Exchange of oxidized cholesteryl linoleate between LDL and HDL mediated by cholesteryl ester transfer protein

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Abstract This study examines the cholesteryl ester transfer protein (CETP)-mediated exchange of cholesteryl linoleate hydroperoxide (Ch18:2-OOH) and cholesteryl linoleate hydroxide (Ch18:2-OH) between low density lipoprotein (LDL) and high density lipoprotein (HDL). When [^{3}H]Ch18:2-OOH- and [^{3}H]18:2-OH-labeled LDL were incubated at 37°C for 0–24 h with unoxidized HDL and purified CETP, Ch18:2-OOH and Ch18:2-OH accumulated in the HDL. Similarly, when incubations were carried out with [^{3}H]Ch18:2-OOH- and [^{3}H]Ch18:2-OH-labeled HDL, unoxidized LDL, and CETP, Ch18:2-OOH and Ch18:2-OH accumulated in the LDL. Comparable results were obtained for the CETP-mediated transfer of [^{3}H]Ch18:2-OH alone from LDL to HDL. Transfer to HDL of oxidized cholesteryl linoleate from [^{3}H]Ch18:2-OOH- and [^{3}H]Ch18:2-OH-labeled LDL was comparable to that of unoxidized cholesteryl linoleate (Ch18:2). However, the rate of transfer of [^{3}H]Ch18:2-OOH and [^{3}H]Ch18:2-OH from LDL to HDL increased linearly as the molar ratio of acceptor (HDL) to donor (oxidized LDL) particles in the incubation increased from 0.5:1 to 10:1. This increased rate of exchange was accompanied by an increased proportion of the oxidized Ch18:2 being present as the hydroxide rather than hydroperoxide. Further increases in the molar ratio of HDL to oxidized LDL particles neither affected the transfer rate nor the extent of reduction of Ch18:2-OOH to Ch18:2-OH. We therefore conclude that *i)* CETP mediates bidirectional transfers of Ch18:2-OOH and Ch18:2-OH between HDL and LDL; *ii)* CETP does not distinguish between Ch18:2-OOH, Ch18:2-OH, and Ch18:2 as it mediates their exchange between HDL and LDL; and *iii)* association with HDL hastens the reduction of Ch18:2-OOH to Ch18:2-OH.—**Christison, J. K., K-A. Rye, and R. Stocker.** Exchange of oxidized cholesteryl linoleate between LDL and HDL mediated by cholesteryl ester transfer protein. *J. Lipid Res.* 1995. **36:** 2017–2026.

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Oxidative modification of low density lipoproteins (LDL) is generally thought to contribute to the development of atherosclerosis (1, 2). Oxidatively modified LDL may be taken up by macrophages at an enhanced rate and in an uncontrolled fashion, thereby causing intracellular accumulation of lipids and formation of "foam cells" (3–5). Oxidation of LDL lipids is thought to be important because the primary lipid oxidation products, i.e., lipid hydroperoxides, can decompose to reactive secondary products capable of modifying apoB, the process ultimately leading to "high-uptake" forms of LDL (6, 7). The precise molecular events leading to oxidative LDL modification in vivo remain poorly understood. Enzymic (8–11) and/or non-enzymic (4, 12) oxidation processes are likely to be involved. Although often assumed to occur on LDL directly, transfer of preformed oxidized lipids from cells and/or lipoproteins may contribute to or even be required for these processes, as in the case of the putative oxidative modification of extracellular LDL by cellular 15-lipoxygenase (1).

Lipoproteins undergo continuous remodelling during their transit in plasma and extravascular compartments (e.g., ref. 13). Lipid transfer and exchange processes relevant to the composition of lipoproteins are mediated by specialized proteins, including lecithin:cholesterol acyltransferase (14), phospholipid transfer protein, and cholesteryl ester transfer protein (CETP) (15, 16). With regards to the oxidation theory of atherosclerosis (1), a possible involvement of CETP in

Abbreviations: AAPH, 2,2'-azo*bis*(2-amidinopropane) dihydrochloride; CE, cholesteryl ester(s); CETP, cholesteryl ester transfer protein; Ch18:2, cholesteryl linoleate; Ch18:2-OH, cholesteryl linoleate hydroxide(s); Ch18:2-OOH, cholesteryl linoleate hydroperoxide(s); Ch18:2-O(O)H, oxidized Ch18:2 comprised of Ch18:2-OH plus Ch18:2-OOH; HDL, high density lipoproteins; LDL, low density lipoproteins; LDL_{OH}, low density lipoproteins containing Ch18:2-OH but not Ch18:2-OOH; $t_{1/2}$, half life.

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the transfer of oxidized lipids, including those of cholesteryl linoleate (Ch18:2), is of potential interest. Ch18:2 is quantitatively the major single substrate for oxidation in human LDL and high density lipoproteins (HDL). Relatively large quantities of hydroperoxides (Ch18:2-OOH) and some hydroxides of Ch18:2 (Ch18:2-OH) are formed during the early stages of in vitro peroxidation of LDL and HDL (17–19), and these two forms of oxidized Ch18:2 are present in human atherosclerotic lesions (20, 21).

