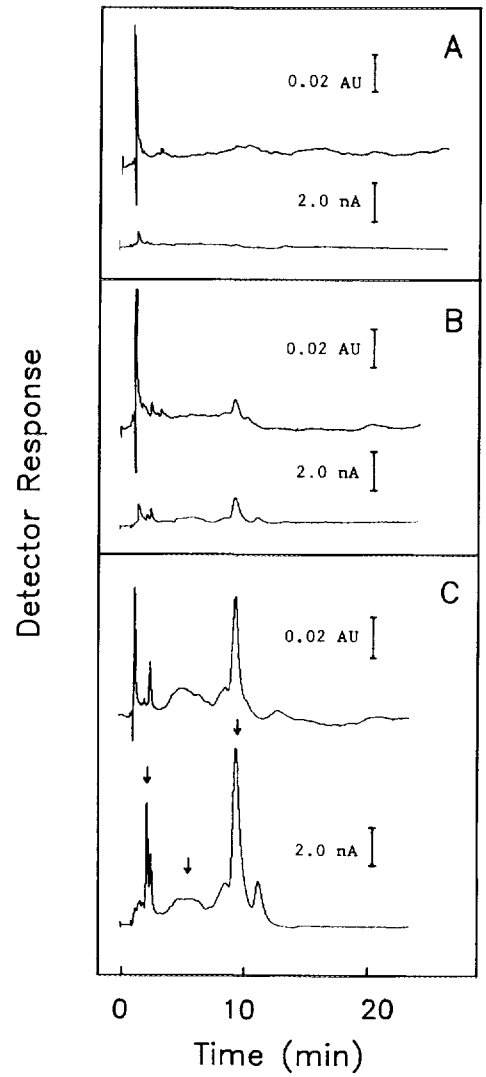

FIG. 3. HPLC-EC of lipid hydroperoxides in photooxidized LDL. LDL (1.0 mg protein/ml in Chelex-treated PBS) was sensitized with 10 μM $AlPcS_4$ and either held in the dark for 30 min (A) or irradiated for 5 min (B) and 30 min (C), after which lipids were extracted and analyzed by HPLC. Top trace in each panel represents uv (234 nm) detection at 0.1 AU full scale; bottom trace represents EC detection at 20 nA full scale. Total amount of injected LOOH (iodometrically determined) was as follows: <0.3 nmol (A), 2.8 nmol (B), 14.6 nmol (C). Total LDL lipid was ~150 μg per injection (A–C). Vertical arrows in C show retention times of separately chromatographed LOOH standards (EC detection): 7α/7β-OOH (2.5 min), PLPC-OOH (5.5 min), CLOOH (9.8 min).

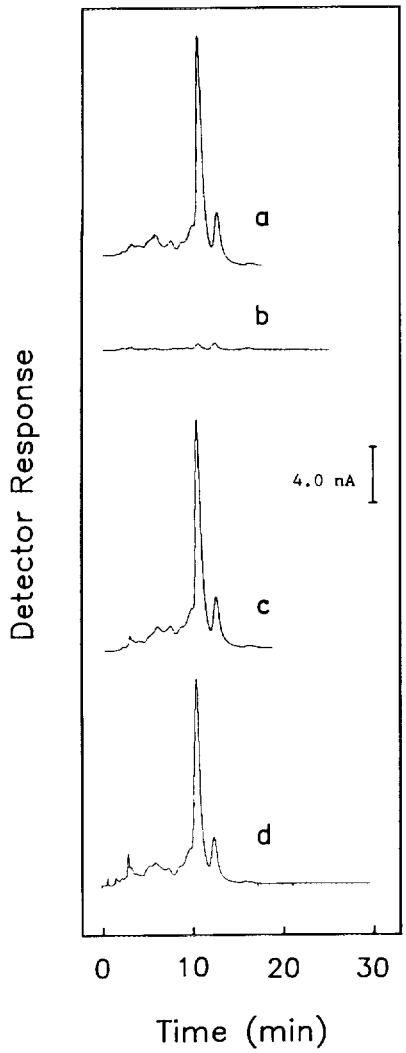

FIG. 4. Enzymatic reduction of lipid hydroperoxides in photooxidized LDL. LDL (1.0 mg protein/ml) in Chelex-treated PBS was photoperoxidized as described in the legend to Fig. 3. Lipids were extracted and analyzed by HPLC-EC before (a) and after (b) incubation of the preparation (29 μM total LOOH) for 30 min at 37°C in the presence of DFO (50 μM), GSH (5.0 mM), and PHGPX (10 mU/ml). A control incubated for 30 min in the presence of only DFO/PHGPX (c) or only DFO/GSH (d) was also analyzed. Incubations were carried out in the dark to prevent any additional photooxidation. Total LDL lipid per injection (a–d) was 56 μg. Full-scale detector sensitivity was 20 nA.

