Spontaneous Transfer of Phospholipid and Cholesterol Hydroperoxides between Cell Membranes and Low-Density Lipoprotein: Assessment of Reaction Kinetics and Prooxidant Effects†

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ABSTRACT: Under oxidative pressure in the vascular circulation, erythrocytes and phagocytic cells may accumulate membrane lipid hydroperoxides (LOOHs), including cholesterol- and phospholipid-derived species (ChOOHs, PLOOHs). LOOH translocation from cells to low-density lipoprotein (LDL) might sensitize the latter to free radical-mediated oxidative modification, an early event associated with atherogenesis. To test this, we examined the spontaneous transfer kinetics of various ChOOH species (5α-OOH, 6α-OOH, 6β-OOH, 7α/7β-OOH) and various PLOOH groups (PCOOH, PEOOH, PSOOH, SMOOH) using photoperoxidized erythrocyte ghosts as model donors and freshly prepared LDL as an acceptor. LOOH departure or uptake was monitored by reverse-phase HPLC with reductive electrochemical detection. Mildly peroxidized ghost membranes transferred overall ChOOH and PLOOH to LDL with apparent first-order rate constants ∼60 and ∼35 times greater than those of the respective parent lipids. Individual ChOOH rate constants decreased in the following order: 7α/7β-OOH > 5α-OOH > 6α-OOH > 6β-OOH. Kinetics for reverse transfer from LDL to ghosts followed the same trend, but rates were significantly higher for all species and their combined activation energy was lower (41 vs 85 kJ/mol). PLOOH transfer rate constants ranged from 4- to 15-fold lower than the composite ChOOH constant, their order being as follows: PCOOH ≃ PEOOH ≃ PSOOH > SMOOH. Similar PLOOH transfer kinetics were observed when LDL acceptor was replaced by unilamellar liposomes, consistent with desorption from the donor membrane being the rate-limiting step. The susceptibility of transfer LOOH-enriched LDL to Cu^{2+}-induced chain peroxidative damage was assessed by monitoring the accumulation of conjugated dienes and products of free radical-mediated cholesterol oxidation. In both cases, transfer-acquired LOOHs significantly reduced the lag time for chain initiation relative to that observed using nonperoxidized ghosts. These findings are consistent with the idea that LDL can acquire significant amounts of "seeding" LOOHs via translocation from various donors in the circulation.

The human low-density lipoprotein (LDL)[1] particle contains a single molecule of apoB-100 protein (∼25 wt %) and several different classes of lipids, including cholesteryl esters, phospholipids, triacylglycerols, and cholesterol (collectively ∼75 wt %). Unsaturated LDL lipids, like those in cell membranes, are subject to peroxidative degradation, which can markedly alter LDL structure/function with potentially pathologic consequences (*1*). Specifically, there is extensive evidence implicating oxidative modification of LDL as an early event in the development of atherosclerotic lesions and coronary artery disease (*2*−*4*). A key finding was the identification of autoantibodies against oxidized LDL in human plasma, suggesting that this material exists in vivo (*5*). LDL that has been oxidized by exposure to trace metal ions (Cu^{2+}, Fe^{2+}), lipoxygenases, or endothelial cells in vitro

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[1] Abbreviations: $AlPcS_2$, aluminum phthalocyaninedisulfonate; LDL, low-density lipoprotein; Ch, cholesterol, ChOOH(s), cholesterol hydroperoxide(s); CEOOH(s), cholesteryl ester hydroperoxide(s); ChOX, cholesterol oxidation product(s); BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DFO, desferrioxamine; DCP, dicetyl phosphate; nsLTP, nonspecific lipid transfer protein; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; HPLC-EC(Hg), high-performance liquid chromatography with mercury cathode electrochemical detection; HPTLC-PI, high-performance thin-layer chromatography with phosphorimaging detection; LOOH(s), lipid hydroperoxide(s); PBS, Chelex-treated phosphate-buffered saline (125 mM NaCl, 25 mM sodium phosphate, pH 7.4); PBS/DFO/EDTA, Chelex-treated PBS containing 0.1 mM DFO and 0.1 mM EDTA; SUV(s), small unilamellar vesicle(s); PL, phospholipid; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PEOOH, phosphatidylethanolamine hydroperoxide; PS, phosphatidylserine; PSOOH, phosphatidylserine hydroperoxide; SM, sphingomyelin; SMOOH, sphingomyelin hydroperoxide; PLOOH(s), phospholipid hydroperoxide(s); 5α-OOH, 3β-hydroxy-5α-cholest-6-ene 5-hydroperoxide; 6α-OOH, 3β-hydroxycholest-4-ene 6α-hydroperoxide; 6β-OOH, 3β-hydroxycholest-4-ene 6β-hydroperoxide; 7α-OOH, 3β-hydroxycholest-5-ene 7α-hydroperoxide; 7β-OOH, 3β-hydroxycholest-5-ene 7β-hydroperoxide; 7α/7β-OOH, undefined mixture of 7α-OOH and 7β-OOH.

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is rapidly taken up by monocytes/macrophages via the scavenger receptor (*6*, *7*). Unlike normal uptake, this process is not subject to feedback inhibition, so there is a dramatic influx of LDL lipid which, in vivo, could lead to formation of foam cells and thence atherosclerotic plaques (*8*). Chemical antioxidants which decrease LDL oxidizability in vitro (e.g., ascorbate, probucol) have been shown to exert significant antiatherogenic effects in experimental animals (*2*), consistent with the oxidative modification hypothesis.

There is evidence that LDL's susceptibility to metal ion-catalyzed free radical peroxidation depends largely on its content of preexisting lipid hydroperoxides (LOOHs) on one hand (*9*, *10*) and protective antioxidants on the other (*1*, *2*). One-electron reduction or oxidation of these LOOHs can trigger waves of damaging chain peroxidation in which new LOOHs are continually formed and reduced (*11*, *12*). Resident antioxidants such as α-tocopherol and *β*-carotene (*1*) or nitric oxide derived from phagocytic cells (*13*) can oppose such reactions by intercepting chain-carrying radicals. LDL isolated from normal human plasma invariably contains LOOHs, some of which may arise adventitiously during the isolation process (*1*, *9*). In a previous study (*10*), LDL prepared with the utmost care to prevent autoxidation and rapidly analyzed by HPLC with electrochemical detection was found to contain 10 ± 2 pmol of cholesteryl ester hydroperoxide (CEOOH)/mg of protein, which sensitized it to Cu^{2+}-induced peroxidation. Evidence pointed to the CEOOH (and lesser amounts of other LOOHs) being present in the LDL prior to isolation, i.e., preexisting in the plasma source (*10*). How LDL might be peroxidized and/or acquire LOOHs in vivo has been the subject of much investigation and debate. It is reported that LDL can be oxidatively modified by reactive oxygen (*14*, *15*) or reactive nitrogen oxide (*16*) species produced by leukocytes that it encounters in the circulation; under these circumstances, preexisting LOOHs would derive mainly from LDL lipids. Other evidence suggests that cellular lipoxygenases, most notably the 15-lipoxygenase of monocytes and macrophages, play a crucial role (*17*−*19*). In this case, LOOH formation might reflect some level of physical contact between LDL and cells (*19*) with LDL lipids accessing the lipoxygenase active site. Another possibility is translocation of lipoxygenase-generated LOOHs from cells to LDL. This would not necessarily require cell−LDL contact but could occur via an aqueous transit pool, as demonstrated for unoxidized cholesterol and phospholipids (*20*). Spontaneous LOOH transfer might also occur from erythrocytes or activated neutrophils, each of which is under relatively high peroxidative pressure in the circulation. Erythrocytes are also deficient in enzymes that specifically detoxify LOOHs (*21*, *22*). Although the prospect of LOOH transfer from cells to LDL has been considered previously (*19*), no studies dealing specifically with this have been reported. In addressing this issue, we recently described the intermembrane translocation of unesterified cholesterol hydroperoxides (ChOOHs) (*22*, *23*) and now report on the kinetics and damaging effects of ChOOH and phospholipid hydroperoxide (PLOOH) movement from cell membranes to LDL.