Comparing the relative oxidizability of different lipoproteins, we observed that during in vitro oxidation of human plasma, HDL cholesteryl esters (CE) are oxidized before those in LDL (18) and likely (22) very low density lipoprotein. Also, plasma of healthy humans contains very small amounts of Ch18:2-OOH (23), of which most are associated with HDL (18). Furthermore, HDL-associated Ch18:2-OOH are reduced to Ch18:2-OH, and both forms of oxidized Ch18:2 are rapidly removed via selective uptake and detoxified by human hepatoma HepG2 cells (24). This has led us to suggest (18) that HDL may act as a detoxifying sink for potentially atherogenic oxidized lipids. Such putative anti-atherogenic effect of HDL could be enhanced if it was shown to acquire Ch18:2-OOH from LDL.

We therefore examined whether CETP can mediate the transfer of Ch18:2-OOH from LDL to HDL, using purified human CETP and in vitro mildly oxidized or [^{3}H]Ch18:2-OOH-labeled LDL as "donor" and HDL as acceptor lipoprotein. Our results show that CETP does transfer Ch18:2-OOH from LDL to HDL to an extent similar to that of unoxidized Ch18:2 and Ch18:2-OH. However, this transfer does not show specificity for its direction as CETP also transfers the two forms of oxidized Ch18:2 from oxidized HDL to LDL.

MATERIALS AND METHODS

Materials

The azo initiators 2,2′-azo*bis*(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azo*bis*(2,4-dimethylvaleronitrile) were purchased from Polysciences (Warrington, PA). Chelex-100 was from Bio-Rad laboratories (Richmond, CA), desferrioxamine was from Ciba-Geigy (Basel, Switzerland), bovine serum albumin and egg yolk lecithin were from Sigma (St. Louis, MO), cholesteryl [9-hydroxy]linoleate was from Cayman Chemicals (Ann Arbor, MI), and [cholesteryl-1,2,6,7-^{3}H]linoleate ([^{3}H]Ch18:2; specific activity 74–91 Ci/mmol) was from DuPont, New England Nuclear (Boston, MA). Ebselen was a generous gift from Rhône-Poulenc-Nattermann (Cologne, Germany). Ch18:2-OOH standard was prepared from purified Ch18:2 (Sigma) by oxidation with 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (25, 26). All other chemicals were from Merck (Darmstadt, FRG). Ethanol (Analytical grade) was obtained from British Drug House (BDH, Poole, England), while all other organic solvents were from Mallinckrodt Inc. (Clayton, VIC, Australia) and were of HPLC quality. Before use, all aqueous solutions were stored over Chelex-100 to remove trace metal contaminations. Centricon-30 concentrators were purchased from Amicon (Beverly, MA) and PD-10 Sephadex G-25M columns were from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Isolation of lipoproteins

Venous blood, anticoagulated with EDTA (1 mg/mL), was obtained from healthy male or female donors (age 22–40 years) and the plasma was separated from blood cells by centrifugation for 10 min at 4°C (500 *g*). The density of the plasma was then adjusted to 1.21 g/mL by dissolving the appropriate amount of solid KBr and the lipoproteins (HDL and LDL) were isolated by two-step density gradient ultracentrifugation in a TL-100 table-top ultracentrifuge equipped with a TL-100.4 rotor (Beckman, CA) centrifuged at 100,000 rpm and 15°C for 2 h (26). The aspirated lipoproteins were pooled from two to three lipoprotein preparations and concentrated at 2,800 *g* for 1 h (for HDL) and 2 h (LDL) using Centricon-30 concentrators. Unless subjected to oxidation, lipoproteins were gel-filtered using a PD-10 column equilibrated with 50 mM phosphate buffer, pH 7.4, and the protein content was estimated (27). Lipoprotein concentrations were expressed in (particle) molarity, assuming an average protein contribution of 50 and 20% to the total mass of HDL and LDL, respectively, and apparent particle masses of 2×10^5 Da and 3×10^6 Da for HDL (mean of HDL_2 and HDL_3) and LDL, respectively (28).

Labeling of lipoproteins with tritiated cholesteryl linoleate

Labeling of lipoproteins was achieved by exchange with donor liposomes prepared by sonication of egg yolk lecithin (160 μg/mL 50 mM phosphate buffer, pH 7.4) containing 50–100 μCi of [^{3}H]Ch18:2. To enhance the exchange of the radiolabel, the concentrated lipoprotein was incubated at 37°C overnight under argon in the presence of an equal volume of lipoprotein-deficient plasma and 0.5–1 mL donor liposomes. The labeled lipoproteins were then re-isolated by two-step ultracentrifugation as described above, and found to contain ≈1 mCi/mmol Ch18:2.

Peroxyl radical-mediated oxidation of lipoproteins

Controlled and limited oxidation of isolated native LDL and HDL or [^{3}H]Ch18:2-labeled LDL was carried

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out by incubation of the lipoproteins with the peroxyl radical generator AAPH (50 mM) at 37°C for 1.5 h (for LDL) or 2 h (HDL). AAPH decomposes thermally to yield aqueous peroxyl radicals at constant and known rates that oxidize the lipoproteins via tocopherol-mediated peroxidation (29). Lipoprotein oxidation was terminated by gel-filtration of the reaction mixtures through three successive PD-10 columns, a procedure that removed all AAPH. Lipoproteins were then concentrated using Centricon-30 concentrators spun at 2,800 *g* for 30 min and their protein content was determined. Oxidized LDL and HDL solutions prepared in this manner contained, on average, 54 and 12 μM Ch18:2-OOH, respectively, corresponding to 180 and 4 molecules of Ch18:2-OOH per LDL and HDL particle, respectively. The corresponding values for Ch18:2-OH (see ref. 19) were 8 and 7 μM for LDL and HDL, respectively, corresponding to 27 and 2 molecules of Ch18:2-OH per particle. Thus, in oxidized LDL and HDL a total of 9 and 4% of the lipoprotein's Ch18:2 were oxidized, respectively. Oxidation did not change the apparent densities of LDL and HDL, as judged by the positions of the lipoprotein bands in density gradients after ultracentrifugation.

