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Oxidation of LDL enhances the cholesteryl ester transfer protein (CETP)-mediated cholesteryl ester transfer rate to HDL, bringing on a diminished net transfer of cholesteryl ester from HDL to oxidized LDL

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Abstract

Cholesteryl ester transfer protein (CETP) plays a controversial role in atherogenesis by contributing to the net transfer of high density lipoprotein (HDL) cholesteryl ester (CE) to the liver via apolipoprotein-B-containing lipoproteins (apoB-LP). We evaluated in vitro the CETP-mediated bidirectional transfer of CE from HDL to the chemically modified pro-atherogenic low density lipoprotein (LDL) particles. Acetylated or oxidized (ox) LDL, either unlabeled or [^{3}H]-CE labeled, were incubated with [^{14}C]-CE-HDL in the presence of the lipoprotein-deficient plasma fraction (d>1.21 g/ml) as the source of CETP. The amount of radioactive CE transferred was determined after dextran sulfate/$MgCl_2$ precipitation of LDL. The results showed a 1.4–2.8-fold lower HDL-CE transfer to acetylated LDL while no effect was observed on the CE transfer to oxidized LDL. However, the reverse transfer rate of [^{3}H]CE-LDL to HDL was 1.4–3.6 times greater when LDL was oxidized than when it was intact. Overall, HDL_2 was better than HDL_3 as donor of CE to native LDL, probably reflecting the relatively greater CE content of HDL_2. Oxidation of LDL enhanced the CETP-mediated cholesteryl ester transfer rate to HDL, bringing on a reduced net transfer rate of cholesteryl ester from HDL to ox LDL. This may diminish the oxLDL particle's atherogenic effect.

Keywords: Oxidized LDL; Acetylated LDL; Cholesteryl ester transfer protein; HDL; Reverse cholesterol transport

1. Introduction

Plasma cholesteryl ester transfer protein (CETP) mediates the net transfer of cholesteryl ester (CE) from HDL to apolipoprotein-B-containing lipoproteins (apoB-LP) [1]. This process could lead to an overall increased rate of plasma CE removal since the apoB-LP are rapidly taken up by specific hepatic receptors [2], bringing about a lower risk

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of atherosclerosis [3]. However, the role of CETP in the development of atherosclerosis has not as yet been completely elucidated [4]. For instance, as compared to control nontransgenic animals, transgenic mice overexpressing CETP [5] develop diet-induced atherosclerosis more severely. Also, the expression of CETP in LDL receptor knockout and apoE knockout mice worsens the atherosclerosis in these animals [6]. On the other hand, there are conflicting data in human population studies, where an increased coronary heart disease incidence was associated with a genetic CETP deficiency [7–9], as well as with increased CETP plasma levels [10].

CETP is a plasma glycoprotein secreted mainly by the liver but with a widespread pattern of tissue expression [11–13], suggesting that the protein may have a local role in the redistribution of cholesterol. Apart from liver, adipose tissue is considered an important source of CETP in several species [14,15].

In vivo, chemically modified LDL is able to trigger a whole cascade of events leading to the development of atherosclerosis [16]. Modified LDL, especially oxidized LDL, is found in atherosclerotic lesions [17,18]. It is also present at low concentrations in the plasma of primates and humans [19,20], including patients with diabetes mellitus [21], chronic renal failure [22], and in smokers [23]. Furthermore, plasma LDL particles from dyslipidemic [24,25] or CETP-deficient patients [1] are more susceptible to oxidation than those of controls.

Previous studies had indicated that CETP-mediated transfer between lipoproteins is altered when lipoproteins are chemically modified [3,26–30]. In this regard, several factors, such as lipoprotein electrical charges, quantity of fatty acids as well as chemical modifications, may interfere with the amount of cholesteryl ester transferred to LDL from HDL particles.

In this study, we investigated the effects of LDL acetylation or oxidation on the in vitro CETP-mediated bidirectional flow of CE between HDL and LDL particles. This process probably occurs in the plasma compartment as well as in the microenvironment of the arterial intima.