MATERIALS AND METHODS

General Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), and brain sphingomyelin (SM) were obtained from Avanti Polar Lipids (Birmingham, AL). Sigma Chemical Co. (St. Louis, MO) supplied the cholesterol (Ch), cholesteryl linoleate, dicetyl phosphate (DCP), butylated hydroxytoluene (BHT), desferrioxamine (DFO), fatty acid-depleted bovine serum albumin (BSA), and Chelex-100 (50−100 mesh). Bovine liver nonspecific lipid transfer protein (nsLTP), also known as sterol carrier protein-2, was isolated as described (*24*); identity was confirmed by N-terminal amino acid analysis (*25*). Chromatographic solvents were obtained from Burdick and Jackson Corp. (Muskegon, MI). Aluminum phthalocyaninedisulfonate ($AlPcS_2$), an amphiphilic photosensitizing dye, was obtained from Dr. J. Van Lier (University of Sherbrooke) as a gift. Stock solutions of $AlPcS_2$ (1 mM in DMSO) were stable indefinitely when stored in the dark at 4 °C. The following ChOOH species, prepared by photooxidation of Ch and characterized as described (*27*, *28*), were used as HPLC standards: 3*β*-hydroxy-5α-cholest-6-ene 5-hydroperoxide (5α-OOH), 3*β*-hydroxycholest-4-ene 6α-hydroperoxide (6α-OOH), 3*β*-hydroxycholest-4-ene 6*β*-hydroperoxide (6*β*-OOH), 3*β*-hydroxycholest-5-ene 7α-hydroperoxide (7α-OOH), and 3*β*-hydroxycholest-5-ene 7*β*-hydroperoxide (7*β*-OOH). Cholesteryl linoleate hydroperoxide was generated similarly (*10*). PLOOH standards (POPC hydroperoxide, POPE hydroperoxide, POPS hydroperoxide, and brain SM hydroperoxide) were prepared by photooxidation of the respective phospholipids in liposomal form and isolated by normal-phase HPLC with UV detection (*10*). Peroxide content of the different standards was determined by iodometric analysis, as described (*28*). [*oleoyl*-1-^{14}C]POPC (58 mCi/mmol in toluene/ethanol, 1:1 v/v) was obtained from NEN Life Sciences, Inc. (Boston, MA) and [4-^{14}C]Ch (46 mCi/mmol in toluene) from Amersham Life Sciences, Inc. (Arlington Heights, IL).

Preparation and Radiolabeling of Erythrocyte Ghosts. Unsealed erythrocyte membranes (white ghosts) were prepared from freshly drawn human blood in 2 mM EDTA. Red blood cells (RBCs) were pelleted, washed four times with PBS, and then lysed using at least 60 volumes of ice-cold 5 mM sodium phosphate/1 mM EDTA, pH 8.0 (*29*). After three washes with this buffer, the membranes were given a final wash with PBS containing 0.1 mM each of DFO and EDTA, resuspended in this medium, and stored under argon at 4 °C, typically being used for experiments within 2 weeks. Preparations were quantitated on the basis of total protein content (*30*). Membranes isolated in this fashion were substantially depleted of redox iron (*22*, *23*) so that iron-catalyzed LOOH formation/turnover during transfer incubation was usually insignificant. In preparation for measuring transfer of unoxidized PC and Ch for comparison with PCOOH and ChOOH, ghosts were labeled with [^{14}C]POPC and [^{14}C]Ch, using nsLTP to facilitate lipid exchange (*24*, *31*). Immediately before use, the [^{14}C]Ch was separated from preexisting oxidation products by means of normal-phase HPLC, as described (*22*). Nonradioactive Ch, being <0.1% oxidized, was used without further purification. [^{14}C]POPC was mixed with a 20-fold excess of carrier POPC and separated from oxidation products by normal-phase HPLC with UV (206 nm) detection using 2-propanol/hexane/

water (100/75/20 by volume) as the mobile phase, a slight modification of a previously described system (*32*). Eluting well before peroxides and other oxidized forms, [^{14}C]POPC was recovered, dried under nitrogen, and mixed immediately with Ch and purified [^{14}C]Ch for constitution into small unilamellar vesicles (50 nm SUVs) by an extrusion technique (*26*, *33*). The typical SUV preparation contained 0.1 mM [^{14}C]POPC (~7 nCi/mL) and 0.08 mM [^{14}C]Ch (~0.2 μCi/mL) in PBS/DFO/EDTA. Ghosts (1.0 mg of protein/mL; ~1.3 mM total lipid) were labeled by incubating with SUVs (~60 μM total lipid) at 37 °C in the presence of nsLTP (75 μg/mL) in PBS/DFO/EDTA plus 10 μM BHT, a chain-breaking antioxidant. (BHT was included as an added precaution to prevent membrane lipid oxidation during incubation.) After 2 h, when ~65% of the [^{14}C]Ch and ~30% of the [^{14}C]POPC had been transferred, the ghosts were pelleted, washed twice to remove SUVs and SCP-2, and used immediately for measuring the kinetics of parent lipid transfer to LDL.

Preparation and [^{14}C]Ch Labeling of LDL. Whole plasma was prepared from freshly drawn blood obtained from an overnight-fasted donor. The LDL fraction (density 1.019−1.063 g/mL) was isolated by flotation ultracentrifugation in KBr, as described (*34*). All preparative solutions were dialyzed against Chelex-100 and saturated with argon before use. Immediately after harvesting, the LDL was dialyzed against argon-sparged PBS/DFO/EDTA and stored under argon at 4 °C, typically being used for experiments within a 2 week period. ApoB-100 protein content was determined according to Lowry et al. (*30*), using bovine serum albumin as the standard.

In preparation for assessing endogenous chain peroxidation via [^{14}C]ChOX formation, LDL was labeled with [^{14}C]Ch using a modified procedure in which agarose-conjugated BSA serves as an easily removable donor (*35*). A 1 mL aliquot of BSA solution (10 mg/mL in PBS/DFO) was added to 1 mL of PBS-washed Affi-Gel agarose beads (Bio-Rad, Richmond, CA). The slurry was gently mixed for 1 h at 37 °C, after which the BSA-linked beads were washed with PBS to remove unbound protein and then sparged with argon. A 50 μL aliquot of [^{14}C]Ch (1 μCi) in acetone was added, followed by 4 h of slow mixing at 37 °C under argon. The [^{14}C]Ch-charged BSA beads were then washed with and resuspended in PBS/DFO. To a 1 mL portion of the slurry was added 1 mL of stock LDL (2 mg of protein/mL), followed by further incubation for 1 h under argon with gentle agitation. The [^{14}C]Ch-labeling efficiency for LDL was typically ~60%, as determined by scintillation counting.

Photoperoxidation of Ghost Membranes. Phospholipid (PL) and Ch account for most of the erythrocyte membrane lipid, i.e., ~48 and ~42 mol %, respectively (*36*). Isolated membranes were "loaded" with PLOOHs and ChOOHs by means of photodynamic action, using $AlPcS_2$ as a singlet oxygen-generating sensitizer (*37*). A typical reaction mixture in a thermostated beaker at 25 °C consisted of ghosts [1.0 mg of protein/mL or 0.88 mg of lipid/mL (0.57 mM Ch, 0.67 mM PL)] and 20 μM $AlPcS_2$ in PBS/DFO. The membranes were irradiated from above using a quartz−halogen source (light fluence ~150 mW/cm^2) and slow magnetic stirring to optimize aeration and ensure uniform photoperoxidation. HPLC-analyzed ChOOHs and PLOOHs accumulated linearly with irradiation time out to at least 20 min, the aggregate rate being ~2.2 μM/min for the former and ~8.2 μM/min for the latter. Thus, a light fluence of 180 J/cm^2 (20 min of irradiation) produced ~45 μM total ChOOH in bulk suspension, which was apportioned approximately as follows: 68% 5α-OOH, 10% 6α-OOH, 16% 6β-OOH, and 6% unresolved 7α-OOH and 7β-OOH. The same light fluence typically gave ~165 μM total PLOOH, 45% of which was PCOOH, 43% PEOOH, 6% SMOOH, and 6% PSOOH. PC and PE exist at approximately the same level and degree of unsaturation in erythrocyte membranes (*36*), which may account for the similar PCOOH and PEOOH yields. Though slightly lower in content, SM on average is much less unsaturated, which probably explains the relatively low SMOOH yield. On the other hand, PS is the least abundant of these phospholipids (*36*), and the low PSOOH yield probably reflects this. Other classes, including glycolipids and phosphatidylinositol, are even less abundant; their peroxides were not studied.

Lipid Transfer Conditions. A typical reaction mixture for studying spontaneous ChOOH and PLOOH transfer from ghosts to LDL contained photoperoxidized ghost membranes (0.2 mg of protein/mL), LDL (0.53 mg of protein/mL), 10 μM BHT, and 0.5% ethanol (the BHT solvent) in PBS/DFO/EDTA. [At the indicated concentrations, neither BHT nor ethanol had any significant effect on transfer kinetics (*23*).] The concentration of total ghost lipid in bulk suspension was 0.25 mM (typically 40−50 μM as LOOH), whereas the concentration of LDL lipid was either 1.25 or 2.5 mM, giving an acceptor:donor lipid mol ratio of 5:1 or 10:1. Transfer mixtures (2.0 mL each) in 13 × 100 mm Pyrex tubes capped with glass marbles were incubated at 37 °C in a thermostated block, with periodic gentle agitation. At various time points over a 2 h period, a 0.1 mL sample was removed from each tube and quenched with 0.15 mL of ice-cold PBS. [It was shown previously (*22*) that LOOH transfer is negligibly slow at 4 °C.] Ghost membranes were then pelleted at 4 °C (16000*g*, 10 min), washed twice with PBS to remove residual LDL, and then resuspended to 0.25 mL with PBS/1 mM EDTA and extracted with chloroform/methanol (2:1 v/v), as described (*28*). For determining total systemic ChOOH or PLOOH during transfer, matching samples were quenched and extracted directly, i.e., without separating LDL from ghosts. A 0.2 mL aliquot from each organic phase was dried under nitrogen and stored at −20 °C for HPLC-EC(Hg) analysis; this was typically completed within 48 h, during which the stored LOOHs were completely stable. LOOH departure from ghosts was examined by this approach. In some instances, LOOH uptake by LDL was monitored using the same reaction conditions. In this case, timed 0.15 mL samples were recovered during incubation, quenched with an equal volume of cold PBS, and centrifuged. A 0.15 mL aliquot of LDL-containing supernatant was diluted to 0.25 mL with PBS/1 mM EDTA and extracted for HPLC-EC(Hg) examination, as described above, along with matching noncentrifuged samples for monitoring overall ChOOH and PLOOH. Back-transfer of LOOHs from LDL to ghost membranes was also examined using methodology similar to that for monitoring LOOH acquisition. Back-transfer was carried out at the same acceptor:donor lipid ratio as forward-transfer to avoid any possible changes in mechanism, e.g., shift from aqueous diffusion to contact-mediated transfer. DMPC/DCP (100:1 mol/mol) SUVs were used as LOOH

acceptors in some experiments. Total SUV lipid and ghost lipid in these reactions was set at 1.25 and 0.25 mM, respectively. Otherwise, conditions were the same as when LDL was the acceptor.

