CETP and lipid transfer inhibitor protein are uniquely affected by the negative charge density of the lipid and protein domains of LDL

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Abstract Lipoprotein surface charge influences cholesteryl ester transfer protein (CETP) activity and its association with lipoproteins; however, the relationship between these events is not clear. Additionally, although CETP and its regulator, lipid transfer inhibitor protein (LTIP), bind to lipoproteins, it is not known how the charge density of lipoprotein protein and lipid domains influences these factors. Here, the electronegativity of the protein (by acetylation) and surface lipid (oleate addition) domains of LDL were modified. LDL-only lipid transfer assays measured changes in CETP and LTIP activities. CETP activity was stimulated by <10 μM oleate but completely suppressed by >20 μM. The same electronegative potential induced by acetylation mildly stimulated CETP. Modification-induced enhanced binding of CETP did not correlate with CETP activity. LTIP activity was completely blocked by ~10 μM oleate but only mildly suppressed by acetylation. LTIP binding to LDL was not decreased by oleate. Thus, the negative charge of LDL surface lipids, but not protein, is an important regulator of CETP and LTIP activity. Altered binding could not explain changes in CETP activity, suggesting that the extent of CETP binding is not normally rate limiting to its activity. Physiologic and pathophysiologic conditions that modify the negative charge of lipoprotein surface lipids will suppress LTIP activity first, followed by CETP.—Morton, R. E., and D. J. Greene. **CETP and lipid transfer inhibitor protein are uniquely affected by the negative charge density of the lipid and protein domains of LDL. *J. Lipid Res.* 2003. 44: 2287–2296.**

Supplementary key words surface charge • binding kinetics • cholesteryl ester transfer protein

Cholesteryl ester transfer protein (CETP) mediates the exchange and net transfer of cholesteryl ester (CE) and triglyceride among lipoproteins (1, 2). In so doing, CETP dramatically influences the composition and catabolism of lipoproteins (3–6). Although this process is not fully understood, kinetic analyses strongly suggest that CETP mediates lipid transfer by a carrier mechanism (7–10). The initial obligatory step in lipid transfer is binding of CETP to the surface of a donor lipoprotein. Several studies suggest that this binding occurs with the lipoprotein's phospholipids (7, 11–13). Once bound, CETP interacts with CE and triglyceride dissolved in the phospholipid surface (14) through specific amino acid residues in the transfer protein's carboxy-terminal region (8). After CETP, with its lipid cargo, dissociates from the donor particle, it binds to an acceptor lipoprotein, where the complementary half of the reaction proceeds. Several lines of evidence suggest that CETP can dissociate from the lipoprotein surface without binding lipid and that the rate of lipid transfer is strongly influenced by the concentration of substrate lipids dissolved in the phospholipid surface (14, 15).

CETP activity is regulated by lipid transfer inhibitor protein (LTIP), which has been identified as apolipoprotein F (16). LTIP regulates CETP activity by disrupting the interaction of CETP with the lipoprotein surface (11). LTIP preferentially binds to LDL (16, 17), although it can also bind to HDL (11). As a result, LTIP, instead of lowering CETP activity in general, primarily inhibits lipid transfer events involving LDL (17) and thus alters the overall pattern of transfer reactions mediated by CETP between VLDL, LDL, and HDL, and actually stimulates lipid flux between VLDL and HDL (17, 18).

Given the profound influence of diet and metabolic processes on lipoprotein composition, there has been considerable interest in determining how changes in the composition of lipoproteins may influence their capacity to serve as substrates for CETP. Based on the original observation of Pattnaik and Zilversmit (12) that increased lipoprotein negative charge enhances the binding of CETP to all lipoproteins, and the observation that CETP binding

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Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; LTIP, lipid transfer inhibitor protein; REM, relative electrophoretic mobility.

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is essential for lipid transfer (8, 11–13, 19), many of these studies have focused on the influence of lipoprotein charge modification on CETP function (19–27). The bulk of these studies have concentrated on variations in free fatty acid levels, which occur under both physiologic and pathophysiologic conditions, and derivatization of apolipoproteins to mimic the modifications that attend oxidative events. A common observation is that increased negative charge density promotes the formation of isolatable CETP-lipoprotein complexes and CETP activity is altered to varying degrees depending on the nature of the modification.