mg protein. This and three other fresh preparations gave the following composite value: 9.8 ± 2.2 pmol CEOOH/mg protein (mean ± SD, n = 4). This translates to approximately 1 CEOOH for every 200 LDL particles, assuming a molecular weight of 515 kDa for the apoB-100 protein (7). Based on reported sensitivity limits, it would be difficult to determine this low level of peroxide by other high performance methods currently in existence (10), and impossible by conventional iodometric analysis (22). Pristine LDL preparations were found to contain 16.7 ± 3.4 nmol α-tocopherol/mg protein (mean ± SD, n = 3), which agrees well with published values for non-vitamin E-supplemented donors (7). That the α-tocopherol content was within the normal range argues against the possibility that our preparations contained CEOOH because their vitamin E levels were either abnormally low or depleted during work-up. When freshly isolated LDL was stored at 4°C in 1 mM EDTA/Chelex-treated PBS, there was a progressive increase in the peak area of CEOOH relative to ubiquinone (compare top traces in Figs. 7A–7C). For this particular LDL preparation, the CEOOH content increased to 25 pmol/mg protein after 2 weeks (Fig. 7B) and to 296 pmol/mg protein after 6 weeks (Fig. 7C). Thus, over a 6-week interval, the peroxide level increased nearly 30-fold, despite the precautions taken to prevent autoxidation. Though elevated to this extent in the aged LDL, total [LOOH] was still too low to be measured by conventional iodometric analysis. In addition, there was no detectable signal in a TBARS assay.

Enzymatic reduction of lipid hydroperoxides in freshly prepared LDL: Effect on Cu^{2+}-stimulated peroxidation. When the freshly isolated LDL represented in Fig. 7A (top trace) was incubated with GSH/PHGPX under

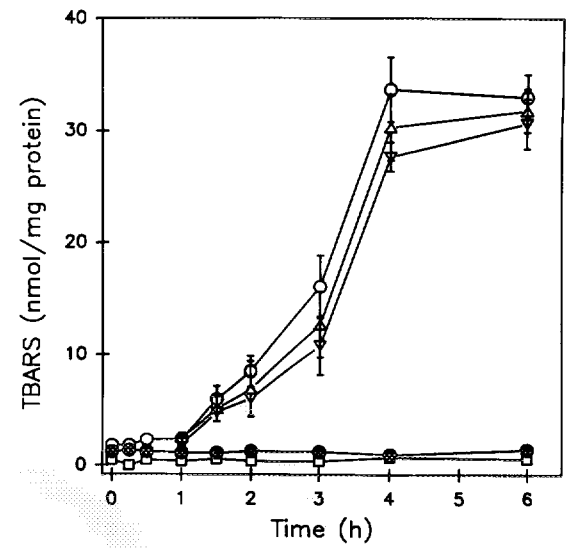

FIG. 5. Cu^{2+}-stimulated lipid peroxidation in photoperoxidized LDL. LDL was charged with LOOHs by irradiating for 30 min in the presence of $AlPcS_4$ (cf. Fig. 3). The preparation was exposed to Cu^{2+} before and after treatment with GSH and PHGPX; material from the experiment described in the legend to Fig. 4 is represented. After mixing with DFO, the peroxidized LDL (1.0 mg protein/ml) was incubated in the dark with GSH/PHGPX, PHGPX alone, or GSH alone for 30 min at 37°C. Samples were then dialyzed to remove GSH, diluted to 0.1 mg protein/ml, and incubated at 37°C in the presence of 0.2 μM $CuCl_2$. At the indicated times, samples equivalent to 50 μg of LDL protein were removed and analyzed for thiobarbituric acid-reactive substances (TBARS): nonphotooxidized LDL (●), photooxidized LDL before (○) and after treating with PHGPX (△), GSH (▽), or GSH plus PHGPX (□). Data points with error bars are means ± deviation of values from duplicate experiments. The results are representative of those obtained with at least three different LDL preparations.