Conditions for studying ghost-to-LDL transfer of unoxidized PC and Ch (both lipids being monitored simultaneously) were similar to those described for hydroperoxide derivatives. [^{14}C]Ch- and [^{14}C]POPC-labeled ghosts (0.2 mg of protein/mL) in PBS containing DFO, EDTA, and BHT were incubated at 37 °C with a 10-fold lipid molar excess of LDL. At various time points out to 65 h, samples were drawn, quenched with cold PBS, and centrifuged. The membrane pellets were washed free of LDL and extracted. Recovered lipid fractions were analyzed for [^{14}C]Ch and [^{14}C]POPC by means of thin-layer chromatography (see below), using conditions that allowed both lipids to be determined on a single chromatographic plate.

Determination of Propagative Lipid Peroxidation in Transfer LOOH-Enriched LDL. The ability of transfer-acquired LOOHs to sensitize LDL to Cu^{2+}-catalyzed lipid peroxidation was examined. For these experiments an aliquot of stock LDL was dialyzed free of DFO and EDTA immediately before use, and transfer incubation was carried out in argon-sparged PBS lacking chelators and BHT. A typical Cu^{2+} reaction mixture contained LDL (0.125 mg of protein/mL; ~0.6 mM total lipid) and 20 μM $CuCl_2$ in PBS at 37 °C. LDL preincubated with nonperoxidized ghosts was incubated alongside as a control. LDL peroxidation was assessed by three different methods: (i) determination of conjugated dienes, as measured by increasing absorbance at 233 nm, using untreated LDL as a blank (*1*, *3*); effects of transfer incubation time on the conjugated diene kinetic profile were examined; (ii) TLC-based detection of free radical-derived Ch oxidation products; in this case LDL-borne [^{14}C]Ch (see above) served as an in situ probe of chain peroxidation activity as manifested by accumulation of characteristic [^{14}C]ChOX, viz., 7α-OOH, 7β-OOH, 7α-OH, 7β-OH, and 5,6-epoxide (*22*, *38*); (iii) HPLC-based determination (*10*) of CEOOH generated by oxidation of cholesteryl ester, the most abundant lipid class in LDL (*1*). In approaches ii and iii, samples recovered during incubation with Cu^{2+} were extracted, and lipid fractions were subjected to the respective chromatographic analyses.

HPLC-EC(Hg) Analysis of LOOHs. LOOHs in lipid extracts from LDL or ghost membranes were analyzed by reverse-phase high-performance liquid chromatography with mercury cathode electrochemical detection [HPLC-EC(Hg)]. The basic system consisted of a model 2350 pump (Isco Inc., Lincoln, NE) and a model 420 electrochemical detector from EG&G Instruments (Oak Ridge, TN). The detector was equipped with a glass capillary that dispensed a hanging mercury drop set at −150 mV vs a Ag/AgCl reference (*27*, *39*). A 400 MHz Dell Dimension computer (Round Rock, TX) and EZChrom Elite software (Scientific Software, Inc., Pleasanton, CA) were used for data acquisition and processing. ChOOH chromatography was carried out at room temperature using an Ultrasphere XL-ODS column (4.6 × 70 mm; 3 μm particles) from Beckman Instruments (San Ramon, CA). The mobile phase consisting of (by volume) 72% methanol, 11% acetonitrile, 8% 2-propanol, and 9% aqueous 1 mM sodium perchlorate was thoroughly sparged with argon and pumped at a rate of 1.0 mL/min. ChOOH standards were used for establishing retention times and EC responsiveness; detection limits at 3:1 signal to noise ratio were in the 150−200 fmol range (*39*). Under the conditions indicated, 5α-OOH, 6α-OOH, and 6β-OOH were well separated from one another and from 7α-OOH and 7β-OOH (*39*); however, the latter two were not resolved and are thus referred to collectively as "7α/7β-OOH".

Chromatography of CEOOH in LDL was performed on the same column as used for ChOOHs. However, a less polar mobile phase was employed, viz., 54% 2-propanol, 23% acetonitrile, 14% methanol, and 9% aqueous 1 mM sodium perchlorate (*10*). Iodometrically determined cholesteryl linoleate hydroperoxide served as a standard (*10*).

PLOOH separations were carried out using a Supelcosil LC-NH_2 column (4.6 × 150 mm; 3 μm particles) from Supelco (Bellefonte, PA). The mobile phase used with this column consisted of (by volume) 61% methanol, 30% 2-propanol, and 9% aqueous 40 mM NaH_2PO_4; like the mobile phase for ChOOHs and CEOOHs, it was subjected to extensive argon sparging before and during a chromatographic run. Four major PLOOH populations were resolved with this system, their order of increasing retention times being as follows: PCOOH < SMOOH < PEOOH < PSOOH. The detection limits for PCOOH and PEOOH were in the 0.8−1.0 pmol range. PCOOH, PEOOH, and PSOOH showed identical EC responsiveness, which was ~45% greater than SMOOH responsiveness. Immediately before chromatographing, an experimental lipid extract was dissolved in 50 μL of 2-propanol, 10 μL of which was injected. This typically represented ~5 nmol of total lipid in the case of LDL extracts and ~15 nmol of total lipid in the case of ghost membrane extracts.

Analysis of Radiolabeled Lipids by HPTLC-PI. Normal-phase high-performance thin-layer chromatography with phosphorimaging radiodetection (HPTLC-PI) was used for monitoring [^{14}C]Ch and [^{14}C]POPC transfer from ghosts to LDL. High-performance silica gel 60 plates (10 × 20 cm) were obtained from EM Science (Gibbstown, NJ). A lipid extract was solubilized in 20 μL of hexane/2-propanol (97:3 v/v) and applied to the plate in a thin nitrogen steam using a Linomat IV programmable applicator (Camag Scientific, Inc., Wilmington, NC). Ch and POPC were resolved from one another and from any oxidation products generated during transfer, using two different irrigations interposed by air-drying, the first with benzene/ethyl acetate (1:1 v/v) and the second with chloroform/methanol/acetic acid/water (25:15:4:2 by volume). The second irrigation was timed so that the distance reached by its front was about half that of the first irrigation. Step 1 moved only Ch (R_f ~0.54) and any of its oxides, whereas step 2 left these species in place and moved only POPC (R_f ~0.38) and any of its oxides. After final development and air-drying, the plate was analyzed for radioactive species using a Storm 860 storage phosphor system with ImageQuant 4.2 software (Molecular Dynamics, Sunnyvale, CA). Additional details were reported previously (*22*, *23*, *38*). HPTLC-PI was also used for detecting [^{14}C]ChOX species generated by Cu^{2+}-stimulated degradation of transfer-acquired LOOHs in [^{14}C]Ch-labeled LDL. Lipid extracts from Cu^{2+}-treated LDL were chromatographed as described above using a single irrigation with benzene/ethyl acetate (1:1 v/v). After development, the plate was scanned for radioactive Ch and ChOX species (7α-OOH, 7β-OOH,

7α-OH, 7β-OH, 5,6-epoxide), the latter being well separated from one another and from parent Ch (*38*). Since chain peroxidation typically resulted in <1% conversion of Ch to ChOX, the former could be used as a convenient internal standard to correct for any extraction or loading discrepancies. Molar yield of ChOX species was based on their image intensities relative to Ch and the absolute molar amount of Ch in LDL, as determined by HPLC with UV (212 nm) detection (*22*).