It is often inferred that charge modification of the lipid or protein component of lipoproteins elicits similar changes in CETP binding and activity, although this has not been rigorously tested. This seems unlikely, however, because CETP is a lipid surface-active protein (7) and its activity does not require interaction with other apolipoproteins. Furthermore, LDL and HDL, which have large differences in negative surface charge density, owing to their different apolipoproteins (28), are equivalent CETP substrates when compared on an equal phospholipid basis (18). To clarify the importance of negative charge, we have compared the dose-dependent effects of lipid-phase versus protein-phase charge modification on the activity of CETP and its binding to modified lipoproteins. In addition, given the important role of LTIP in regulating CETP and its known interaction with lipoproteins (11, 16, 17), we have also investigated the influence of these charge modifications on its activity.

EXPERIMENTAL PROCEDURES

Preparation, modification, and radiolabeling of lipoproteins

Lipoproteins were isolated from fresh plasma by differential ultracentrifugation (29). LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml), isolated in NaBr solutions containing 0.02% EDTA, were extensively dialyzed against 0.9% NaCl, 0.02% NaN_3, 0.02% EDTA (pH 7.4) and stored in the dark at 4°C. The protein content of lipoproteins was determined using a modified Lowry protein assay (30). Triglyceride and total cholesterol content were assayed using enzymatic colorimetric kits (Sigma, St. Louis, MO). Phospholipid was quantitated by phosphorus analysis (31).

The electronegativity of LDL, or biotinylated LDL (prepared as described below), was increased by either enrichment with oleate or acetylation of lysyl groups. A stock solution (33.3 mM) and working dilutions of sodium oleate (Sigma) were prepared in deionized water and briefly heated to 60°C before use. Oleate was incorporated into LDL during a 2 h incubation at room temperature (32). The free fatty acid content of native and oleate-enriched LDL was determined by an enzymatic assay (Wako Diagnostics, Richmond, VA). Lysyl groups on LDL were derivatized by repetitive addition of acetic anhydride (33). To achieve variable acetylation, LDL was incubated with different concentrations of acetic anhydride, which were prepared by diluting the anhydride in saturated sodium acetate just prior to use. After derivatization, LDL was extensively dialyzed against 0.9% NaCl, 0.05% EDTA (pH 7.4) and filtered (0.45 μm). The extent of derivatization was determined by trinitrobenzene sulfonic acid reactivity using BSA as standard (58 mol lysine/mol) (34). The electrophoretic mobilities of oleate-enriched and acetylated LDL were determined by agarose gel electrophoresis (35). Lipoproteins were visualized by lipid (Fat Red B) and protein (Coomassie) staining (35). Relative electrophoretic mobilities (REMs), with native LDL assigned a value of 1, are reported. To compare mobilities between LDL preparations and electrophoretic runs, transferrin was used as a standard. The average mobility of native LDL preparations was 0.83 of that for transferrin.

For lipid transfer assays and select binding studies, LDL, and in some instances acetyl LDL, was biotinylated by reaction with *N*-hydroxysuccinimidobiotin (NHS-biotin) (Pierce, Rockford, IL) as previously described (36). Following extensive dialysis against 0.9% NaCl, 0.02% EDTA, 0.02% NaN_3 (pH 7.4), biotinylated LDL was filtered (0.45 μm) and quantitated by cholesterol assay. On average, biotinylation reduced the lysine content of LDL by 14% and increased its relative REM to 1.20 ± 0.07 ($n = 8$). Native LDL and HDL were labeled with ^{3}H-CE by incubation of plasma with [^{3}H-CE]phosphatidylcholine dispersions as previously detailed (37).

CETP and LTIP preparation

Partially purified CETP and LTIP were isolated from lipoprotein-deficient plasma (38) by hydrophobic and ion exchange chromatography as previously described (17, 37, 39). CETP preparations, which are free of phospholipid transfer protein and lecithin cholesterol acyltransferase activities, are functionally identical to homogenous CETP (18, 40). CETP was stored in 0.27 mM EDTA (pH 7.4) to stabilize its activity (41). LTIP preparations, stored in 150 mM NaCl, 0.01% EDTA, 0.02% NaN_3 (pH 7.4), were devoid of CETP activity and were very similar to highly purified LTIP and recombinant LTIP in their capacity to selectively suppress CETP activity involving LDL (16, 17).