Preparation of LDL_{OH}

Oxidized LDL prepared by AAPH-mediated oxidation of the [^{3}H]Ch18:2 labeled or unlabeled lipoprotein, was incubated at 37°C for 30 min in the presence of the glutathione peroxidase mimic ebselen (68 μM) and reduced glutathione (408 μM). This procedure leads to catalytic conversion of all Ch18:2-OOH to Ch18:2-OH (30). The resulting LDL_{OH} was then passed through two sequential PD-10 columns to remove ebselen and glutathione (31). Lipoprotein preparations were stored under argon at 4°C and used within 72 h (for native, unoxidized lipoproteins) or 24 h (oxidized lipoproteins).

Purification of CETP

Human CETP was purified from plasma donated by the Transfusion Service (Royal Adelaide Hospital). The purification procedure was carried out exactly as described elsewhere (32). The CETP isolated was pure, as judged by polyacrylamide gel electrophoresis and Coomassie staining, where the purified protein appeared as a single band. Activity of the preparations was determined as the transfer of [^{3}H]CE from [^{3}H]CE-HDL_3 to LDL (33, 34). One unit of activity represents the transfer activity of 1 mL of a pooled sample of human lipoprotein-deficient plasma.

CETP-mediated transfer experiments

For these experiments gel-filtered LDL and HDL were used in 50 mM phosphate-buffered saline, pH 7.4, at a final concentration of 0.3 and 3 μM, respectively, to mimic the approximate ratio of these lipoproteins in human plasma. The reaction mixtures were prepared on ice and desferrioxamine (200 μM) was added to all samples to complex any remaining transition metals from the reaction mixture. CETP was then added to a final concentration of 1 unit/ml. For control incubations, an equal volume buffer (20 mM Tris–150 mM NaCl, pH 7.4, containing 1 mg/mL bovine serum albumin) was added in place of CETP. Incubations were carried out at 37°C for 24 h under an atmosphere of argon to prevent autoxidation. At the time points indicated, an aliquot of the reaction mixture was removed, its density was adjusted to 1.21 g/mL by addition of solid KBr, and lipoproteins were re-isolated by ultracentrifugation for 45 min in a TL-100.2 rotor centrifuged at 100,000 rpm (26). As oxidized lipoproteins (particularly oxidized HDL) were often nearly colorless, a reference tube containing a mixture of unoxidized lipoproteins was included to allow accurate aspiration of the lipoprotein bands. The entire fraction of the appropriate lipoprotein was aspirated. Separate experiments showed that the LDL obtained in this way was essentially devoid of contaminating HDL, as judged by the absence of apoA-I and apoA-II. Similarly, the HDL obtained was devoid of contaminating apoB. Aliquots (200 μL) of the lipoprotein solutions removed were used for analysis of unoxidized and oxidized Ch18:2-O(O)H as described below.

Analysis of ^{3}H-labeled and unlabeled Ch18:2, Ch18:2-OOH, and Ch18:2-OH

After their re-isolation, lipoprotein samples (200 μL) were extracted with methanol (2 mL) and hexane (10 mL), the hexane phase was removed and evaporated, and the sample was resuspended in ethanol. Separation and analysis of [^{3}H]Ch18:2, [^{3}H]Ch18:2-OOH, and [^{3}H]Ch18:2-OH present in the extracts was performed by reversed-phase HPLC on a 25 × 0.46 cm LC-18 column (Supelco, Bellefonte, PA) eluted with acetonitrile–isopropanol–water 22:27:1 (v/v/v) at 1 mL/min (35). Detection was via UV at 210 and 234 nm and, in the case of radiolabeled materials, flow-through radiometric detection (Canberra Packard, series A100 Radiomatic detector, Canberra A.C.T., Australia). Results were quantified by area comparison with appropriate standards.

RESULTS

Incubation of native, unoxidized HDL with oxidized LDL in phosphate-buffered saline for 24 h at 37°C in the absence of added CETP resulted in the time-depend-

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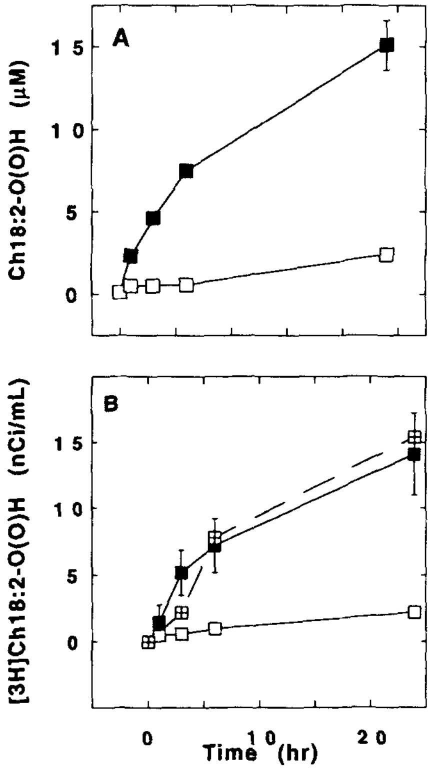