2. Material and methods

2.1. Isolation, chemical modification and labeling of lipoproteins

LDL (d=1.006–1.063 g/ml), HDL_2 (d=1.063–1.125 g/ml) and HDL_3 (d=1.125–1.21 g/ml) fractions were isolated from fresh plasma of healthy normolipidemic human donors (total cholesterol= 177±24 mg/dl; triglycerides=126±51.4 mg/dl and HDL=42±8 mg/dl) by sequential ultracentrifugation [31] in an L8 Beckman ultracentrifuge at 4°C in a 50 Ti rotor at 100,000 *g*. Preservatives were added to the plasma pools: aprotinin (0.1 trypsin inhibitor unit/ml), 2 mmol/l benzamidine (5 μl/ml), gentamicin plus 0.25% chloramphenicol (20 μl/ml) and 0.5 mmol/l phenylmethyl-sulfonylfluoride (0.5 μl/ml). The lipoproteins were dialyzed against phosphate-buffered saline (PBS) with EDTA, at pH 7.4. The LDL used in the oxidation experiments was dialyzed in PBS without EDTA.

[1,2,(*N*)-^{3}H-cholesteryl-oleoyl-ether] (50 μCi), which was previously dissolved in ethanol, was incubated with human LDL and lipoprotein-deficient plasma (d>1.21 g/ml) for 24 h at 37°C in a rotary shaker at 20 rpm [32]. The density was then adjusted to 1.063 g/ml and the LDL-[^{3}H]-CE was separated by ultracentrifugation.

LDL was acetylated as previously described [33] using 1.5 μl of acetic anhydride per mg of LDL protein. The LDL was oxidized as previously described [34]. Briefly, LDL (100 μg protein) was incubated with $CuSO_4$ (final concentration of 10 μmol/l) over 8 h at 37°C in an opened tube in a shaking water bath. After oxidation, LDL was dialyzed against PBS with EDTA, to prevent further oxidation. The oxidation level was determinate by TBARS [35]. The efficiency of the LDL chemical modification was determined by 1% agarose gel electrophoresis in barbital buffer, pH 8.6, for 30 min. LDL was visualized by Fat Red 7B staining.

Whole HDL fraction was incubated with 25 μCi of [cholesteryl-4-^{14}C]-oleate ([^{14}C]-CE) in lipoprotein-deficient plasma (d>1.21 g/ml) for 24 h at 37°C in a rotary shaker [36]. After the incubation, the solution density was adjusted to 1.125 g/ml with solid KBr, and the HDL_2 [^{14}C]-CE was obtained by

ultracentrifugation for 40 h at 100,000 *g* at 4°C. The density of the infranatant was adjusted to 1.21 g/ml and the HDL_3 [^{14}C]-CE was again obtained by ultracentrifugation. HDL_2 and HDL_3 were then dialyzed against PBS/EDTA.

The chemical composition of the HDL was (mean values of three preparations, % by weight): 3.2% free cholesterol (FC); 5.3% triglycerides (TG); 15% CE; 33.1% phospholipids (PL) and 43.3% proteins (PR) for HDL_2 and 1.7% FC; 2.5% TGs; 5% CE; 36.7% PL; 54.1% PR for HDL_3 and 2.6% FC; 7.2% TG; 30.8% CE; 33.4% PL and 25.4% PR for LDL. The contents of cholesterol, triglycerides, phospholipids and proteins were not altered in the chemically modified LDL.

2.2. Cholesteryl ester transfer assay

LDL protein (100 μg of native, acetylated or oxidized), either unlabeled or [^{3}H-CE] labeled, was incubated with 10,000 dpm of HDL_2 or HDL_3 [^{14}C]-CE in the presence of 50 μl of plasma infranatant (d>1.21 g/ml) as the source of CETP. All incubations contained 0.25 mmol/l of DTNB (5,5-dithiobis-2-nitrobenzoic acid), which was needed to inhibit the lecithin cholesterol acyl transferase activity. The final volume was adjusted to 250 μl with Tris-buffered saline, pH 7.4. Incubations were carried out simultaneously at 37 and 4°C (blank reaction) for 2, 4 and 6 h. ApoB-containing lipoproteins were then precipitated with 1.6% dextran-sulfate–1 mol/l $MgCl_2$ solution (1:1, v/v) and the radioactivity, [^{14}C]-CE and [^{3}H]-CE, was measured in the supernatant using an LS 6000 Beckman Beta Counter.