CETP and LTIP activity assays

During the preparation of CETP and LTIP, lipid transfer activities were routinely measured in assays containing [^{3}H-CE]LDL and HDL as donor (42, 43). This assay was also used to quantify the CETP content of fractions derived from CETP-LDL binding experiments. In experiments that evaluated the influence of oleate enrichment or acetylation on CETP and LTIP activities, lipid transfer assays contained only LDL (36). For these assays, donor [^{3}H-CE]LDL was incubated with biotinylated LDL as acceptor, a source of CETP and LTIP, and assay buffer without the 1% BSA normally present in these assays. As indicated in the figure legends, for some studies, the donor and/or acceptor LDL was also acetylated. After incubation, 1% BSA was added, and the donor and acceptor were separated by incubation of the assay mixture at room temperature with avidin-Sepharose as previously described (36). CETP activities, calculated as previously described (42), are reported as percent transfer. LTIP activities, determine by the capacity to suppress a standard amount of CETP, are reported as percent inhibition. Blank values (absence of CETP or LTIP) were determined for each assay condition to correct for minor changes in donor/acceptor separation caused by oleate or acetylation.

Binding studies

LDL, acetylated LDL, and HDL were coupled to CNBr-Sepharose as previously detailed (11). This method was also used to generate solid-phase oleate-enriched LDL. However, in this instance, to decrease the loss of oleate, the normal acid/base washing steps used to remove noncovalently bound lipoprotein were modified to use only the acid step. Recovery of oleate was >94%. More commonly, to prepare oleate-LDL linked to a solid phase,

biotinylated LDL was enriched with oleate as described above, and then this modified lipoprotein was incubated with streptavidin-Sepharose (Sigma) for 2 h at room temperature. With either the CNBr-Sepharose or the streptavidin-Sepharose approach, >96% of the lipoprotein was bound to the resin and final preparations contained ~2 mg lipoprotein protein/ml gel.

Non-steady-state binding. To measure the binding of CETP to native and modified lipoproteins, ~1 ml columns containing 2 mg lipoprotein (protein) were prepared at room temperature and equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 0.02% EDTA, and 0.02% NaN_3 (pH 7.4) at a flow rate of 8 ml/h. CETP, in the same buffer, was applied, and the column was washed with 1 ml of the same buffer. The amount of CETP applied (450–900 μg protein) varied between CETP preparations and was chosen to result in a CETP-to-solid-phase lipoprotein ratio that was similar to that used in lipid transfer assays. Subsequently, columns were eluted with 0.25% EDTA and 10 mM NaCl (pH 7.4), and 1 ml fractions were collected into tubes containing 50 μg HDL cholesterol. CETP in each fraction was quantitated by a standardized [^{3}H-CE]LDL-to-HDL transfer assay (42, 43). Although the NaCl concentration in these transfer assays is higher than that used in the binding protocol, CETP activity differs by <25% under these two conditions (22, 44). The binding of CETP to Sepharose or streptavidin-Sepharose lacking lipoprotein was very low. CETP binding to LDL-Sepharose and to biotinylated LDL coupled to streptavidin-Sepharose was very similar, indicating that the derivatization of LDL by biotin did not affect CETP binding. Non-steady-state binding of LTIP to native and modified LDL followed essentially the same protocol as for CETP, except as noted in the figure legends.

Steady-state binding. To measure the equilibrium binding of CETP to lipoproteins, Sepharose-bound LDLs (150 μg lipoprotein cholesterol) were incubated with varying amounts of CETP in 50 mM Tris-HCl, 150 mM NaCl, 0.02% EDTA, and 0.02% NaN_3 (pH 7.4) containing 0.025% BSA. Samples were mixed at room temperature for 3 h, which exceeds the time required for equilibrium to be established (11). Subsequently, samples were centrifuged briefly and the supernatant removed for determination of its CETP content by activity assay. Bound CETP was determined from the difference between the amount of CETP applied and that recovered in the supernatant after binding with the solid-phase LDL. CETP binding to Sepharose alone was low and constant over the CETP dose range used.