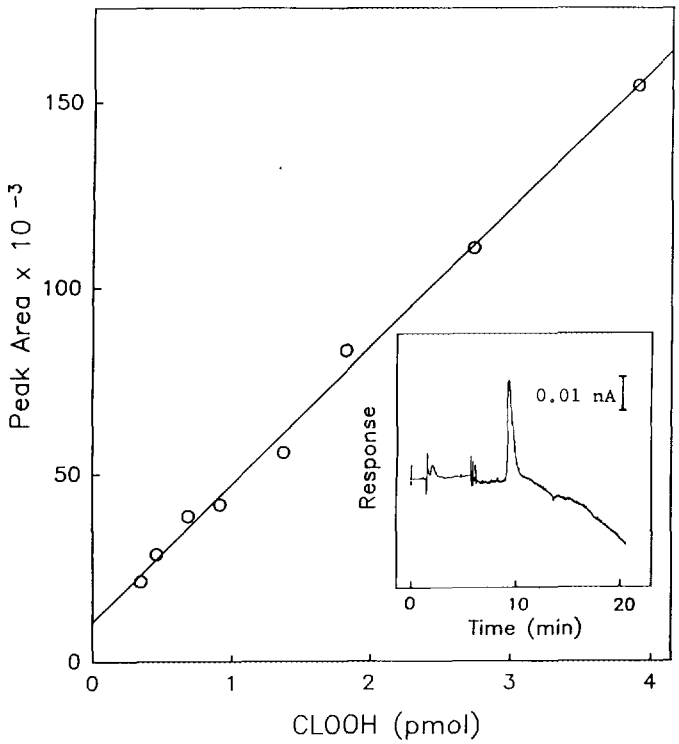

FIG. 6. Amperometric response curve for cholesteryl linoleate hydroperoxide standard. Responses shown are integrated HPLC peak areas obtained with increasing amounts of an iodometrically determined CLOOH standard. Peak areas were normalized to a detector sensitivity of 1 nA. Parameters from linear regression analysis are as follows: R = 0.997; slope = 36.9×10^3; y-intercept = 10.5×10^3. Each data point represents the mean of three determinations. Inset shows chromatogram for an injection containing 820 fmol of CLOOH.

the conditions described, its CEOOH content was reduced by more than 85% (Fig. 7A, lower trace). The CEOOH in 2-week and 6-week LDL was similarly reduced (Figs. 7B and 7C, lower traces). In contrast, there was no change in the ubiquinone content of these samples, which is not surprising, since ubiquinone is not a substrate for PHGPX. Thus, ubiquinone served as a convenient internal marker for confirming that sample loads were well matched. The ubiquinone peak, like the LOOH peaks, disappeared upon triphenylphosphine treatment (results not shown).

We determined whether GSH/PHGPX treatment of freshly isolated LDL would affect Cu^{2+}-sensitized peroxidation, as measured by TBARS assay. The LDL was incubated with GSH/PHGPX for 1 h and then dialyzed to remove the GSH. [Recycling assay (25) indicated that less than 0.5% of the GSH remained after dialysis.] As shown in Fig. 8, the GSH/PHGPX-treated LDL was much more resistant to Cu^{2+}-stimulated peroxidation than a control that had been incubated with PHGPX alone; the lag time before rapid TBARS formation was at least 6-times longer for the former. [Starting material and a GSH control showed essentially the same TBARS profile as the PHGPX control (data not shown).] The Cu^{2+} concentration in this experiment (Fig. 8) was five times greater than that used in the Fig. 5 experiment, which explains the differences in lag time shown by the controls. It is clear from the results shown in Figs. 7 and 8 that LOOHs (mainly CEOOH) preexist in carefully prepared LDL and can act as powerful sensitizers of free radical-mediated oxidative modification.