Fig. 1. CETP mediates the transfer of oxidized Ch18:2 from oxidized LDL to native HDL. Radiolabeled, oxidized LDL (final concentration 0.3 μM) prepared as described in Materials and Methods, was incubated with HDL (3.0 μM) at 37°C for 24 h in the presence (■, ⊞) or absence (□) of purified CETP. At the time points indicated, 600- to 800-μL aliquots of the reaction mixture were removed, the HDL was re-isolated, extracted, and analyzed for Ch18:2-OOH and Ch18:2-OH by UV_{234nm} detection (A) and for [^{3}H]Ch18:2-OOH and [^{3}H]Ch18:2-OH by radiometric detection (B) (see Materials and Methods). Similar results were obtained for the unlabeled and labeled Ch18:2-OOH and Ch18:2-OH. (B) shows the combined values for [^{3}H]Ch18:2-OOH plus [^{3}H]Ch18:2-OH obtained with LDL containing both forms of radiolabeled oxidized Ch18:2 (■), as well as the time-dependent transfer of [^{3}H]Ch18:2-OH (⊞) obtained with LDL_{OH} containing [^{3}H]Ch18:2-OH only. All results shown represent the means ± SEM of three independent experiments.

ent accumulation of small amounts of oxidized Ch18:2 (Ch18:2-O(O)H, i.e., Ch18:2-OOH plus Ch18:2-OH) in HDL (**Fig. 1A**). Similarly, incubation of HDL with [^{3}H]Ch18:2-O(O)H-labeled oxidized LDL resulted in the accumulation of small quantities of [^{3}H]Ch18:2-O(O)H in HDL (Fig. 1B). To confirm that oxidized LDL was the principal source of oxidized Ch18:2 in HDL in these experiments, native HDL was incubated for up to 24 h in the absence of oxidized LDL and under argon. This did not result in accumulation of detectable amounts of Ch18:2-OOH in HDL, though occasionally traces of Ch18:2-OH (<1 μM) were seen (data not shown). Addition of purified CETP, at a final concentration of 1 unit/mL resulted in a 10- and 7.5-fold increase in the initial rate and total amounts, respectively, of Ch18:2-O(O)H accumulating in HDL after incubation with oxidized LDL for up to 24 h (Fig. 1 and **Table 1**). In contrast, addition of CETP did not promote formation of Ch18:2-O(O)H in HDL when incubated in the absence of oxidized LDL (not shown).

The total amounts of [^{3}H]Ch18:2-O(O)H lost from oxidized LDL during incubation with HDL were 2.1 ± 5.9 and 15.9 ± 5.3 nCi/mL (mean ± SEM, n = 3) in the absence and presence of CETP, respectively (Table 1). These losses correlated well with the gains of Ch18:2-O(O)H in HDL, i.e., 2.2 ± 0.4 and 14.1 ± 3.1 nCi/mL in the absence and presence of CETP, respectively. The kinetic of transfer of Ch18:2-O(O)H from oxidized LDL to HDL (Fig. 1) was not significantly different from that of the unoxidized Ch18:2 contained in oxidized LDL (not shown). Over 24 h and in the presence of CETP, 18.5 ± 4.0 and 30.8 ± 12.8% (mean values ± SEM) of the oxidized and unoxidized Ch18:2, respectively, initially contained in the oxidized LDL, were transferred to HDL (Table 1). In both cases, approximately half of the 24-h amount had been transferred by 6 h, consistent with previous findings on the CETP-mediated transfer of unoxidized CE between HDL and LDL (36, 37). In the

TABLE 1. Balance sheet of CETP-mediated transfer of Ch18:2-O(O)H from oxidized LDL to HDL

	Oxidized LDL		HDL	
	[^{3}H]Ch18:2-O(O)H	[^{3}H]Ch18:2	[^{3}H]Ch18:2-O(O)H	[^{3}H]Ch18:2
CETP				
0 h	76.1 ± 8.7	648 ± 74	0 ± 0	0 ± 0
24 h	74.0 ± 9.5	611 ± 172	2.2 ± 0.4	53 ± 21
Δ	-2.1 ± 5.9	-36 ± 99	+2.2 ± 0.4	+53 ± 21
+CETP				
0 h	76.1 ± 8.7	648 ± 74	0 ± 0	0 ± 0
24 h	60.2 ± 4.4	457 ± 157	14.1 ± 3.1	200 ± 85
Δ	-15.9 ± 5.3	-191 ± 84	+14.1 ± 3.1	+200 ± 85

Radiolabeled, oxidized LDL (final concentration 0.3 μM) prepared as described in Materials and Methods was incubated with native, unoxidized HDL (3.0 μM) at 37°C for 24 h in the presence or absence of purified CETP (1 unit/mL). Before and after the incubation, (re-isolated) lipoproteins were extracted and the hexane phase was analyzed for [^{3}H]Ch18:2, [^{3}H]Ch18:2-OOH, and [^{3}H]Ch18:2-OH as described in Materials and Methods. Results are given in nCi/mL and represent the total oxidized Ch18:2 (i.e., Ch18:2-O(O)H defined as Ch18:2-OOH plus Ch18:2-OH) and unoxidized Ch18:2. The results shown are the means ± SEM of three independent experiments.