RESULTS

ChOOH Transfer between Membranes and LDL. Most transfer kinetic experiments were carried out with photooxidized ghosts containing <10% of their starting Ch as ChOOH. Overall ChOOH consisted of 5α-OOH, 6α-OOH, 6β-OOH, and 7α/7β-OOH, a typical yield distribution being 67%, 9%, 15%, and 9%, respectively. 5α-OOH, 6α-OOH, and 6β-OOH are characteristic singlet oxygen ($^1O_2$) adducts (*40*), whereas 7α- and 7β-OOH derive from free radical reactions or 5α-OOH rearrangement (*41*, *42*). The observed predominance of 5α-OOH in our preparations indicates that photoperoxidation was mediated primarily by $^1O_2$. The ability of photooxidized ghosts to transfer ChOOHs was examined by incubating the ghosts with a large excess of freshly prepared LDL at 37 °C and tracking peroxide in the membrane and LDL compartments by means of HPLC-EC(Hg). As shown in Figure 1A, there was a progressive loss of donor 7α/7β-OOH, 5α-OOH, 6α-OOH, and 6β-OOH during 1 h of incubation with LDL at a 10:1 lipid mol ratio and corresponding accumulation of each of these species in the lipoprotein. The loss of each ChOOH from ghosts appeared to be stoichiometric with accumulation in LDL at each time point. The LDL contained negligible amounts of these peroxides at zero time (at least at the EC sensitivity used), indicating that its Ch had not been unduly oxidized in situ or during isolation and handling. The peak area for each ChOOH in the overall system did not change significantly over at least 1 h (Figure 1A, memb + LDL), demonstrating that these peroxides were quite stable under the conditions used and essentially totally recoverable. First-order plots depicting ChOOH decay in the membrane compartment are shown in Figure 1B. The plots remain linear down to approximately 20% residual peroxide and then begin to level off, suggesting an approach to equilibrium in favor of acceptor LDL. The observed linearity implies that a single kinetic pool of ChOOH existed in the ghosts. As shown previously when SUVs were used as acceptors (*23*), individual ChOOHs differed significantly in their intrinsic transfer kinetics. Thus, the apparent first-order rate constant for 7α/7β-OOH departure from ghosts was approximately twice that of 5α-OOH and eight times that of 6β-OOH (Table 1). The absolute values of these rate constants are nearly identical to those determined for ghost-to-SUV transfer (*23*), which suggests that desorption from the ghost membrane rather than uptake by LDL was the primary rate determinant under the conditions used. This is consistent with the aqueous transit pool model described for parent lipid transfer (*20*), where egress from the donor is slow and rate-limiting. The kinetics for ChOOH acquisition by LDL at 37 °C were also found to be first order, the rate constants being similar to those determined for departure from ghosts and decreasing in the same rank order: 7α/7β-OOH (7.89×10^{-2} min^{-1}) > 5α-OOH (3.15×10^{-2} min^{-1}) > 6β-OOH (0.82×10^{-2} min^{-1}). This similarity again supports the notion that the rate-determining step in ChOOH translocation was desorption from the membrane surface (*20*). One should note that the order of reverse-phase retention times for the ChOOHs (7α/7β-OOH < 5α-OOH < 6α-OOH < 6β-OOH) (Figure 1A) is diametrically opposite to that of their transfer rate constants (Table 1). We attribute this to an enlargement of the aqueous transit pool as hydrophilicity increases (*20*, *43*−*45*).

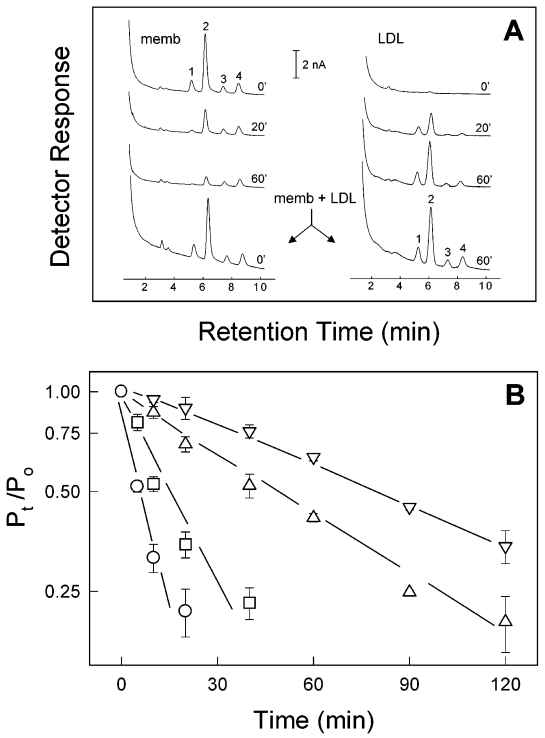

FIGURE 1: Spontaneous transfer of ChOOH species from ghost membranes to LDL. Reaction mixtures contained photoperoxidized ghosts (0.25 mM total lipid), LDL (2.5 mM total lipid), and 10 μM BHT in PBS/DFO/EDTA at pH 7.4. Initial concentrations of individual ChOOHs in bulk suspension were as follows: 5α-OOH (5.8 μM), 6α-OOH (0.8 μM), 6β-OOH (1.4 μM), and 7α/7β-OOH (1.0 μM). At various times during incubation at 37 °C, samples were removed, mixed with cold PBS to quench transfer, and centrifuged. LDL-containing supernatant fractions were extracted directly, whereas membrane pellets were first washed with PBS and then extracted. Noncentrifuged samples, representing the entire reaction system at selected times, were also extracted. Recovered lipid fractions were analyzed for ChOOHs by HPLC-EC(Hg). (A) Chromatographic profiles depicting the progressive loss of ChOOHs from membranes and acquisition by LDL over a 60 min incubation period. Total lipid per injection: 9.2 nmol (memb); 18.6 nmol (LDL). The entire system (memb + LDL) at 0 and 60 min is also represented (21.7 nmol of lipid per injection). Peak assignments and retention times are as follows: (1) 7α/7β-OOH (4.86 min); (2) 5α-OOH (5.70 min); 6α-OOH (6.80 min); 6β-OOH (7.72 min). (B) First-order kinetic plots for ChOOH decay in ghosts. P_0 and P_t denote peroxide content at zero time and time *t*, respectively. Key: 7α/7β-OOH (○), 5α-OOH (□), 6α-OOH (△), and 6β-OOH (▽). Data points are means ± deviation of values from duplicate experiments, one of which is represented in (A).

In separate experiments, we measured transfer of parent Ch to LDL, using [^{14}C]Ch-labeled ghosts and HPTLC-PI methodology (Table 1). The apparent first-order rate constant for Ch was found to be ~5.0×10^{-4} min^{-1} at 37 °C, which is <1% of the value determined for 7α/7β-OOH and ~5% of that for 6β-OOH. Nonperoxidized ghosts were used for determining the Ch rate constant, but otherwise the reaction

Table 1: Rate Constants for Ch and ChOOH Transfer between Ghost Membranes and LDL[a]

	$k \times 10^2$ (min^{-1})[b]		
	ghost → LDL		LDL → ghost
analyte	37 °C	20 °C	20 °C
Ch	0.050 ± 0.003	nd[c]	nd
7α/7β-OOH	7.43 ± 1.08	2.52 ± 0.23	4.57 ± 0.47
5α-OOH	3.68 ± 0.40	1.17 ± 0.06	2.75 ± 0.32
6α-OOH	1.38 ± 0.08	0.32 ± 0.03	1.16 ± 0.09
6β-OOH	0.92 ± 0.05	0.17 ± 0.01	0.94 ± 0.13

[a] For measuring Ch transfer, reaction mixtures contained [^{14}C]Ch-labeled ghosts (0.18 mg of lipid/mL, ~25 nCi/mL), LDL (2.12 mg of lipid/mL), and BHT (10 μM) in PBS/DFO/EDTA at 37 °C. Total ghost and LDL lipid concentrations in bulk suspension were 0.25 and 2.50 mM, respectively. Samples recovered during incubation were centrifuged; membrane pellets were washed free of LDL and extracted. Residual [^{14}C]Ch in lipid fractions was analyzed by HPTLC-PI. ChOOH data at 37 °C are from the experiments described in Figure 1; ChOOH data at 20 °C are from the experiments described in Figure 2. Transfer direction is denoted by the arrow. [b] Apparent first-order rate constants for the various analytes were determined from their decay kinetics in ghosts or LDL. Means ± deviations of values from duplicate experiments are shown. [c] nd = not determined.

system was identical to that used for the ChOOH measurements (Figure 1, Table 1). In earlier studies involving a ghost/SUV system (*22*), we found that donor membrane photooxidation to the level represented in Figure 1 did not affect Ch transfer kinetics. The composite rate constant for overall ChOOH transfer to LDL at 37 °C was (2.95 ± 0.18) × 10^{-2} min^{-1}, i.e., ~60 times greater than the Ch constant. For the Figure 1 experiment, total [ChOOH] at the start of transfer was 9 μM, or ~8% of the native membrane [Ch] (110 μM in bulk suspension). Knowing this, we calculated the initial transfer rates for ChOOH and Ch to be 0.27 and 0.05 μM/min, respectively. Thus, the ChOOH rate exceeded the Ch rate by ~5-fold despite the fact that [Ch] exceeded [ChOOH] by 11-fold. A similar deduction applies for the individual ChOOHs; e.g., 5α-OOH at <6% the Ch concentration exhibited a 4.3-fold greater initial transfer rate. Rate calculations for the ChOOHs gave the following values (cf. Figure 1, Table 1): 431 nM/min (5α-OOH); 37 nM/min (7α/7β-OOH); 13 nM/min (6β-OOH); 11 nM/min (6α-OOH). Therefore, among the ChOOHs monitored in the Figure 1 experiment, 5α-OOH exhibited by far the highest initial transfer rate (12-fold greater than that of 7αβ-OOH and 18-fold greater than that of 6α-OOH and 6β-OOH together). It is clear from these results that peroxidation of cell membrane Ch strongly enhances its ability to translocate to LDL.