DISCUSSION

Oxidative modification of LDL has been proposed to be an important early event in atherogenesis (1–7, 31). The precise mechanism of oxidative LDL modification is still unclear, but most of the proposed schemes suggest that peroxidative degradation of unsaturated LDL lipids plays a central role (7). Rigorous investigation into the role of lipid peroxidation is difficult because important intermediates such as LOOHs are relatively unstable and often exist at exceedingly low levels in carefully prepared LDL. Recent advances in high-performance liquid chromatography with chemiluminescence detection (10, 32) and electrochemical detection (13, 14) have permitted not only the separation and specific identification of different LOOHs in LDL and other biological materials, but also their quantitation at high sensitivity levels.

Endogenous preexisting LOOHs are known to be initiators of chain peroxidation in a wide variety of artificial and natural membrane systems (21–23). This process is described as LOOH-dependent initiation of lipid peroxidation (33). Redox metal ions such as iron and copper typically play important catalytic roles, cycling between reduced and oxidized states. One-electron reduction of preexisting LOOHs is a key triggering event in the process (21, 22). The ultimate reductant might be superoxide radical (O_2^-), ascorbate, or some other electron donor. By analogy with HO$^\bullet$ (hydroxyl radical) formation via the Fenton reaction (33), one-electron reduction of LOOH gives LO$^\bullet$ (lipid oxyl radical) (Eqs. [1] and [2]). Subsequently, LO$^\bullet$, or OLOO$^\bullet$ (epoxyallylic peroxyl radical), a rearrangement/oxidation product of LO$^\bullet$ [Eq. [3], Ref. (34)], can abstract a hydrogen from unoxidized lipid (LH) to initiate chain peroxidation (Eqs. [4]–[7]). Three im-

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad [1]$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^\bullet + OH^- \quad [2]$$

$$LO^\bullet + O_2 \rightarrow OLOO^\bullet \quad [3]$$

$$LO^\bullet + LH \rightarrow LOH + L^\bullet \quad [4]$$

$$OLOO^\bullet + LH \rightarrow OLOOH + L^\bullet \quad [5]$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad [6]$$