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absence of added CETP, only 3 and 8% of Ch18:2-O(O)H and unoxidized Ch18:2, respectively, were transferred in a near-linear fashion from oxidized LDL to HDL in 24 h (Table 1). This small extent of transfer is likely due to the presence of low concentrations of endogenous CETP that is known to associate with HDL (38).

Figure 2 shows representative chromatograms of the reversed-phase HPLC analysis of hexane extracts of the re-isolated HDL and oxidized LDL before (0 h) and after (24 h) incubation with CETP for the experiments described in Fig. 1 and Table 1. As can be seen, the chromatographic conditions used (35) separated Ch18:2-OOH from Ch18:2-OH and Ch18:2. Radiometric detection allowed tracing of [^{3}H]Ch18:2, [^{3}H]Ch18:2-OH, and [^{3}H]Ch18:2-OOH between LDL and HDL. As can be seen from the 0-h radiometric trace of oxidized LDL in Fig. 2, most of the radiolabeled Ch18:2 in oxidized LDL was not oxidized, demonstrating the limited degree of oxidation of the "donor" lipoprotein used for the transfer experiments. Endogenous and in vitro-transferred Ch18:2 were oxidized at identical rates when LDL was exposed to AAPH (24). Typically, approximately 15 and 85% of oxidized Ch18:2 in such oxidized LDL were initially present as Ch18:2-OH and Ch18:2-OOH, respectively (Fig. 2). After incubation with HDL for 24 h at 37°C in the absence of CETP, approximately 30 and 70% of the oxidized Ch18:2 of oxidized LDL were present, on average, as Ch18:2-OH and Ch18:2-OOH, respectively (not shown). In the presence of CETP, oxidized LDL contained, on average, 35 and 65% of its oxidized Ch18:2 as Ch18:2-OH and Ch18:2-OOH, respectively, after the incubation. This slightly higher hydroxide proportion of Ch18:2-O(O)H in the "donor" lipoprotein in the presence versus absence of CETP was not statistically different. The proportion of Ch18:2-OH was even higher in HDL, where approximately 80% of the Ch18:2-O(O)H was present as the hydroxide after a 24-h incubation with oxidized LDL, as assessed by both UV_{234nm} and radiometric detection (Fig. 2). HDL has a higher relative Ch18:2-OOH reducing activity compared to LDL (19). More importantly, the results clearly demonstrate that CETP was able to transfer oxidized Ch18:2 from oxidized LDL to HDL. However, the results did not allow us to distinguish whether CETP transferred Ch18:2-OOH or Ch18:2-OH, as the latter could have been formed from the hydroperoxides subsequent to their transfer (see below).

Increasing the ratio of acceptor to donor lipoprotein (i.e., HDL:oxidized LDL) by increasing the HDL concentration from 0.15 to 15 μM while maintaining the concentration of oxidized LDL at 0.3 μM, led to a 10-fold increase in the CETP-mediated transfer of Ch18:2-O(O)H to HDL, with a clear plateau reached at a particle

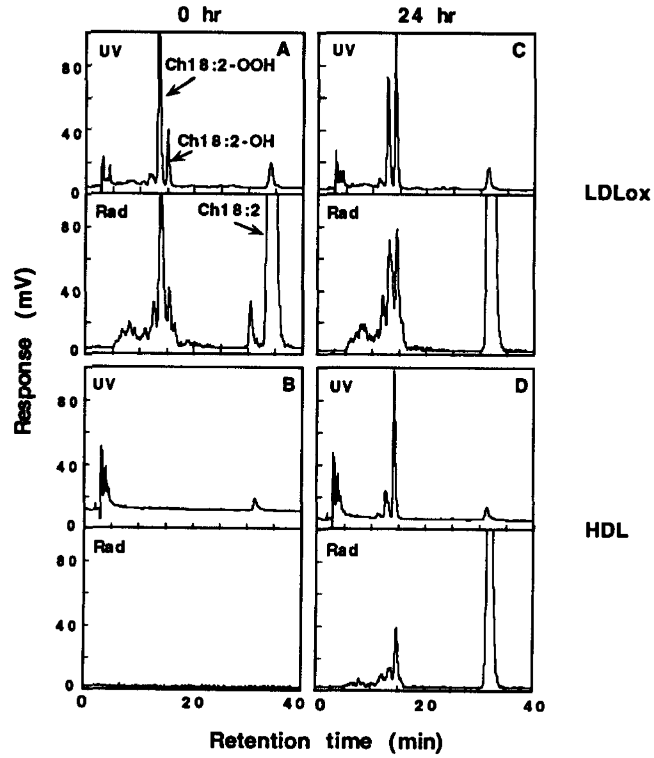

Fig. 2. Chromatography of CETP-mediated transfer of oxidized Ch18:2 from oxidized LDL to native HDL. Radiolabeled, oxidized LDL (final concentration 0.3 μM) was incubated with HDL (3.0 μM) at 37°C for 24 h in the presence of purified CETP. Representative chromatograms of the analysis of re-isolated oxidized LDL (A, C) and native HDL (B, D) are shown at 0 h (A, B) and 24 h (C, D). In each case the upper panel shows total oxidized Ch18:2 as determined by detection with UV_{234nm} absorption, while the lower panel shows the presence and form of the radiolabeled Ch18:2 as determined with radiometric detection. For experimental procedures see Materials and Methods. Note that at the wavelength used (i.e., 234 nm) Ch18:2 absorbs much less than equimolar amounts of Ch18:2-OOH and Ch18:2-OH. The proportions of Ch18:2-OOH and Ch18:2-OH in oxidized LDL (LDLox) after incubation for 24 h varied among different experiments and, on average, were 65 and 35%, respectively. The nature of the radiolabeled compound present in oxidized LDL at the beginning of the incubation and eluting at around 31 min is unknown.