RESULTS

Previous studies investigating the influence of charge modification on CETP have used heterogeneous assay systems, i.e., donor and acceptor lipoproteins of different classes. However, the influence of charge modification on CETP activity is dependent on the two lipoproteins used in these assays and on which particle serves as the donor (27, 32). This likely occurs because CETP not only mediates the transfer event that will be measured (donor-to-acceptor lipid flux), but also facilitates competing reactions (donor-to-donor and acceptor-to-acceptor transfers). To avoid complications in data interpretation that may arise when charge modifications alter the relative rates of these competing reactions, we have measured CETP and LTIP activities in assays containing only LDL (36). Thus, in most instances, donor and acceptor particles differ only in that the acceptor has been biotinylated to facilitate its separation at the end of transfer reactions. Several lines of evidence show that biotinylation itself does not alter the transfer properties of LDL and that the LDL-only transfer assay provides a physiologically relevant readout. For example, we have shown that CETP activity in the LDL-only assay is the same as that in LDL-to-HDL transfer assays (36) and that the substrate properties of LDL and biotinylated-LDL are indistinguishable in CETP mass transfer assays (45).

Effect of charge modification on CETP binding and activity

The electronegative potential of LDL was altered by acetylation of its apolipoproteins or the addition of sodium oleate to modify the negative charge of its surface lipids. Following variable acetylation or addition of oleate, lipoproteins were chemically characterized and examined by agarose electrophoresis to determine changes in their electronegative surface potential. For either modification, electrophoretic mobility correlated directly with the amount of oleate incorporated or the loss of amino groups as determined by trinitrobenzene sulfonic acid reactivity.

To assess the effect of charge modification on CETP activity, acetylated [^{3}H-CE]LDL and biotinylated LDL or native [^{3}H-CE]LDL and biotinylated LDL plus oleate were incubated with CETP and the extent of CE transfer determined. As previously reported by us and others (23, 26, 27, 46–48), the addition of low levels of oleate stimulated CETP activity up to 60% (**Fig. 1A**). However, higher levels of fatty acid inhibited CETP activity almost completely. A similar inhibitory effect has been observed in assays containing different classes of lipoproteins as donor and acceptor (27). In contrast, progressive acetylation, which altered LDL electronegativity to the same extent as oleate, mildly stimulated CETP transfer activity. At an REM of 1.7, which gave maximal stimulation of CETP by oleate, acetylation elicited only a 10% increase in CETP activity. Similar results were obtained when both the donor and acceptor LDL particles were acetylated (data not shown). This differs from that seen in HDL-to-LDL assays, in which acetylation of 40% of LDL lysines reduced CETP activity by 65% (Fig. 1A, inset). Consistent with the mild stimulation of CETP activity in LDL-to-LDL assays by increased protein negative charge, when LDL was made significantly less electronegative (REM decreased from 1.0 to 0.4) by removing sialic acid residues with Sepharose-bound *N*-acetyl neuraminidase, CETP activity was only slightly lower, compared with native LDL (11.4 ± 1.3 vs. 9.5 ± 0.7% transfer).

The above results show that CETP activity is highly sensitive to changes in the negative charge of the lipid phase but relatively unaffected by similar elevations in apolipoprotein charge. To investigate whether charge modification of these two environments is additive, we compared the influence of oleate addition to a lipid transfer assay containing native LDL as both donor and acceptor to one containing acetylated LDL (REM = 2.1) as both donor and acceptor. As seen in Fig. 1B, although initially more electronegative, acetylation of donor and acceptor particles did not alter the dose response of CETP to oleate.