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet \quad [7]$$

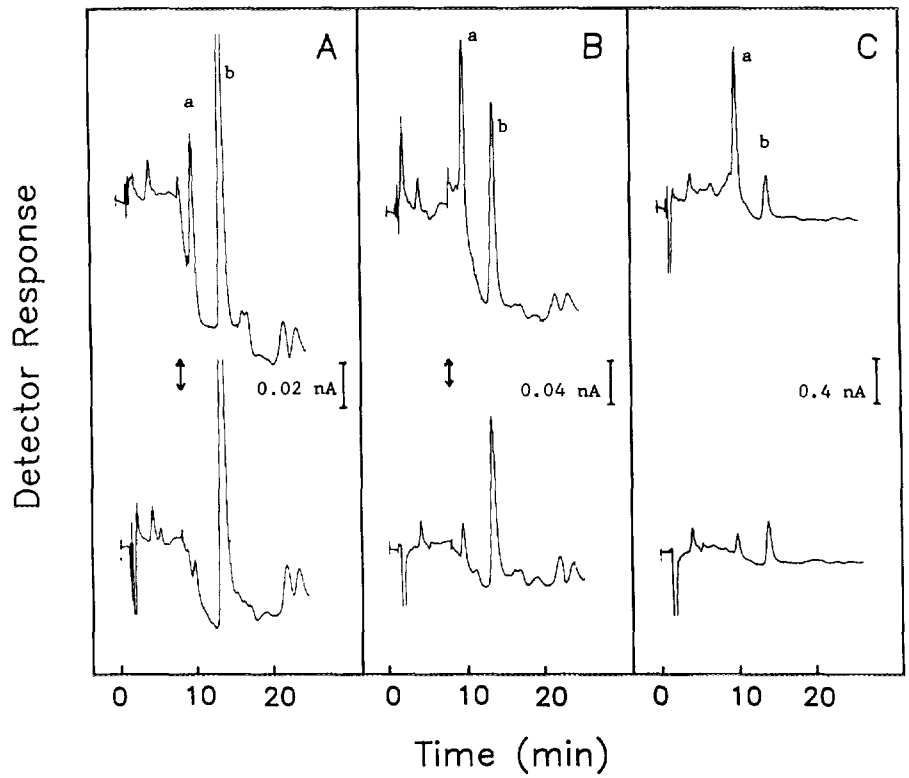

FIG. 7. Lipid hydroperoxides in freshly prepared and aged LDL: susceptibility to enzymatic reduction. A single preparation of LDL was analyzed by HPLC-EC: (A) within 24 h after final preparative centrifugation, (B) after a 2-week storage period at 4°C, and (C) after a 6-week storage period at 4°C. Each LDL sample (in Chelexed PBS containing 1 mM EDTA) was diluted from a protein concentration of ~5 mg/ml to 1.0 mg/ml, and either analyzed directly or after a 60-min incubation at 37°C in the presence of GSH (5.0 mM) and PHGPX (10 mU/ml). Top trace in each panel represents starting LDL; bottom trace represents GSH/PHGPX-treated LDL. Total LDL lipid per injection (A–C) was 112 μg. Full-scale sensitivity settings were as follows: (A) 0.5 nA over the first 7 min, then a switch to 0.1 nA (arrow); (B) 0.5 nA over the first 7 min, then a switch to 0.2 nA (arrow); (C) 2.0 nA throughout. Peak assignments based on retention times of standards: (a) CEOOH (9.9 min), (b) ubiquinone-10 (14.1 min).

portant factors governing the rate and extent of LOOH-dependent initiation of lipid peroxidation are (a) availability of redox-active metal ions,typically chelated in some fashion; (b) level and localization of preexisting LOOHs; and (c) availability of α-tocopherol and other chain-breaking antioxidants (7). In the specific case of Cu^{2+}-induced LDL oxidation, it has been proposed (7) that an electron donor on LDL (presently undefined) first reduces Cu^{2+} to Cu^{+}, which reacts rapidly with LOOH to initiate a peroxidation chain similar to that described for iron in membrane systems (cf. Eqs. [2]–[6]).

We have demonstrated that the level of preexisting LOOHs in LDL is a critical determinant of its susceptibility to more extensive, free radical-mediated peroxidation triggered by Cu^{2+}. The LOOHs were separated and determined by reverse-phase HPLC with mercury drop electrochemical detection, a high-sensitivity/specificity approach that was recently developed in this laboratory (13, 14). The detection limit for CEOOH, the most abundant lipid hydroperoxide class in LDL, was found to be ~0.1 pmol (~1 pmol/mg protein), which is lower than that reported by other workers, using state-of-the-art methods (10, 35, 36). For example, Lynch *et al.* (36), using HPLC with chemiluminescence detection, attained a limit of ~10 pmol CEOOH/mg LDL protein. It has been pointed out (37–39) that ubiquinol in LDL can interfere with the chemiluminescence determination of CEOOH, which might explain why the value of Lynch *et al.* (36) is much higher than ours.

Differences in LDL oxidizability among individuals is commonly assessed by comparing the duration of lag phases in Cu^{2+}-treated samples. Thus, LDL from a patient with coronary artery disease typically exhibits a shorter lag than LDL from a normal individual (40). However, whether this reflects a difference in preexisting "priming" LOOH levels has not been assessed. Ultrasensitive and specific LOOH analysis, such as that achieved with HPLC-EC, is crucial for a better understanding of the early events in LDL autoxidation, e.g., changes in LOOH levels relative to antioxidant levels during the lag period