ratio of HDL:oxidized LDL of 10:1 (**Fig. 3A**). Despite this net increase in transfer, the number of molecules of Ch18:2-O(O)H transferred to each HDL particle actually decreased steadily as the concentration of HDL increased (Fig. 3B). This may suggest that at the higher HDL concentrations, acceptor particles were not saturated with Ch18:2-O(O)H within the time frame of our experiments.

With increasing HDL:oxidized LDL ratios, the proportion of Ch18:2-O(O)H present as Ch18:2-OH at the end of the 24-h incubation period in the presence of CETP increased in HDL but not in oxidized LDL (**Table 2**). Thus, the 10-fold increase in the total amounts of oxidized Ch18:2 transferred (Fig. 3A) together with the 2-fold increase in the proportion of Ch18:2-OH led to an overall 20-fold increase in HDL Ch18:2-OH by in-

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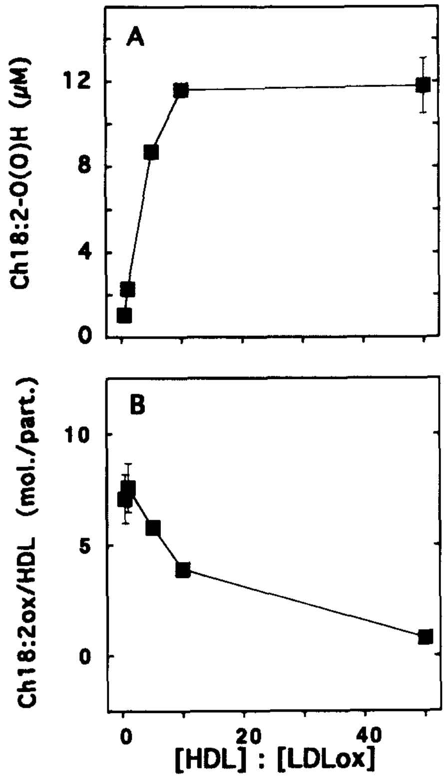

Fig. 3. CETP-mediated transfer of Ch18:2-O(O)H from oxidized LDL to HDL increases with increasing concentration of the acceptor particle. A) Oxidized LDL (final concentration 0.3 μM) was incubated with increasing concentrations of HDL (0.15–15 μM) at 37°C for 6 h in the presence of purified CETP. After incubation, 600- to 800-μL aliquots were removed, and HDL was re-isolated, extracted, and analyzed for Ch18:2-OOH and Ch18:2-OH by HPLC with UV_{234nm} detection as described in Materials and Methods. Similar results were obtained for Ch18:2-OOH and Ch18:2-OH, and the corresponding values were therefore combined and are shown as Ch18:2-O(O)H. The results represent means ± SEM of three independent determinations. Comparable results were obtained when radiolabeled oxidized LDL was used as the "donor" lipoprotein and the extent of transfer was determined by radiometric analysis of [^{3}H]Ch18:2-OOH and [^{3}H]Ch18:2-OH. B) Increasing the HDL:LDLox ratio decreases the number of Ch18:2-OOH plus Ch18:2-OH molecules transferred to each HDL particle. The results shown were calculated from the results obtained in A).

creasing the HDL:oxidized LDL ratio 100-fold. That the proportion of Ch18:2-OH in LDL oxidized Ch18:2 did not increase with increasing HDL is further support for the notion (19) that HDL is not able to directly reduce Ch18:2-OOH in oxidized LDL.

To test whether CETP was able to transfer Ch18:2-OH, we prepared LDL_{OH} (which contained Ch18:2-OH but no detectable Ch18:2-OOH) by reduction of oxidized LDL with ebselen and GSH (see Materials and Methods). Comparison of the CETP-mediated transfer of Ch18:2-OH (not shown) and [^{3}H]Ch18:2-OH with Ch18:2-O(O)H from LDL_{OH} to HDL showed no differences in the kinetics and absolute amounts of oxidized Ch18:2 transferred (Fig. 1B). These results demonstrate that CETP was able to transfer Ch18:2-OH from oxidized LDL to native HDL and, in fact, did not appear to distinguish between Ch18:2-OOH and Ch18:2-OH.

TABLE 2. HDL concentration-dependent increase in the proportion of Ch18:2-OH of Ch18:2-O(O)H in HDL but not oxidized LDL after incubation in the presence of CETP

[HDL]	Ratio Ch18:2-OH:Ch18:2-O(O)H	
	Oxidized LDL	HDL
μM		
0.15	0.27	0.39
0.30	0.30	0.45
1.5	0.31	0.61
3.0	0.31	0.68
15.0	0.30	0.64

Oxidized LDL (final concentration 0.3 μM in all incubations) prepared as described in Materials and Methods, was incubated at 37°C for 6 h in the presence of purified CETP (1 unit/mL) with increasing concentrations of HDL (0.15–15 μM). After incubation, 600- to 800-μL aliquots of the reaction mixture were removed, the lipoproteins were re-isolated, and HDL was extracted and analyzed for Ch18:2-OOH and Ch18:2-OH by UV_{234nm} detection as described in Materials and Methods. The results shown represent the means of three separate experiments.