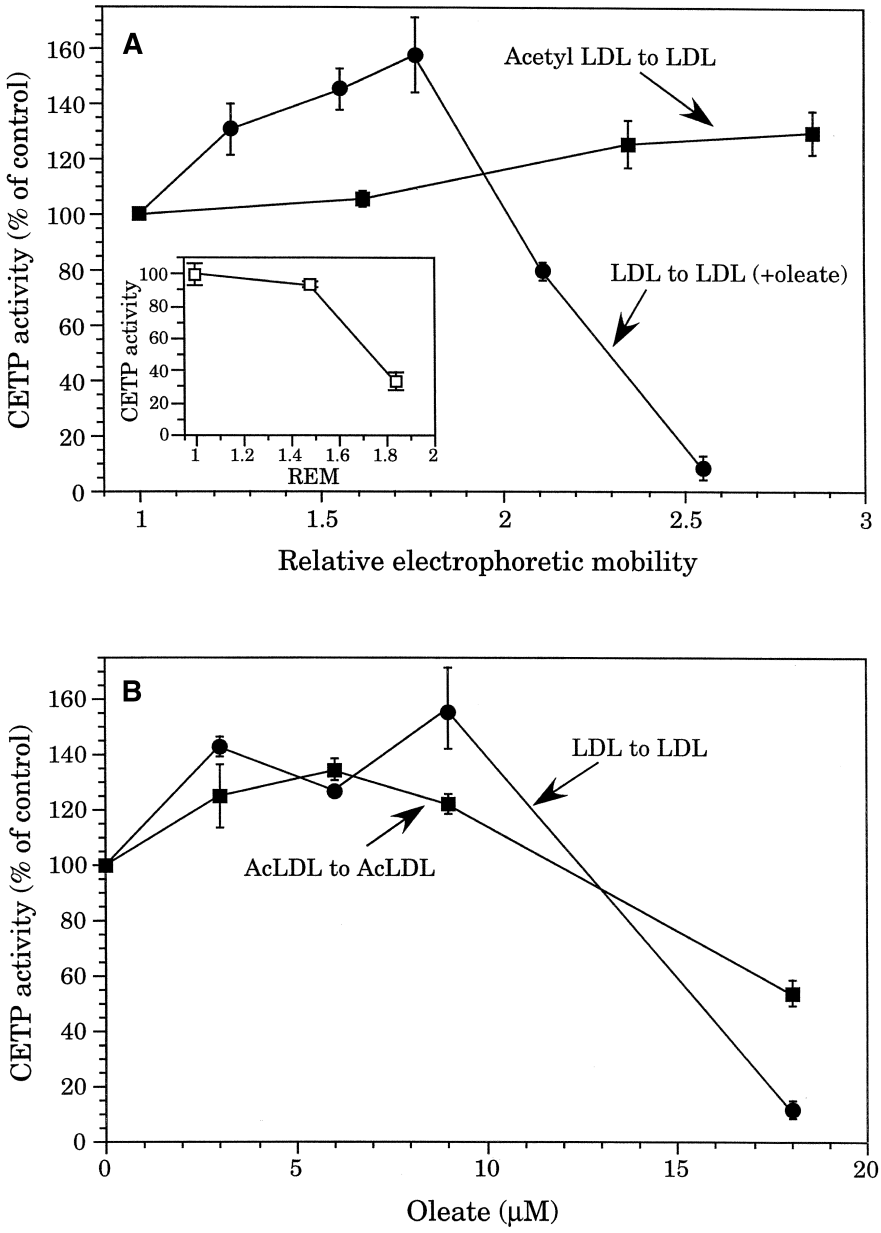

Fig. 1. Effect of protein and lipid charge modification on cholesteryl ester transfer protein (CETP) activity. The influence of oleate enrichment or acetylation on CETP activity was measured under standardized assay conditions, as described in Experimental Procedures. In general, assays contained 10 μg cholesterol each of the indicated donor and acceptor, and 10.6 μg CETP in a total volume of 0.5 ml. A: Effect of oleate and acetylation on CETP activity. CETP activity between native labeled LDL and biotinylated LDL ± oleate, or influence of acetylation on transfer activity between radiolabeled, acetylated LDL and biotinylated LDL is shown relative to the electrophoretic mobility change induced by these modifications. Inset: Effect of LDL acetylation on cholesteryl ester (CE) transfer from HDL to LDL. CETP activity was 19.6% with native LDL as acceptor. B: Nonadditivity of oleate and acetylation charge modifications. Oleate was added at the indicated final concentration to lipid transfer assays containing either radiolabeled, native LDL and biotinylated LDL acceptor, or radiolabeled, acetylated LDL and biotinylated, acetylated LDL acceptor. The acetylated donor and acceptor LDL used in this study had 40% of its lysyl groups acetylated and a relative electrophoretic mobility (REM) of 2.15. CETP activity in the absence of oleate was 9.8% and 12.5% for transfer reactions between LDL and biotinylated LDL versus acetyl LDL and biotinylated acetyl LDL, respectively. All data are the mean ± SD of duplicate determinations. These results are typical of three or four similar experiments.