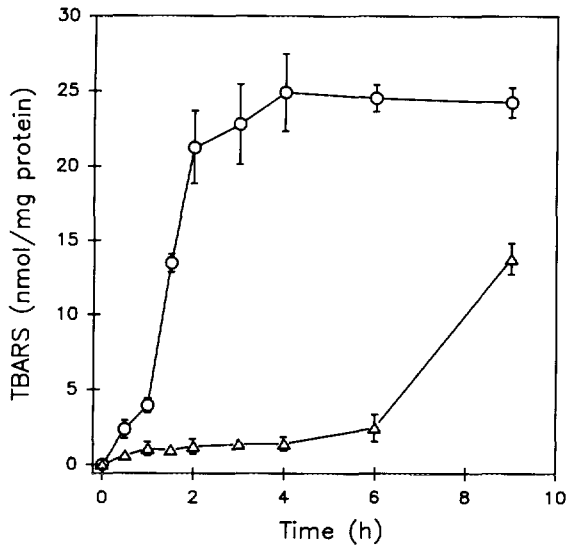

FIG. 8. Enzymatic reduction of lipid hydroperoxides in freshly prepared LDL: effects on Cu^{2+}-stimulated peroxidation. LDL (1.0 mg/ml) in Chelex-treated PBS was preincubated for 60 min with PHGPX alone (10 mU/ml) or PHGPX (10 mU/ml) plus GSH (5.0 mM), followed by overnight dialysis against argon-saturated PBS. After dilution to 0.1 mg protein/ml, the two solutions were incubated at 37°C in the presence of 1 μM $CuCl_2$. At the indicated times, samples were removed for determination of TBARS: (○) PHGPX-treated LDL, (△) GSH/PHGPX-treated LDL. Data points are means ± deviation of values from duplicate experiments. The results are representative of those obtained with at least three different LDL preparations.

that precedes rapid chain peroxidation. Conventional TBARS or conjugated diene assays clearly lack the sensitivity and/or specificity to provide such information. Although highly sensitive, chemiluminescence approaches have also not permitted lag phase LOOH determinations (10, 35–37).

The effect of preexisting LOOHs on Cu^{2+}-stimulated oxidation was tested initially on LDL that had been subjected to dye-sensitized photooxidation to increase its initial peroxide content. The primary oxidant is believed to have been singlet molecular oxygen, since the dye used, $AlPcS_4$, can generate this species in high quantum yield (27). Exposure of photoperoxidized LDL to $CuCl_2$ resulted in rapid formation of TBARS after a short lag. This was markedly delayed in material that had been depleted of LOOHs by GSH/PHGPX treatment, consistent with a requirement for priming LOOHs. Unlike autoxidation of LDL, singlet oxygen-mediated photooxidation does not necessarily depend on prior depletion of α-tocopherol. Singlet oxygen can react simultaneously with unsaturated lipids and α-tocopherol (28, 29). Thus, in the experiment represented in Fig. 4, we cannot rule out the possibility that some antioxidant loss due to photooxidation contributed to the enhanced peroxidizability with Cu^{2+}. It has been reported that treating LDL with arachidonate hydroperoxides enhances its sensitivity to Cu^{2+} oxidation without affecting antioxidant content (41). We were unable to repeat this experiment with exogenous CLOOH because of solubility difficulties.

After establishing that photochemically generated LOOH families could be readily monitored by HPLC-EC, we used this technique to determine whether freshly isolated LDL contains measurable LOOHs. Working at maximal EC sensitivity, we found that LDL prepared with meticulous care to prevent autoxidation contained small amounts of LOOH (mainly CEOOH) when analyzed within 1 day after the final centrifugation step. The average CEOOH content of four different preparations of LDL from normal male donors was ~10 pmol/mg protein. This corresponds to approximately 1 CEOOH in 320,000 cholesteryl ester molecules, or 1 CEOOH for every 200 LDL particles. Other LOOHs in virgin LDL (e.g., cholesterol, phospholipid, and triacylglycerol hydroperoxides) have not been determined; however, based on relative levels of parent lipids (30), their aggregate content would probably not exceed that of CEOOH. That freshly isolated LDL contained far less than 1 LOOH per particle suggests that the peroxide-dependent peroxidation observed with this material required some type of transfer of LOOHs or other reactive intermediates among particles. If operational, this mechanism could suggest new strategies for suppressing LDL oxidation *in vivo*, e.g., direct interception of LOOHs and/or other propagating species in plasma or subendothelial spaces. Although there have been numerous prior attempts at determining LOOH in "pristine" LDL, most have proven to be unsatisfactory, due to (among other things) interfering substances and inadequate sensitivity. For example, most iodometrically determined values are suspect because measurements were made near the detection limit of the assay (7). On the other hand, fresh LDL has been reported to be devoid of detectable LOOHs when analyzed by HPLC with chemiluminescence detection (10, 36, 38, 42). Although this approach is far more sensitive than iodimetry, its reported detection limit for CEOOH, ~2 pmol (10), exceeds that of EC (cf. Fig. 6) by at least 20-fold. Thus, peroxide levels similar to those we have measured could easily have been missed by chemiluminescence detection. The trace levels of CEOOH that we have consistently observed in freshly isolated LDL cannot be attributed to vitamin E deficiency in the donor pool or large-scale loss of vitamin E during isolation, since α-tocopherol levels in all of our preparations were within the normal range. Still, we cannot rule out the possibility that at least some of the CEOOH observed in fresh LDL was generated during preparation. In attempting to resolve this, we determined that freshly extracted plasma from our donor pool contained higher levels of CEOOH than isolated LDL on a mole peroxide per mole cholesteryl ester basis (J. P. Thomas and A. W. Girotti, unpublished observation). Although this does not prove that the CEOOH could not have arisen during LDL isolation, it at least establishes that the peroxide was already present in the plasma source. On the other hand,