We tested the reversibility of ChOOH transfer in a common reaction system by first examining movement from ghosts to LDL and then back to fresh ghosts, maintaining the same mol ratio of acceptor to donor lipid (5:1) throughout. A preliminary experiment carried out at 37 °C indicated that back-transfer was too fast to be measured accurately, so the temperature was lowered to 20 °C for both phases of the reaction. As shown in Figure 2A, overall ChOOH accumulated progressively in LDL during transfer incubation at 20 °C, reaching a maximum of ~65% transferred after 4 h. When the LDL was separated from donor ghosts after 7 h and presented with unoxidized ghosts (now acting as acceptors) there was a rapid and substantial loss of acquired ChOOH back to the membrane compartment, confirming that transfer was reversible. A striking difference was observed

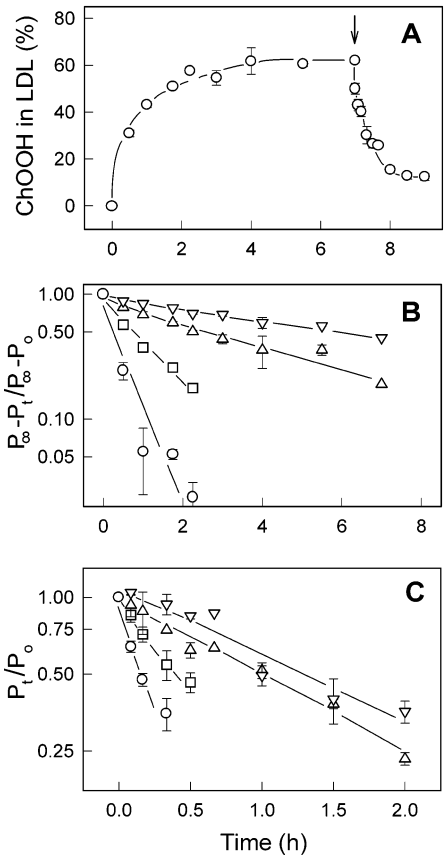

FIGURE 2: Forward and reverse transfer of ChOOHs between membranes and LDL. Forward (ghost-to-LDL) transfer mixtures contained photooxidized ghosts (0.25 mM total lipid, 14 μM total ChOOH), LDL (1.25 mM total lipid), and 10 μM BHT in PBS/DFO/EDTA. Reactions were carried out at 20 °C with periodic sampling, centrifugation, and recovery of LDL for HPLC-EC(Hg) determination of ChOOH uptake. Immediately after 7 h of incubation, the entire remaining reaction mixture was centrifuged. LDL in the supernatant fraction was recovered and mixed with nonoxidized ghosts to give 0.25 and 1.25 mM of total LDL and ghost lipid, respectively, in PBS/DFO/EDTA. The mixture was incubated for an additional 2 h at 20 °C, during which the LDL compartment was sampled for ChOOH analysis. (A) Time courses for total ChOOH acquisition by LDL and subsequent departure to nonoxidized ghosts (arrow). (B) First-order kinetic plots for LDL acquisition of individual ChOOH isomers. P_0, P_t, and P_∞ denote peroxide content at zero time, time *t*, and infinite time, respectively. Key: 7α/7β-OOH (○), 5α-OOH (□), 6α-OOH (△), and 6β-OOH (▽). (C) First-order plots for departure of ChOOHs from LDL. P_0 and P_t represent peroxide remaining at time zero and time *t*, respectively. ChOOHs are denoted as in panel B. Mean values from duplicate experiments are plotted in panels A−C.

in the kinetics of ChOOH uptake and release, the latter occurring ~2.5 times faster (0.42 vs 1.08 h^{-1}). First-order plots for the acquisition and release of individual ChOOHs by/from LDL at 20 °C are shown in panels B and C of Figure 2, respectively, and rate constants derived from these data are listed in Table 1. Though the acquisition (ghost → LDL) constants are 3−5-fold lower than at 37 °C, they exhibit the same rank order (7α/β-OOH > 5α-OOH > 6α-OOH > 6β-OOH). The same is true for the departure (LDL → ghost) constants at 20 °C, which vary from ~2- to ~5.5-fold greater than the respective acquisition constants at 20 °C (Table 1). It is relevant to note that Ch desorption from intact RBCs has also been reported to be slower than from LDL (*k* ~0.15 vs 0.95 h^{-1} at 37 °C) (*46*, *47*). LDL is ~250 times smaller in diameter than the RBC, and its Ch and PL are in an outer

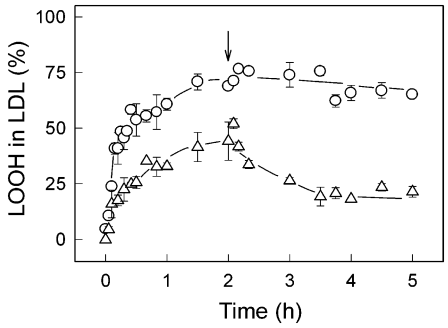

FIGURE 3: ChOOH and PLOOH transfer to LDL and relative stability therein. Ghost membranes were photoperoxidized and incubated with LDL as described in Figure 1. The initial concentrations of total ChOOH and PLOOH in bulk suspension were 5.5 and 20.7 μM, respectively, 7.5 μM of the latter being PCOOH. At various time points, LDL was recovered by centrifugation and extracted, and lipid fractions were analyzed for ChOOH and PCOOH levels. After 2 h (arrow), LDL in the remaining reaction mixture was recovered and incubated without ghosts for an additional 3 h at 37 °C with periodic sampling. The percent of ChOOH (○) and PCOOH (△) found in LDL is plotted as a function of incubation time. Mean values from duplicate experiments are plotted.

monolayer rather than a bilayer. Accordingly, LDL's outer lipids would be more loosely packed than RBC membrane lipids, and this might account for the more rapid Ch efflux from the lipoprotein. Moreover, the overall level of unsaturated lipid relative to Ch is substantially greater in LDL than in RBCs (*1*, *36*), which would also favor Ch departure in the former (*20*). Faster ChOOH release from LDL than from ghosts (Figure 2) can be explained along similar lines.

PLOOH Transfer between Membranes and LDL. We found that photoperoxidized ghosts could transfer PLOOHs to LDL in addition to ChOOHs. Kinetic experiments were typically carried out with ghosts in which ~20% of the total PL had been converted to PLOOH. Before obtaining detailed kinetic information for the different PLOOH families (PCOOH, PEOOH, PSOOH, SMOOH), we examined their stabilities under experimental transfer conditions. Whereas ChOOHs in a complete system (ghosts + LDL) were fully recoverable after long reaction periods (cf. Figure 1A), PLOOHs underwent a slow progressive loss over time. This was attributed to factor(s) associated with LDL because the decay was negligible when photooxidized ghosts were incubated alone or with SUVs as acceptors instead of LDL (data not shown). As shown in Figure 3, LDL accumulated both ChOOH and PLOOH (represented here by PCOOH) during incubation with peroxidized ghosts at 37 °C. After 2 h in this experiment, the LDL was separated from the membranes and incubated by itself for an additional 3 h, during which PCOOH and ChOOH contents were measured. Unlike [ChOOH], which dropped by <10% over this interval, [PLOOH] decreased by ~55%, beginning rapidly and reaching an apparent nadir after ~2 h. While the ChOOH loss was too small to matter for most experiments, the PLOOH loss had to be corrected for in order to obtain accurate rate constants. This was accomplished by determining overall (LDL + ghost) PLOOH in addition to ghost PLOOH at each experimental time point. HPLC-EC(Hg) profiles depicting the progressive loss of individual PLOOHs in photooxidized ghosts and appearance in LDL during transfer incubation are shown in Figure 4A. As in the case of ChOOHs (Figure 4A), starting LDL (0 min) exhibited

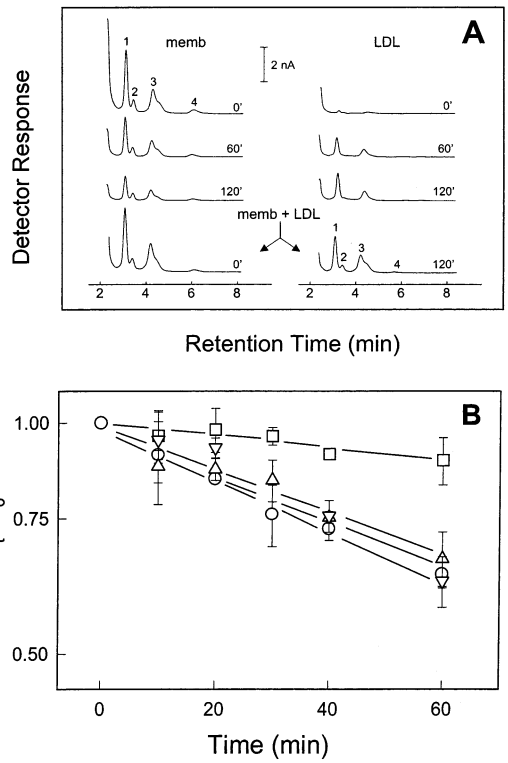

FIGURE 4: Spontaneous transfer of various PLOOH families from photooxidized ghost membranes to LDL. Reaction mixtures were composed and incubated as described in Figure 1. Initial PLOOH concentrations in bulk suspension were as follows: PCOOH (11.8 μM), PEOOH (15.1 μM), SMOOH (3.6 μM), and PSOOH (2.2 μM). Samples were removed at the indicated times and centrifuged, and the supernatant and membrane fractions (after washing) were then extracted. Duplicate samples at the same time points were extracted directly, i.e., without centrifugation, and thus were used to monitor the level of each PLOOH group in the entire reaction system. Recovered lipid fractions were analyzed for PLOOHs by HPLC-EC(Hg). (A) Chromatographic profiles showing the progressive disappearance of PLOOHs in membranes and appearance in LDL over a 2 h incubation period. Total lipid per injection: 9.2 nmol (memb); 18.5 nmol (LDL); 21.6 nmol (memb + LDL). Peak assignments and retention times are as follows: (1) PCOOH (2.89 min); (2) SMOOH (3.17 min); (3) PEOOH (4.11 min); (4) PSOOH (6.02 min). (B) First-order kinetic plots for PLOOH decay in the membrane compartment. Plotted values for each PLOOH group were corrected for LDL-mediated degradation, as determined by complete system analysis. Key: PCOOH (○), PEOOH (△), PSOOH (▽), and SMOOH (□). Data points are means ± deviation of values from duplicate experiments, one of which is represented in (A).