The [^{14}C]-CE transfer from HDL to modified LDL was calculated as: 1−(dpm remaining in HDL at 37°C/dpm remaining in HDL at 4°C)×100.

The [^{3}H]-CE transfer rate from modified LDL to HDL was calculated as: [(dpm remaining in HDL at 37°C minus dpm remaining in HDL at 4°C)/total initial radioactivity (dpm) in LDL]×100.

2.3. Other analyses

Total cholesterol (Chod-Pap, Merck S/A, São Paulo, Brazil), unesterified cholesterol (Boehringer, Mannheim, Germany) and triglycerides (Enz-Color, Biodiagnostica, Pinhais, PR, Brazil) were determined by enzymatic methods; protein was measured according to the Lowry procedure [37] and phospholipids by the Bartlett method [38].

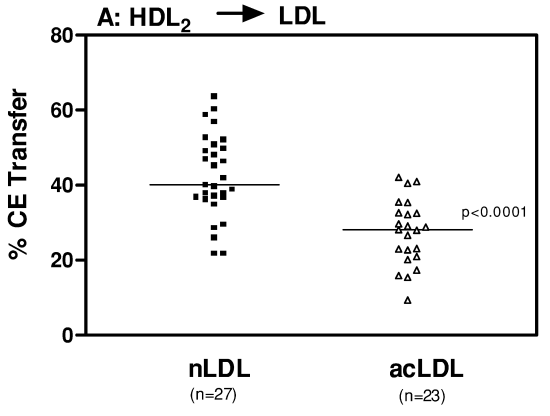
The size of LDL was measured by electron microscopy using the cryofracture method [39]. All LDL samples were frozen in the liquid phase of nitrogen slush (−210°C). Freeze-fracture was carried out at −100°C at 2×10^{-6} Torr, in a Blazers 301 freeze–etch apparatus. The replicas were examined in a Joel 1010 model at 80 kV. The mean diameter was estimated in approximately 100 particles of each LDL type in four electron micrographs.

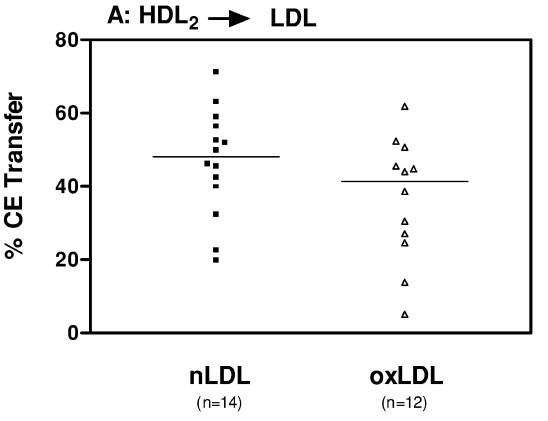
The Mann–Whitney *U* test was used for statistical comparison of the medians of the CE transfer rates (%) between lipoproteins.

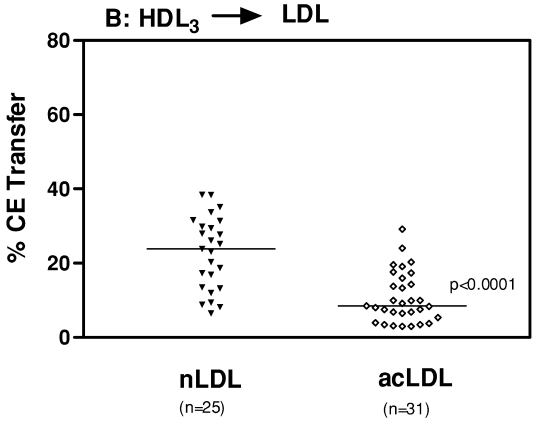
3. Results

The modified LDL particles used in this study presented, as expected, a differential migration pattern on 1% agarose gel electrophoresis. The migration distances to the cathode were, in decreasing order: acetylated>oxidized>native LDL. The average degree of oxidation produced was 22.9 nmol/l of TBARS/mg of LDL protein, while no oxidation was detected in acetylated and native LDL preparations. The contents of cholesterol, triglycerides, phospholipids and proteins were not altered in the chemically modified LDL. The mean±S.D. diameters of the LDL particles showed statistically significant differences when acetylated (144±8 Å) and oxidized (253±47 Å) LDL were compared to native LDL (231±36 Å), $P<0.05$.