it has been reported (10) that most of the CEOOH in plasma resides on high density lipoprotein rather than LDL.

Our previous experiments based on qualitative chromatographic analysis showed that PHGPX can catalyze the reduction of all major LOOH classes in photoperoxidized LDL (16, 17). This was confirmed recently by Sattler *et al.* (43), who showed by HPLC with uv (234 nm) detection that CEOOH in LDL that had been "mildly" oxidized with an azo initiator (~25 nmol CEOOH/mg protein) could be stoichiometrically converted to hydroxide (CEOH) during GSH/PHGPX treatment. Using HPLC-EC, we have now demonstrated that primordial CEOOHs in fresh LDL are also reduced by GSH/PHGPX. [Analysis of hydroxide formation was not attempted because no known detection method, including uv (234 nm), is sensitive enough to accomplish this.] It is important to note that the LDL used in these experiments contained less than 0.1% of the CEOOH found in the mildly oxidized material used by Sattler *et al.* (43). Most, if not all, of the CEOOH in pristine or photooxidized LDL disappeared after sufficient incubation with GSH/PHGPX. That the CEOOH did not reappear after extraction and chromatography argues against the possibility that it arose artifactually during these manipulations. LDL that had been "purged" of preformed LOOHs was found to be markedly more resistant to Cu^{2+}-induced oxidation. Residual GSH or PHGPX was ruled out as a contributing factor in this resistance. These findings confirm those of Thomas and Jackson (11), who treated LDL with GSH and Ebselen, a lipophilic selenoperoxidase mimetic. However the interpretation of their results is somewhat uncertain because residual Ebselen (not checked for after dialysis) could have reduced newly formed LOOHs stoichiometrically, i.e., in the absence of GSH. Our findings add support to the notion that preexisting LOOH levels, along with polyunsaturated fatty acid and endogenous antioxidant levels, are crucial factors in determining LDL's oxidizability.

If one assumes on a tentative basis that preexisting LDL–LOOHs are not generated during LDL preparation, i.e., plasma isolation and centrifugation, then how might they originate? One attractive possibility is that they arise via primary (initiation) reactions taking place in the circulation. Alternatively, the peroxides might first appear in membrane components of parenchymal or circulating cells (erythrocytes, leukocytes) and then be transferred or exchanged into LDL. Whether either of these mechanisms is operative *in vivo* remains to be seen.

ACKNOWLEDGMENTS

The advice and assistance of Witold Korytowski and Gary Bachowski on the application of HPLC-EC to LDL–LOOH analysis is greatly appreciated. Neil Hogg's assistance in the fluorometric determination of α-tocopherol is also appreciated, as is the technical help of Peter Geiger and Andrew Struck.