little detectable PLOOH, and this remained the case during incubation with nonphotooxidized ghosts (data not shown). A comparison of the complete system (memb + LDL) profiles at 0 and 120 min indicates that all PLOOH peaks were significantly diminished after incubation; thus, the degradation appeared to be nonselective. First-order kinetic plots representing the corrected losses of individual PLOOHs in the membrane compartment are shown in Figure 4B. The plots are linear over a 1 h reaction period, PCOOH, PEOOH, and PSOOH each decaying to ~65% of its starting level and SMOOH to ~90%. The apparent first-order rate constants for PCOOH, PEOOH, and PSOOH were found to be essentially the same ($\sim 7.4 \times 10^{-3}$ min^{-1}) and approximately 4 times greater than the SMOOH constant (Table 2). Similar rate constants were determined for ghost-to-SUV PLOOH transfer (Table 2); no corrections were necessary in this case because PLOOHs were stable in the ghost/SUV

Table 2: Rate Constants for PC and PLOOH Transfer from Ghost Membranes to LDL or SUVs[a]

	$k \times 10^3$ (min^{-1})[b]	
analyte	ghost → LDL	ghost → SUV
POPC	0.22 ± 0.03	nd[c]
PCOOH	7.57 ± 0.90	7.85 ± 1.20
PEOOH	7.09 ± 0.94	7.92 ± 1.13
PSOOH	7.60 ± 0.85	6.83 ± 1.08
SMOOH	1.87 ± 0.32	1.68 ± 0.43

[a] Reaction mixtures for measuring POPC transfer to LDL contained [^{14}C]POPC-labeled ghosts (0.18 mg of lipid/mL, ~0.5 nCi/mL), LDL (2.12 mg of lipid/mL), and BHT (10 μM) in PBS/DFO/EDTA at 37 °C. Timed incubation samples were centrifuged, and membrane pellets were washed free of LDL and extracted. Recovered lipid fractions were analyzed for [^{14}C]POPC by means of HPTLC-PI. Ghost-to-LDL transfer data for the various PLOOHs are from the experiments described in Figure 4. Ghost-to-SUV transfer was examined using photoperoxidized ghosts (0.25 mM lipid) and DMPC/DCP (100:1 mol/mol) SUVs (1.25 mM lipid). [b] Apparent first-order rate constants for the various analytes were determined from their decay kinetics in the donor compartment. Means ± deviations of values from duplicate experiments are shown. [c] nd = not determined.

system. The observed agreement between ghost/SUV and ghost/LDL values indicates that the latter were corrected properly and are, therefore, valid. Knowing the initial concentration of each membrane PLOOH (Figure 4) and its rate constant, we calculated the following initial rates for transfer to LDL: 90 nM/min (PCOOH); 107 nM/min (PEOOH); 17 nM/min (PSOOH); 6 nM/min (SMOOH). Thus, PCOOH and PEOOH had similar initial rates, which were ~6 times greater than the PSOOH value and ~16 times greater than the SMOOH value. Though relatively fast among the PLOOHs, PCOOH and PEOOH moved at only one-fourth the 5α-OOH rate (see above).

It was of interest to compare the indicated PLOOH transfer kinetics (Table 2) with those of at least one parent phospholipid. For this, we labeled ghosts with [^{14}C]POPC, using SCP-2 as a mediator, and tracked movement to LDL under the same conditions as described for PLOOHs. Typically, the membranes were charged with both [^{14}C]POPC and [^{14}C]Ch, and the lipids were monitored simultaneously using HPTLC-PI (Ch data shown in Table 1). POPC was found to have an apparent first-order rate constant of 2.2×10^{-4} min^{-1} at 37 °C, which is ~3% of the average PLOOH value (Table 2). For the experiment described in Figure 4, the initial concentration of PL in membrane suspension was ~0.14 mM. Assuming that endogenous PLs had the same kinetic constant as POPC, we estimate that the initial rate of overall PL transfer was ~31 nM/min. By comparison, the aggregate PLOOH transfer rate (see above) was 210 nM/min or nearly 7 times the PL rate. For the donor PCOOH/PC group specifically in the Figure 4 experiment, the initial concentrations of PCOOH and PC were ~12 and ~29 μM, respectively. Knowing this and the rate constants shown in Table 2, one finds that PCOOH moved to LDL 14 times faster than parent PC. Clearly, therefore, PLs, like Ch, translocated far more rapidly after being peroxidized. These are the first measurements of PLOOH transfer to be reported for any donor/acceptor system.

Prooxidant Activity of Transfer-Acquired LOOHs. Preexisting LOOHs increase LDL's sensitivity to metal ion-catalyzed chain peroxidative damage (*9*, *10*). Such LOOHs have been detected in freshly isolated LDL or have been

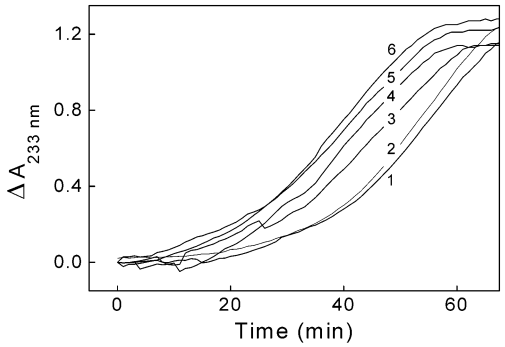

FIGURE 5: Conjugated diene formation in transfer LOOH-enriched LDL. Transfer reactions were carried out as described in Figure 1. The initial concentrations of total membrane ChOOH and PLOOH in bulk suspension were 13.6 and 51.2 μM, respectively. At various points during incubation, 0.4 mL samples were centrifuged to pellet ghost membranes. LDL-containing supernatant solutions were diluted to a final protein concentration of 0.125 mg/mL in PBS and incubated in the presence of 20 μM $CuCl_2$ at 37 °C. The time course for increasing conjugated diene absorbance at 233 nm was monitored, using non-$CuCl_2$-treated LDL as a blank. Numbered traces represent the following: (1) LDL not exposed to ghosts; (2) LDL exposed to nonphotooxidized ghosts for 60 min; (3−6) LDL exposed to photooxidized ghosts for 2, 5, 10, and 30 min, respectively.

enriched in vitro by photochemical (*10*) or cell-mediated means (*7*, *17*−*19*). It was of interest, therefore, to learn how transfer-acquired LOOHs would affect LDL susceptibility to free radical-mediated peroxidation. Reactions were triggered with Cu^{2+}, which acts catalytically, beginning with either the oxidation of LOOH to a chain-initiating peroxyl radical (LOO$^{\bullet}$) or reduction to a chain-initiating oxyl radical (LO$^{\bullet}$) (*48*). We first examined conjugated diene formation as a general indicator of LOOH-induced free radical peroxidation (*49*). Reactions were monitored in real time by measuring increments in absorbance at 233 nm. As shown in Figure 5, LDL preincubated with nonperoxidized ghosts for 1 h at 37 °C displayed the same time course for conjugated diene accumulation as untreated LDL, i.e., a 20−25 min lag, followed by a rapid buildup and maximization after 1 h. Significantly, LDL that had been treated with photoperoxidized ghosts for 2, 5, 10, and 30 min before contacting Cu^{2+} exhibited progressively shorter lags and earlier bursts of conjugated diene formation. These effects are consistent with more rapid depletion of LDL antioxidants (α-tocopherol, β-carotene, ubiquinol) as free radical pressure due to the one-electron turnover of "seeding" LOOHs increased.