Incubation of oxidized HDL with native, unoxidized LDL at 37°C for 24 h also resulted in the accumulation of significant quantities of Ch18:2-O(O)H (6.7 ± 1.2 nCi/mL) in the LDL, and this transfer was increased 4- to 5-fold in the presence of CETP (**Fig. 4**). For reasons presently unknown, the extent of transfer of Ch18:2-O(O)H in 24 h in the absence of CETP, was approximately 3- to 4-fold higher compared to that between oxidized LDL and HDL. This difference was even more pronounced when taking into account that the initial concentrations of Ch18:2-O(O)H were smaller in trans-

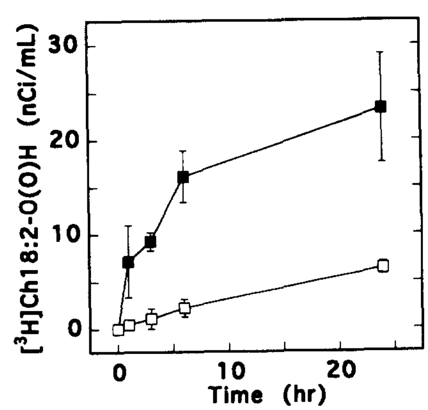

Fig. 4. CETP-mediated transfer of oxidized Ch18:2 from oxidized HDL to native, unoxidized LDL. Radiolabeled, oxidized HDL (final concentration 3.0 μM) was incubated with LDL (0.3 μM) at 37°C for 24 h in the presence (■) or absence (□) of purified CETP. At the time points indicated, 600- to 800-μL aliquots of the reaction mixture were removed, and the LDL was re-isolated, extracted, and analyzed for [^{3}H]Ch18:2-OOH and [^{3}H]Ch18:2-OH using radiometric detection as described in Materials and Methods. The results shown are the means ± SEM of two independent experiments.

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fer experiments using oxidized HDL as the "donor" lipoprotein. Separate experiments showed that Ch18:2-OH were transferred from oxidized HDL to native LDL at comparable rates as Ch18:2-O(O)H and Ch18:2 (not shown).

DISCUSSION

CETP mediates the exchange of core lipids between all lipoprotein classes (reviewed in refs. 13, 39). In this study we demonstrate, for the first time, that purified CETP at a concentration similar to that in human plasma also transfers Ch18:2-OOH and Ch18:2-OH between LDL and HDL in vitro. Ch18:2-OOH (17, 40–42) and, to a lesser extent, Ch18:2-OH (19), are the primary and quantitatively most important lipid products formed during radical-mediated LDL oxidation, and oxidatively modified LDL is thought to be atherogenic (1, 43). Our results, therefore, suggest that CETP could affect atherogenesis through distribution of atherogenic oxidized lipids between lipoproteins. Whether this extends to oxidized CE other than those tested in the present study is not known although this seems feasible given the relatively low degree of substrate specificity of CETP (44, 45).

In vitro the rate of CETP-mediated transfers of core lipids is determined by the ratio of "donor" to "acceptor" particles (46–48). The present study confirms that this is also the case for the CETP-mediated transfer of oxidized core lipids between HDL and LDL (Fig. 3). The observed transfer appears to be nonspecific in its direction and we could not detect a clear difference between the transfer of Ch18:2 and Ch18:2-O(O)H, although the transfer rates of the unoxidized ester tended to be higher than those of the oxidized Ch18:2. However, the fact that oxidized Ch18:2 from LDL containing both Ch18:2-OOH and Ch18:2-OH were transferred to HDL as efficiently as Ch18:2-OH from LDL_{OH} (which contained Ch18:2-OH only) (Fig. 1B) clearly suggests that lipoprotein-associated Ch18:2-OOH, at least at the concentrations used in our experiments, do not inhibit CETP. This is consistent with a previous report demonstrating that emulsions containing esterified fatty acid peroxides do not inactivate and degrade CETP (49). CETP is, however, inactivated by emulsions containing unesterified, peroxidized fatty acids (49) by a mechanism not presently understood.

In vivo, CETP is believed to cause a net transfer of CE from HDL to LDL. It is not known whether CETP could facilitate the exchange of oxidized CE between these two lipoproteins in vivo and, if so, in which direction such a transfer could be. Several factors are important and need to be considered. Perhaps most relevant to atherosclerosis is the situation in the intimal space, where CETP could be, and Ch18:2-OH and Ch18:2-OOH are known to be present (20, 21) (C. Suarna, R. T. Dean, J. May, and R. Stocker, unpublished observation). Unfortunately however, little direct information is presently available on the lipoprotein distribution of these oxidized CE. Indirect evidence suggests that lesion LDL is oxidized (49) and may thus carry oxidized CE, though we are not aware of any investigation specifically examining the extent of HDL oxidation in atherosclerotic lesions in humans (or animals). What is known, however, is that the LDL:HDL ratio in lesion is high compared to plasma (50, 51). We speculate that, if oxidized CE in lesions were located predominantly in LDL, CETP could mediate a local net transfer of these oxidized lipids from LDL to HDL, and this could potentially lead to the removal of oxidized lipids from the lesion via HDL (**Fig. 5**). Thus, CETP-facilitated net transfer of oxidized and unoxidized lipids between LDL and HDL could proceed in opposite directions, depending on the relative concentration gradients.