Thus, the negative charges of the lipid and protein domains of LDL influence CETP activity independently.

Because lipid transfer activity requires the binding of CETP to the lipoprotein surface (8, 11, 13, 19), and because it is well recognized that CETP binding is enhanced by elevated negative charge of lipoproteins (12, 19, 22), we measured the binding of CETP to charge-modified LDL to determine if changes in CETP activity correlate

with CETP binding. Native and modified LDLs were immobilized on Sepharose and packed into columns. CETP was applied, and the columns were continuously eluted. CETP levels were chosen to provide a CETP/lipoprotein ratio near that present in lipid transfer assays. Initially CETP quantitatively bound to all solid-phase lipoproteins. Under these non-steady-state conditions, CETP binding affinity was estimated from the time required for half ($t_{1/2}$) of the applied CETP to dissociate. Acetylation of LDL increased the non-steady-state binding affinity of CETP (**Fig. 2A**). Lysyl modifications of 26%, 37%, and 75% progressively increased the $t_{1/2}$ for CETP elution from 15 min (native LDL) to 118 min (**Table 1**). However, LDL with 26% lysine modification consistently bound CETP slightly better than LDL with 37% lysine modification (Fig. 2A and Table 1). Similar to LDL acetylation, fatty acid modification of LDL increased the association of CETP with LDL in a dose-dependent fashion (Fig. 2B), resulting in a >20-fold increase in the $t_{1/2}$ of CETP elution (Table 1). At 10% oleate, CETP binding to modified LDL was similar to that for native HDL (Fig. 2B). Overall, regardless of the mechanism of negative charge enrichment, the apparent CETP binding affinity increased with electronegativity. However, there was no correlation between increased association of CETP with an LDL and the CETP activity it supported (Table 1).

Non-steady-state binding properties, although the most common measure of CETP-lipoprotein interaction reported in the literature, may not be the most relevant to CETP activity, because transfer events are measured under steady-state conditions. Steady-state CETP binding was measured by incubating solid-phase LDL with nonsaturating levels of CETP at CETP/lipoprotein ratios that encompassed the ratio used in lipid transfer assays. After 3 h incubation, which exceeds the time required to reach equilibrium (11), the solid-phase LDL was rapidly pelleted by brief centrifugation and the supernatant removed and assayed for CETP content. Although LDLs containing 10 wt% oleate (REM = 2.8) completely inhibited CETP activity, and acetylated LDLs (REM = 3.2) stimulated CETP activity by 30%, CETP binding was very similar between native, oleate-enriched, and acetylated LDL over the CETP dose range studied (**Fig. 3**). Together, these binding studies indicate that changes in CETP activity induced by a modified negative charge of the lipid and protein domains of lipoproteins cannot be explained by alteration in the binding of CETP to its target lipoprotein.