More specific information about the ability of transferred LOOHs to induce free radical peroxidation was obtained by applying a technique in which [^{14}C]Ch serves as an in situ probe of free radical activity, as indicated by characteristic oxidation products (ChOX species). Developed in this laboratory, the ChOX approach has been used previously for assessing chain peroxidation in model membranes and cell membranes (*38*). Representative HPTLC-PI profiles depicting product accumulation after 0, 1, 1.5, and 4 h of incubation with Cu^{2+} are shown in Figure 6A. System a represents control LDL (preincubated with native ghosts) and system b experimental LDL (preincubated with photooxidized ghosts). Donor membranes in this experiment were photooxidized twice as long as those in the Figure 1 experiment, which explains their initial ChOOH and PLOOH

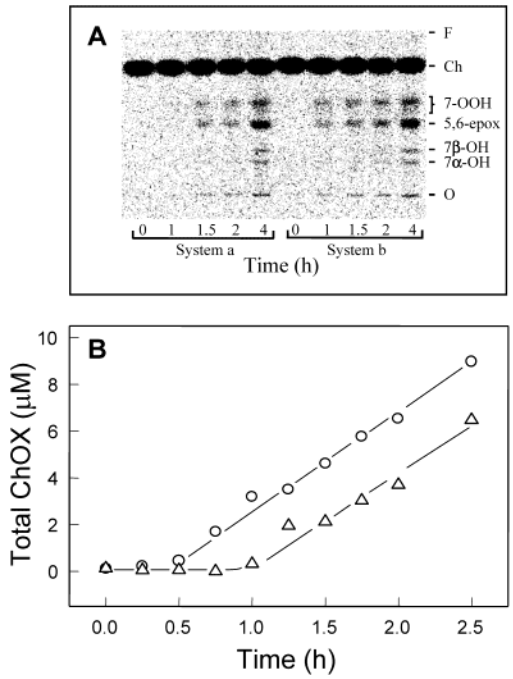

FIGURE 6: Accumulation of free radical-derived cholesterol oxidation products (ChOX species) in transfer LOOH-enriched LDL. [^{14}C]Ch-labeled LDL (2.5 mM total lipid, 0.56 μCi/mL) was incubated for 90 min with photoperoxidized ghosts (0.25 mM total lipid, including 15.5 μM ChOOH and 58.3 μM PLOOH) in argon-sparged PBS at 37 °C. After centrifugation, LDL was recovered and diluted to 0.125 mg of protein/mL (0.58 mM lipid; ~2.8 μM as ChOOH and ~1.8 μM as PLOOH) in PBS. A control prepared alongside with nonphotooxidized ghosts contained <25 nM each of ChOOH and PLOOH. The LDL preparations were incubated in the presence of 20 μM $CuCl_2$ at 37 °C. Samples were removed at the indicated times and extracted; lipid fractions were analyzed by HPTLC-PI (0.11 μg of LDL lipid per lane). (A) HPTLC-PI plate showing parent Ch and various ChOX species (7-OOH, 5,6-epoxide, 7β-OH, 7α-OH); O, origin; F, solvent front; system a, control LDL plus Cu^{2+}; system b, peroxidized ghost-treated LDL plus Cu^{2+}. (B) Time courses for total ChOX accumulation: system a (△); system b (○). Data are from the same experiment with additional time points represented. ChOX concentrations in the bulk reaction suspension are plotted.

concentrations being nearly double those of the latter. Note that the initial [ChOOH] in experimental LDL was 55% higher than initial [PLOOH] (2.8 vs 1.8 μM), whereas the combined levels in control LDL were <0.05 μM (Figure 6). Five ChOX species were identified in Cu^{2+}-treated LDL: 7α-OH, 7β-OH, 5,6-epoxide, and 7-OOH (partially resolved 7α- and 7β-OOH) in order of increasing mobility. All were well separated from one another and from Ch, which moved far ahead of 7-OOH; material near the origin is unidentified. ChOX products were detected much earlier in system b than in system a, consistent with more intense free radical activity in the former (cf. Figure 5). ChOX kinetic plots (Figure 6B) clearly illustrate this point by showing that the lag period was 30 min longer in system a than system b, whereas rates of accumulation after the lag were the same for both systems.

In addition to ChOX products, transfer-acquired ChOOHs and PLOOHs caused a substantial enhancement of cholesteryl ester peroxidation in Cu^{2+}-treated LDL. As shown in Figure 7, control LDL (membrane-treated) contained a small amount of HPLC-EC(Hg)-detectable CEOOH at the outset, which increased ~6-fold after incubation with Cu^{2+} for 20

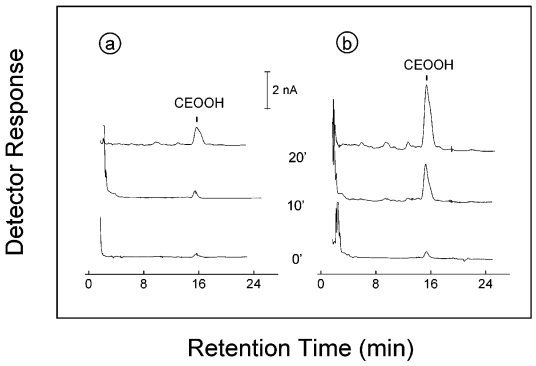

FIGURE 7: Cholesteryl ester (CEOOH) formation in transfer LOOH-enriched LDL. Transfer incubation with peroxidized or nonperoxidized ghosts was carried out as described in Figure 6, but in this case the LDL was not radiolabeled. After separation from ghosts by centrifugation, the LDL at 0.53 mg of protein/mL (2.5 mM total lipid) was reacted with 20 μM $CuCl_2$ at 37 °C. System a, control LDL plus Cu^{2+}; system b, peroxidized ghost-treated LDL plus Cu^{2+}. Initial levels of LDL peroxides in system a: 0.8 μM CEOOH; <0.1 μM PLOOH; <0.1 μM ChOOH. Initial levels of LDL peroxides in system b: 1.1 μM CEOOH; 7.8 μM PLOOH; 11.9 μM ChOOH. At various times during Cu^{2+} treatment, samples were extracted for HPLC-EC(Hg) determination of CEOOH (peak at 15.1 min). The total lipid per injection was 7.4 nmol.

min. By contrast, experimental LDL (peroxidized membrane-treated) showed a 15-fold increase in CEOOH content over the same reaction period. When corrected for starting levels, the differences are quite striking. Thus, net [CEOOH] in the experimental sample was 43 times greater than that in the control after 10 min and 4 times greater after 20 min (Figure 7). The smaller difference at the longer time may reflect increasing one-electron turnover of the hydroperoxide as it reached higher levels. Taken together, the conjugated diene, ChOX, and CEOOH data clearly demonstrate that acquired LOOHs significantly enhance LDL's susceptibility to oxidative modification propagated by free radicals.

DISCUSSION

Preexisting LOOHs sensitize LDL to oxidative modification, which is widely believed to be an early event in the development of atherosclerosis (*2*, *3*). Using HPLC-EC(Hg) analysis, we found previously (*10*) that carefully prepared LDL from normal volunteers contained ~10 pmol of CEOOH/mg of protein, which corresponds to approximately 1 CEOOH molecule for every 200 LDL particles. We reasoned that this low level could not have been an artifact of extraction and workup because it was not detected when the LDL was extracted after treatment with GSH and PHGPX (GPX4), an LOOH-reducing selenoperoxidase (*10*). Other evidence argued against the possibility of adventitious peroxidation during isolation from plasma (*10*). The implication of these findings is that the CEOOH, and presumably other LOOHs derived from lipids less abundant than cholesteryl ester, was already present in LDL before isolation. The in vivo origin of these LOOHs is not entirely clear. It has been reported that reactive oxygen (O_2^-, H_2O_2, $HO^\bullet$) or reactive nitrogen oxide species ($ONOO^-$, $^\bullet NO_2$) produced by endothelial cells (*7*, *50*) or activated leukocytes (*16*) can generate LOOHs de novo by direct attack on LDL. There is also evidence that lipoxygenases (LOXs) play a role, particularly the monocyte/macrophage 15-LOX. Accordingly, several broad-specificity LOX inhibitors have been reported

to inhibit LDL oxidation by murine macrophages (*17*). In a more recent study (*19*), LDL accumulated LOOHs more rapidly when incubated with fibroblast transfectants expressing high levels of 15-LOX than when incubated with low expression controls. Concomitantly, free radical damage was more extensive in the LDL exposed to the high 15-LOX cells. Direct access of the lipoprotein to 15-LOX's active site was offered as one possible explanation (*19*), although this is difficult to imagine on steric grounds and because the enzyme normally exists in intracellular compartments. As an alternative, it was suggested that LOOHs generated by 15-LOX might somehow depart from the cell and translocate to LDL through the medium (*19*). On the basis of the present findings, this would appear to be the more likely possibility. LOOH transfer from cells under relatively high oxidative pressure in the circulation could be a general phenomenon, but up to now this has been given surprisingly little attention. Good examples of donor cells would include neutrophils, macrophages, and red blood cells. In activated neutrophils, LOOHs might accrue from oxidants produced by plasma membrane NADPH oxidase (*51*), whereas in red cells they could accumulate spontaneously and progressively throughout the cell lifetime due to the high O_2 and iron content. Although human red cells contain antioxidant enzymes of the preventative type, viz., catalase, Cu/Zn-superoxide dismutase, and type 1 glutathione peroxidase (GPX1), they lack type 4 glutathione peroxidase (GPX4, PHGPX), which is more important as a reparative enzyme for "back-up" protection (*52*). Unlike GPX1, GPX4 can directly reduce/inactivate ChOOHs and PLOOHs in a membrane bilayer (*53*). Therefore, red cells are deficient in the ability to detoxify LOOHs. Under these circumstances, translocation would be a logical means of relieving LOOH pressure and its potentially damaging effects.