The CETP-mediated transfer of [^{14}C]-CE from HDL_2 to native or acetylated LDL during the 6-h incubation period was 40.1 and 28.1%, respectively ($P<0.0001$; Fig. 1, panel A). When HDL_3 was used as the donor particle, the transfer rates of [^{14}C]-CE to native or acetylated LDL were 23.8 and 8.5%, respectively ($P<0.0001$; Fig. 1, panel B). Therefore, acetylated LDL takes up much less cholesteryl ester from both HDL particles than native LDL does.







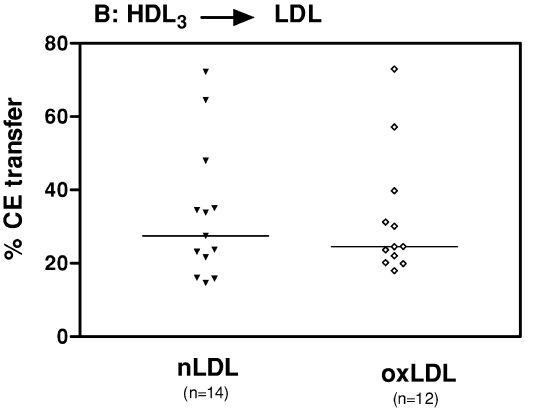


Fig. 1. CETP-mediated transfer from ^{14}C-CE-labeled HDL_2 (panel A) or HDL_3 (panel B) to native (n) or acetylated (ac) LDL. ^{14}C-CE-labeled HDL (10,000 dpm) was incubated with LDL (100 μg protein) and plasma fraction of $d>1.21$ g/ml (50 μl), at 4 and 37°C for 2, 4 and 6 h. Horizontal lines represent the median of individual data. Radioactivity was measured in the supernatant after LDL precipitation with dextran sulfate/$MgCl_2$. Mann–Whitney test, nLDL versus acLDL, $P<0.0001$ (panels A and B); HDL_2 versus HDL_3 for nLDL and acLDL, $P<0.0001$.

Fig. 2. CETP-mediated transfer from ^{14}C-CE-labeled HDL_2 (panel A) or HDL_3 (panel B) to native (n) or oxidized (ox) LDL. ^{14}C-CE-labeled HDL (10,000 dpm) was incubated with LDL (100 μg protein) and plasma fraction of $d>1.21$ g/ml (50 μl), at 4 and 37°C for 2, 4 and 6 h. Radioactivity was measured in the supernatant after LDL precipitation with dextran sulfate/$MgCl_2$. Horizontal lines represent the median of individual data. Mann–Whitney test, HDL_2 versus HDL_3 for nLDL, $P<0.05$.

Furthermore, HDL_2 is a better CE donor particle than HDL_3 ($P<0.0001$), regardless of whether native LDL or acetylated LDL is utilized as acceptor particles, as shown statistically in regard to native or acetylated LDL.

The CETP-mediated transfer from [^{14}C]-CE-HDL_2 to oxidized LDL during 6 h of incubation (Fig. 2, panel A) was not different from the transfer to native LDL. Similarly, when [^{14}C]-CE-HDL_3 was used, no difference in the CE transfer rates to oxidized or native LDL was observed (Fig. 2, panel B). Statistical comparison of the CE donor ability of the two HDL subfractions (Fig. 2, panel A vs. panel B) showed that HDL_2 was better than HDL_3 as CE donor when native LDL was the acceptor particle ($P<0.05$), but when oxidized LDL was utilized, no difference was detected between these HDL subfractions.