REFERENCES

1. Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D., and Witztum, J. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1372–1376.
2. Heinicke, J. W., Rosen, H., and Chait, A. (1984) *J. Clin. Invest.* **4,** 1890–1894.
3. Henrikson, T., Mahoney, E. M., and Steinberg, D. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6499–6503.
4. Wieland, E., Parthasarathy, S., and Steinberg, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5929–5933.
5. Parthasarathy, S., Printz, D. J., Boyd, D., Joy, L., and Steinberg, D. L. (1986) *Arteriosclerosis* **6,** 505–510.
6. Parthasarathy, S., Young, S., Witzum, J. L., Pittman, R. C., and Steinberg, D. (1986) *J. Clin. Invest.* **7,** 641–644.
7. Esterbauer, H., Gebicki, J., Puhl, L., and Jurgens, G. (1992) *Free Radical Biol. Med.* **13,** 341–390.
8. Heinecke, J. W., Rosen, H., and Chait, A. (1986) *J. Clin. Invest.* **77,** 757–761.
9. Esterbauer, H., Striegl, G., and Puhl, H. (1989) *Ann. N.Y. Acad. Sci.* **570,** 254–267.
10. Stocker, R., Bowry, V. W., and Frei, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1646–1650.
11. Thomas, C. E., and Jackson, R. L. (1991) *J. Pharmacol. Exp. Ther.* **256,** 1182–1188.
12. Miyazawa, T. (1989) *Free Radical Biol. Med.* **7,** 209–217.
13. Korytowski, W., Bachowski, G. J., and Girotti, A. W. (1993) *Anal. Biochem.* **213,** 111–119.
14. Korytowski, W., Bachowski, G. J., and Girotti, A. W. (1991) *Anal. Biochem.* **197,** 149–156.
15. Thomas, J. P., Geiger, P. G., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *Biochim. Biophys. Acta* **1045,** 252–260.
16. Maiorino, M., Thomas, J. P., Girotti, A. W., and Ursini, F. (1991) *Free Radical Res. Commun.* **12,** 131–135.
17. Hatch, F. T., and Lees, R. S. (1968) *Adv. Lipid Res.* **6,** 2–63.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
19. Maiorino, M., Gregolin, C., and Ursini, F. (1990) *Methods Enzymol.* **186,** 448–457.
20. Geiger, P. G., Thomas, J. P., and Girotti, A. W. (1991) *Arch. Biochem. Biophys.* **288,** 671–680.
21. Thomas, J. P., and Girotti, A. W. (1988) *Biochim. Biophys. Acta* **962,** 297–307.
22. Bachowski, G. J., Pintar, T. J., and Girotti, A. W. (1991) *Photochem. Photobiol.* **53,** 481–491.
23. Girotti, A. W., and Thomas, J. P. (1984) *J. Biol. Chem.* **259,** 1744–1752.
24. Lehman, J., and Martin, H. L. (1982) *Clin. Chem.* **28,** 1784–1787.
25. Tietze, F. (1969) *Anal. Biochem.* **27,** 502–522.
26. Horner, L., and Hoffman, H. (1956) *Angew. Chem.* **68,** 480–484.
27. Bachowski, G. J., Ben-Hur, E., and Girotti, A. W. (1991) *J. Photochem. Photobiol.* **1991,** 307–321.
28. Girotti, A. W. (1990) *Photochem. Photobiol.* **51,** 497–509.
29. Foote, C. S., Ching, T. Y., and Geller, G. G. (1974) *Photochem. Photobiol.* **20,** 511–513.
30. Fielding, C. J. (1984) *J. Lipid Res.* **25,** 1624–1628.
31. Haberland, M. E., Fong, D., and Cheng, L. (1990) *Eur. Heart J.* **11,** 100–104.
32. Lynch, S. M., and Frei, B. (1993) *J. Lipid Res.* **34,** 1745–1752.
33. Girotti, A. W. (1985) *J. Free Radical Biol. Med.* **1,** 87–95.

34. Gardner, H. W. (1989) *Free Radical Biol. Med.* **7,** 65–86.
35. Mohr, D., and Stocker, R. (1994) *Arterioscler. Thromb.* **14,** 1186–1192.
36. Lynch, S. M., Morrow, J. D., Roberts, L. J., and Frei, B. (1994) *J. Clin. Invest.* **93,** 998–1004.
37. Frei, B., Stocker, R., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9748–9752.
38. Frei, B., Yamamoto, Y., Niclas, D., and Ames, B. N. (1988) *Anal. Biochem.* **170,** 120–130.
39. Bowry, V. W., Stanley, K. K., and Stocker, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10316–10320.
40. Chiu, H-C., Jeng, J-R., and Shieh, S-M. (1994) *Biochim. Biophys. Acta* **1225,** 200–208.
41. O'Leary, V. J., Darley-Usmar, V. M., Russell, L. J., and Stone, D. (1992) *Biochem. J.* **282,** 631–634.
42. Frei, B., England, L., and Ames, B. N. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6377–6381.
43. Sattler, W., Maiorino, M., and Stocker, R. (1994) *Arch. Biochem. Biophys.* **309,** 214–221.