In initial studies dealing with this concept (*22*, *23*), we used photoperoxidized red cell ghosts as LOOH donors and unilamellar liposomes (SUVs) as acceptors. Carried out exclusively with ChOOHs, these studies demonstrated for the first time that these species, like parent Ch (*20*), translocate via an aqueous transit pool and that the rate-limiting step is desorption from the donor membrane. ChOOH loss from ghost membranes obeyed apparent first-order kinetics, the initial rate increasing linearly with peroxide concentration up to at least 10% of the donor Ch oxidized. Thus, even though other lipids besides Ch and also membrane proteins were affected by photooxidation, the rate constant for overall ChOOH transfer remained constant, at least over the indicated range of oxidative modification. This constant was shown to be ~65 times greater than that for parent Ch measured under the same conditions, and this translated into a greater ChOOH initial rate notwithstanding the much higher starting concentration of membrane Ch. Approximately the same difference in Ch and ChOOH transfer kinetics was observed in the present study (Table 1), confirming that these kinetics are determined by properties of the donor rather than acceptor (in this case LDL), assuming that the latter exceeds the former in pool size (*20*). As observed previously with SUVs as acceptors (*23*), we found that four positional isomers in the ChOOH population transferred to LDL with different rate constants, their rank order being 7α/7*β*-OOH > 5α-OOH > 6α-OOH > 6*β*-OOH, which correlates with their observed order of decreasing polarity. At equivalent ChOOH starting concentrations, therefore, 7α/7*β*-OOH would desorb most rapidly from the donor membrane and thus translocate via the largest aqueous transit pool. This could be highly significant for plasma membranes damaged by free radical reactions (e.g., iron-catalyzed chain peroxidation) because 7α- and 7*β*-OOH are the most prominent ChOOHs produced by such reactions (*41*). On the other hand, 5α-OOH is the predominant ChOOH in $^1O_2$-mediated reactions (*40*), and its high yield in our photooxidized ghosts explains why it translocated to LDL with the highest initial rate. A large number of cytotoxic and potentially atherogenic cholesterol oxides have been identified in oxidized LDL, including 5α-OOH (*54*) and 7*β*-OOH (*55*). 7*β*-OOH has been reported to be far more noxious to fibroblasts and endothelial cells than its diol (7*β*-OH) or 7-keto analogue (*55*). For $^1O_2$-producing systems, e.g., activated eosinophils and possibly neutrophils (*16*, *56*), 5α-OOH could be especially dangerous because of its high rate of generation, resistance to metabolic detoxification (*57*), and relatively rapid transfer. That LDL 5α-OOH and 7*β*-OOH might derive at least in part from translocation rather than entirely de novo has been largely overlooked up to now.

No significant net loss or gain of any ChOOH species was observed when transfer to LDL was carried out under redox inhibited conditions, i.e., in the presence of DFO, EDTA, and BHT in Chelex-treated PBS. We found that the LDL-favored equilibrium in ChOOH transfer could be shifted back toward the membrane compartment by exposing the LDL to an excess of unoxidized ghosts. This confirmed that translocation was freely reversible, as reported earlier for the ghost/SUV system (*22*). However, back-transfer from LDL was found to be significantly faster than forward-transfer, the overall ChOOH rate constant being ~3 times greater, whereas the rank order of individual ChOOH constants remained the same. On the basis of earlier findings with SUV donors of varying dimensions (*23*), the more rapid departure from LDL might reflect its smaller average diameter and more loosely packed outer lipids. However, this could be an oversimplification because ghost ChOOHs emanated from a membrane bilayer containing at least one large lytic fissure (*29*), whereas LDL ChOOHs emanated from a surface monolayer of different composition. Moreover, the ghosts had been subjected to photooxidation, which modified proteins and other lipids besides Ch, whereas LDL was unmodified except for the low-level incorporation of LOOHs. We reported previously (*23*) that overall ChOOH desorbed from photooxidized ghosts with an activation energy of 84.7 ± 3.4 kJ/mol. In the present study, we determined that the activation energy for ChOOH departure from LDL was 40.6 ± 5.4 kJ/mol, i.e., <50% of the ghost donor value. This implies that the free energy for transfer activation, i.e., movement of the hydrophilic portion of a ChOOH molecule to the aqueous interface (*23*), was far less in the LDL monolayer than in the ghost bilayer. Transfer of other LOOHs (e.g., PLOOHs and CEOOHs) from LDL has not been examined, but one would expect this to be relatively rapid, based on the ChOOH results. The implication of these results is that peroxidized LDL may be cytotoxic and atherogenic not only through its recognition by the scavenger receptor (*2*) but also through its ability to release LOOHs for direct uptake by vulnerable target cells.

Like ChOOHs, PLOOHs moved spontaneously from peroxidized ghosts to LDL, but with significantly lower first-order rate constants; for example, k_{PCOOH} was about one-tenth k_{7-OOH} at 37 °C. These differences are not surprising, given that parent PLs have been shown to transfer/exchange much more slowly than Ch in various donor/acceptor systems (*20*). Among the PLOOHs studied, PCOOH, PEOOH, and PSOOH moved from ghosts to LDL with the same *k* value, whereas SMOOH's value was one-fourth as large. The direct proportionality between transfer rate constant and retention time-based hydrophilicity, which applied for the ChOOHs, did not hold for the PLOOHs (cf. Figure 4). However, this would not be unexpected because the amino column used for PLOOH separation does not operate under the same principles as the octadecylsilyl column used for ChOOH separation. Therefore, for the PLOOHs it is unclear whether relative hydrophilicity was a key determinant of desorption rate. Sphingomyelin is known to interact strongly with Ch in membrane bilayers (*58*). If this interaction persisted with SMOOH, it is conceivable that this could explain the latter's relatively low rate constant. In evaluating the PLOOH kinetic data, it was necessary (as noted) to correct for a progressive net loss of PLOOH during transfer to LDL (Figure 3). Since LDL was shown to be responsible for this loss, we subjected it to various treatments at 37 °C in an attempt to characterize and possibly prevent the process involved. These treatments included (i) a 1 h incubation with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) to block −SH groups on apoB-100, (ii) 1 h with 0.1 mM chloramine-T to oxidize possible PLOOH-reducing methionine residues on apoB-100 (*59*), (iii) 2 h with 0.1 mM *p*-bromophenacyl bromide to inhibit apoB-100-associated phospholipase A_2 activity (*60*), and (iv) 2 h with 0.05 mM diisopropyl fluorophosphate to inhibit possible acyltransferase (LCAT) contamination in the LDL (*61*). None of these steps followed by dialysis inhibited LDL's ability to slowly degrade PLOOHs, as assessed by incubating photooxidized ghosts with treated vs nontreated lipoprotein (results not shown). Similarly, incubating LDL at 55 °C for 18 h or at 37 °C in the presence of a protease mixture in an attempt to inhibit some unknown protein that might either reduce or hydrolyze PLOOHs was without effect. Thus, we have not identified the responsible factor. That PLOOHs were sensitive to this activity while ChOOHs were resistant is intriguing and might possibly relate to the fact that 5α-OOH, the predominant ChOOH in our system, is a tertiary peroxide, whereas PLOOHs are secondary peroxides. If the factor were some type of peroxidase, it is interesting to note that all PLOOHs are good substrates for type 4 glutathione peroxidase, whereas 5α-OOH is a poor substrate (*57*). A preliminary experiment (not represented) has indicated that 5α-OOH-rich ChOOH is also relatively stable in the presence of whole serum. Thus, incubation of peroxidized ghosts with 20% fetal calf serum in PBS/DFO/EDTA at 37 °C for 1 h resulted in <10% ChOOH degradation. This suggests that ChOOH at least would be sufficiently long-lived in plasma for translocation to occur. Moreover, transit time might be shortened by plasma transfer proteins (*62*). Studies aimed at more closely modeling natural transfer conditions are in progress.

Transfer-acquired LOOHs markedly increased the sensitivity of LDL to Cu^{2+}-induced free radical peroxidation, as assessed by conjugated diene, ChOX, and CEOOH formation (Figures 5−7). Transferred LOOHs consisted of both PLOOHs and ChOOHs, 5α-OOH being the most prominent pro-oxidant. Whereas 7α/7*β*-OOH continually turned over and re-formed from LDL Ch during chain peroxidation, 5α-OOH, 6α-OOH, and 6*β*-OOH only decayed (data not shown), suggesting that $^1O_2$ was not a significant byproduct (*38*). We have not studied this, but it is likely that transfer LOOH-enhanced peroxidation of LDL resulted in greater oxidative modification of the apoB-100 protein, a reaction associated with increased recognition by the scavenger receptor (*1*−*3*). Instead of propagating new chain reactions in the targeted particle, successor (downstream) LOOHs arising from LDL lipids might themselves translocate to other available acceptors and thus propagate damage at some distance from the original donor. Relatively fast departure from LDL, at least for the ChOOHs (Figure 2), would tend to favor this. Although we have not studied CEOOH (the most abundant of the oxidized LDL LOOHs) in this regard, others have demonstrated CEOOH transfer/exchange between LDL and high-density lipoprotein (HDL) and between HDL and liver cells (*62*, *63*). It is clear from our findings that LOOHs which "seed" peroxidative damage in LDL may derive not only from direct attack of oxidants on the lipoprotein but also from transfer uptake.

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