However, the CETP-mediated reciprocal CE trans-

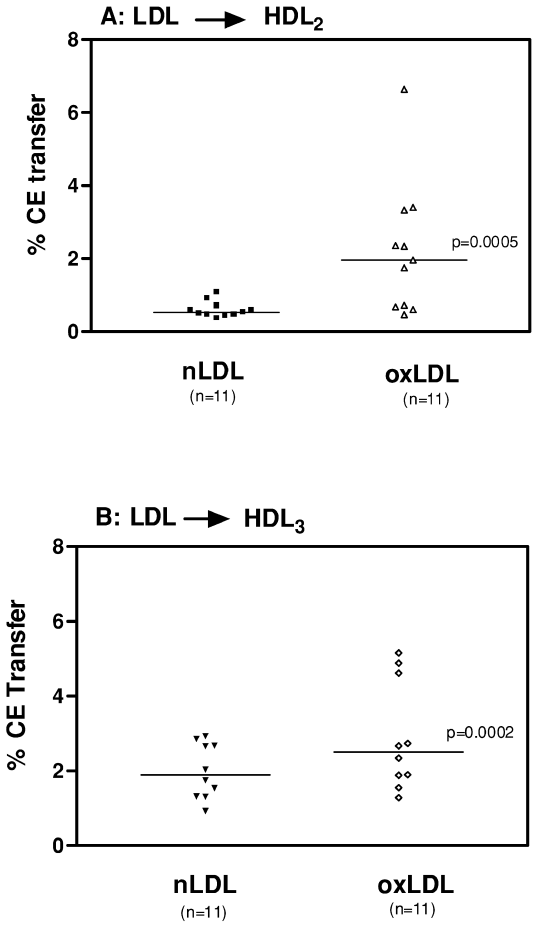


Fig. 3. CETP-mediated transfer from ^{3}H-CE-labeled native (n) or oxidized LDL to HDL_2 (panel A) or HDL_3 (panel B). Experiments were performed as described in Fig. 2. Horizontal lines represent the median of individual data. Mann–Whitney test, LDL to HDL (panel A, $P=0.0005$; panel B, $P=0.0002$); HDL_2 versus HDL_3 for nLDL, $P=0.0002$.

fer rate, i.e., from LDL to HDL (Fig. 3, panels A and B), showed that, when compared to native LDL, CE from the oxidized LDL more effectively shifted to HDL_2 and to HDL_3, respectively, 2.18 vs. 0.46% ($P<0.0005$, panel A), and 2.52 vs. 1.75% ($P<0.035$, panel B). The net effect of the CETP-mediated CE transfer process involving HDL and LDL, shown in Table 1, is an oxLDL-CE content that is smaller than that of nLDL, which helps to explain the antiatherogenic effect of the HDL particles, as mediated by CETP action.

There was a 30% variation of the degree of oxidation of LDL produced in all preparations (22.9±7.7 nmol/l TBARS/mg protein). This could explain the dispersion of the data corresponding to CE transfer from oxidized LDL to HDL (Fig. 3). However, the possibility of a correlation between degree of oxidation and CE transfer was examined and no correlation was found in this range.

Comparing the CE acceptor capacity of the HDL subfractions, HDL_3 was a better CE acceptor than HDL_2 when native LDL was the donor particle (Fig. 3, panel A vs. B, $P=0.0002$). However, when the CE donor particle was the oxidized form of LDL, both HDL subfractions accepted CE equally well.

4. Discussion

In this study, we demonstrated that chemical modifications of LDL significantly alter the CETP-mediated redistribution of CE between HDL and LDL. The CETP-mediated HDL-CE transfer to acetylated LDL was decreased whereas to oxidized

Table 1
Bidirectional transfer of cholesteryl ester mass between HDL_2 or HDL_3 and native or oxidized LDL

	CE transfer			
	HDL_2		HDL_3	
	(CE) mass (μg)[a]		(CE) mass (μg)[a]	
	nLDL	oxLDL	nLDL	oxLDL
HDL→ LDL	5.3	4.6	7.8	6.9
LDL→ HDL	0.4	1.2	1.2	1.7
Net transfer (HDL→ LDL)	4.9	3.4	6.6	5.2

[a] Data calculated from medians of data in Figs. 2 and 3, and the CE masses of HDL and LDL used in the same experiments: $HDL_2=11.1$ μg, $HDL_3=28.2$ μg and LDL=67 μg.