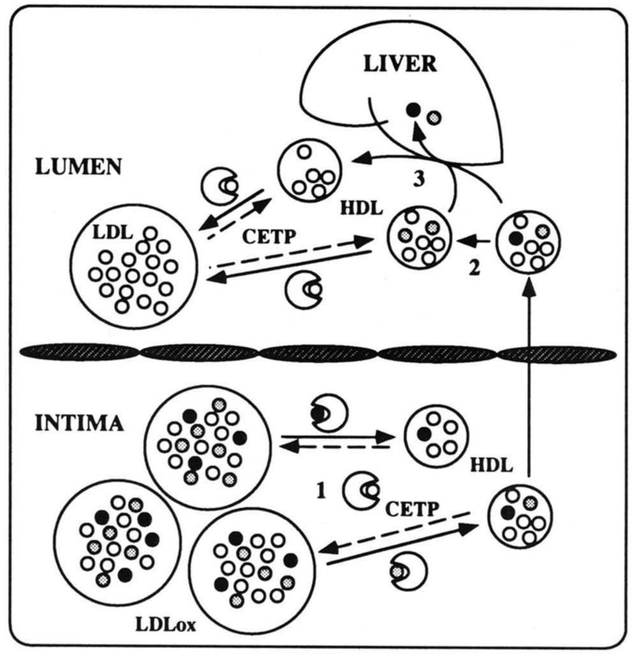

Fig. 5. Cartoon depicting the speculated role of CETP and HDL in the detoxification of potentially atherogenic oxidized lipids associated with lesion LDL. It is assumed that CETP is present in the intimal space where hydroperoxides (●) and hydroxides (dotted circle) of cholesteryl esters are preferentially located in LDL, thereby forcing a CETP-mediated net transfer (solid arrows) of these oxidized cholesteryl esters from LDL to HDL (1). HDL may then reduce acquired hydroperoxides to the corresponding hydroxides (2). This could occur within the intimal space or the lumen, in either case lowering the concentration of potentially harmful hydroperoxides. Once present in the circulation, HDL-associated oxidized cholesteryl esters are removed rapidly by the liver via selective uptake (3), with unoxidized cholesteryl esters (○) remaining quantitatively the major cholesteryl esters for CETP-mediated net transfer to LDL. The broken arrows indicate transfers of unoxidized cholesteryl esters in exchange for oxidized (intima) and unoxidized (lumen) cholesteryl esters.

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Whether CETP-mediated transfer of oxidized CE could be important in vivo is also dependent on the time required for the transfer compared to the half-life ($t_{1/2}$) of these oxidized CE. This study shows that in vitro this transfer of oxidized CE between LDL and HDL is more than one order of magnitude slower than the in vitro 'selective uptake' of HDL Ch18:2-O(O)H by human hepatocytes (52) which itself is substantially faster than 'selective uptake' of unoxidized CE of HDL (24) and hence likely also LDL (53). As HDL- but not LDL-associated Ch18:2-O(O)H are removed rapidly by rat liver in situ (J. K. Christisen, A. Karjalainen, F. Bygrave, R. Stocker, unpublished data), it seems possible that in vivo hepatic removal of HDL oxidized CE could effectively compete with CETP-mediated transfer to LDL. Therefore, HDL could conceivably facilitate hepatic detoxification of these potentially atherogenic oxidized lipids (Fig. 5), even though quantitatively the overall uptake of unoxidized cholesteryl esters may still be much higher from LDL (via receptor and non-receptor-mediated processes) than HDL. Further work is required to assess this possibility.

Another important issue to consider is the nature of the oxidized lipids. Hydroperoxides are unstable products of lipid peroxidation in the presence of transition metals where they give rise to reactive moieties that can oxidatively modify apoB in LDL (6). In contrast, lipid hydroxides are comparatively stable and do not act as precursors for reactive secondary lipid oxidation products. Therefore, the reduction of hydroperoxides of CE to their corresponding hydroxides represents a potentially important step in the antioxidant defense (19). Lipoproteins appear to contain a reducing activity that carries out this reduction, and this activity is more pronounced in HDL than LDL (19). The results reported here (Fig. 2, Table 2) are consistent with and further support these earlier findings: oxidized CE in HDL were predominantly present as the hydroxides, particularly after prolonged incubation periods. Thus, even if CETP were to mediate a bidirectional exchange of oxidized CE between HDL and LDL, HDL may still decrease the concentration of lipid hydroperoxides that could give rise to oxidatively modified LDL that can lead to foam cell formation.

The majority of the literature on CETP suggests that this protein is pro-atherogenic (reviewed in ref. 13) (54). However, the situation is complex in that CETP may also have anti-atherogenic activities. For example, CETP may be involved in reverse cholesterol transport, promoting the efflux of cholesterol from cells and vascular interstitium. The results presented here demonstrate the ability of CETP to transfer oxidized CE between lipoproteins. This, together with our observation that once associated with HDL these oxidized lipids are rapidly reduced and cleared by hepatocytes, perhaps more rapidly than they are transferred (back) to LDL, suggest that CETP could aid HDL in the removal and hepatic detoxification of oxidized lipids from site of high concentration, such as atherosclerotic lesions. Further studies will be required to test whether this process can indeed take place in vivo.

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