LDL, the result was similar to that for intact LDL. On the other hand, the reverse flow of CE to HDL from oxidized LDL was about 1.4- to 3.6-fold greater than from native LDL. Christison et al. [26] also showed that CETP mediates the bidirectional transfer of oxidized lipids between LDL and HDL. A more effective removal of esterified cholesterol from the oxidized than from native LDL, verified in the present work, is in accordance with the finding that, in human plasma, HDL is the major carrier of lipid hydroperoxides [40]. The role of CETP in the removal of oxidized lipids from LDL and their subsequent transfer to HDL is further strengthened by the fact that CETP-deficient patients have significantly higher plasma levels of oxidized LDL [1,41].

In incubations with native LDL, as expected, HDL_2 was a better CE donor than HDL_3 (Fig. 2). Conversely, HDL_3 was a better CE acceptor than HDL_2 (Fig. 3). These findings suggest that the ability of HDL subfractions as CE donor or acceptor depends on the CE content of the HDL. Interestingly, when oxidized LDL was the donor particle, both HDL subfractions efficiently took up esterified cholesterol (Fig. 3).

Several studies have investigated the effect of LDL modification on the CETP reaction [26,29,42,43]. Harada et al. [42] showed that CETP increases the transfer of CE-HDL to the less negatively charged desialylated LDL. Passarelli et al. [43] showed that CETP-mediated CE transfer from control HDL to LDL from diabetic patients is increased. Nishida et al. [29] examined the effect of succinylation and acetylation of LDL on the CETP-mediated CE transfer. They showed that when a low degree of modification was produced, the modified LDL accepted CE more efficiently. Only when $>10\%$ of lysine residues of LDL were modified did LDL become a less efficient CE acceptor. Taking into account the final degree of oxidation and acetylation attained, we estimate that $>10\%$ of our LDL's amino groups must have been modified. However, neither Nishida et al. [29] nor we have analyzed a dose–response curve that would have been obtained with oxidized LDL. Besides the increase in the negative charge of LDL, the oxidation process elicited other chemical alterations. The degree of LDL oxidation in our study was about four- to tenfold higher than that observed in plasma or aortic lesions from humans and apoE knockout mice [17,44]. However, the values reported by Aviran et al. [17] and Shimano et al. [44] fell within the normal range of TBARS in LDL reported by Parthasarathy et al. [45] and Steinbrecher [46], i.e., 4 to 6 nmol/l TBARS/mg protein. Thus, the actual in vivo level of oxidized LDL has not yet been clearly defined.

The physicochemical mechanism whereby modified LDL interferes with CETP activity was not specifically addressed here. However, some possibilities can be excluded. Considering that the CE transfer rate to HDL from oxidized LDL was greater than from native LDL, it is possible to conclude that oxidation of LDL seemingly did not inhibit or degrade the CETP moiety, a fact that, nonetheless, had been reported when CETP was incubated with oxidized lipid emulsions [47]. Rather than modifying the CE transfer efficiency of the CETP moiety, LDL oxidation might have altered some properties of the LDL particle as CE donor since it was previously reported that the negative electrical charges elicited by these chemical modifications do increase the affinity of CETP for lipoproteins [27–29]. However, this is an unlikely explanation because desialylation of LDL, i.e., reduction of its negative charges actually increases the CETP activity [42]. It is unlikely that altered size of the modified LDL particles could play a role in the CE transfer rate because CE transfer was not significantly altered when oxidized LDL was used as acceptor but was increased when oxidized LDL was used as a donor.

Our in vitro results suggest that, in vivo, CETP displaces CE from the atherogenic oxidized LDL towards HDL more efficiently than from native LDL, thus raising the possibility that CETP may have an additional antiatherogenic effect in the microenvironment of the arterial intima.

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