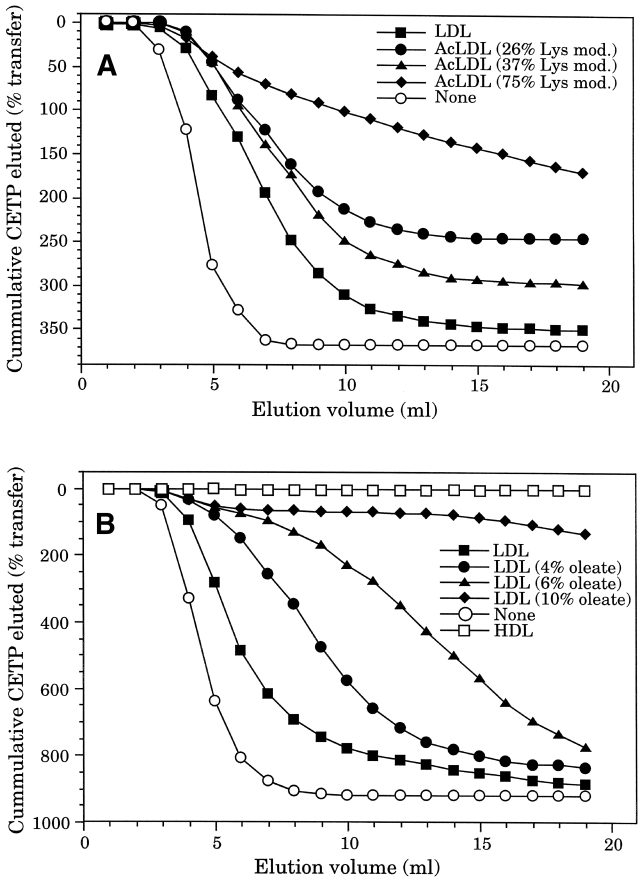

Fig. 2. Changes in CETP non-steady-state binding caused by acetylation or oleate enrichment. Sepharose-bound LDL, acetylated LDL, oleate-enriched biotinylated LDL, HDL, or resin alone were prepared as described in Experimental Procedures. One milliliter columns containing 2 mg LDL protein were prepared and partially purified CETP (685–900 μg protein) was applied, and columns were eluted at 8 ml/hr. Columns were eluted with 0.25% EDTA and 10 mM NaCl (pH 7.4), 1 ml fractions were collected, and their CETP content was determined by activity assay. A: CETP binding to native LDL and LDL acetylated to varying extents. For the acetylated LDL shown, 26%, 37%, and 75% of LDL lysyl groups were derivatized, resulting in electrophoretic mobilities of 1.5, 1.8, and 3.2 relative to native LDL, respectively. Lys mod., lysine modification. B: CETP binding to native HDL bound to CNBr-Sepharose, or native and oleate-enriched, biotinylated LDL bound to streptavidin-Sepharose. Oleate-enriched LDL contained 4%, 6%, or 10% oleate (oleate-phospholipid, wt/wt), resulting in electrophoretic mobility of 1.8, 2.4, and 2.8 relative to native LDL, respectively. For both panels, CETP activity recovery averaged 70%, reflecting mainly a partial inactivation of CETP due to the high degree of dilution involved in the experiment. Adding HDL (50 μg) to the fractions as they were collected helped minimize this denaturation. Data points are the average of duplicates. The results are typical of those obtained in three similar experiments.

Influence of lipid and protein charge modification on LTIP activity

The response of LTIP to increased electronegativity was markedly different from that seen for CETP. When LDL was acetylated, the increased negative charge of the protein domain of LDL caused a small, modification-dependent decrease in LTIP activity (**Fig. 4A**). However, when corrected for the mild stimulation of CETP activity induced by acetylation (Fig. 1A), absolute LTIP activity declined only 10%. Conversely, decreasing the negative charge of LDL protein (REM decreased from 1.0 to 0.4) by neuraminidase treatment modestly increased LTIP activity compared with control (48.9 ± 2.3 vs. 57.5 ± 2.8% inhibition). Again, after correcting for the increased CETP activity caused by this treatment (see above), the effect of decreased LDL protein charge on absolute LTIP activity was minimal. The relative insensitivity of LTIP activity to acetylation of LDL protein was also seen in HDL-

TABLE 1. Relationship between stable CETP-LDL complex formation and CETP activity

Modification	REM	Affinity ($t_{1/2}$ Dissociation)	CETP Activity (% of Control)
		min	
None	1.0	15	100
Acetylation	1.5	31.8	105
Acetylation	1.8	27.8	110
Acetylation	3.2	117.8	133
Oleate	1.8	33.8	150
Oleate	2.4	67.5	33
Oleate	2.8	>300	0

CETP, cholesteryl ester transfer protein; REM, relative electrophoretic mobility. The electronegativity of LDL was increased by variable acetylation or oleate addition to generate lipoproteins with the indicated relative electrophoretic mobilities. CETP binding affinity to native or modified LDL bound to Sepharose was estimated from the time required for half of the bound CETP to dissociate ($t_{1/2}$) after subtracting the time required for CETP to elute from Sepharose alone (see Fig. 2). CETP activities were determined in LDL-only transfer assays as shown in Fig. 1A.

to-LDL transfer assays (Fig. 4A, inset). On the other hand, LTIP activity was inhibited by oleate in a dose-dependent fashion, resulting in complete suppression of LTIP at oleate levels that had minimal inhibitory effect on CETP activity (compare Figs. 1A, 4A). A 2-fold increase in LDL electrophoretic mobility by oleate completely blocked LTIP activity, whereas a similar mobility shift caused by acetylation decreased LTIP activity by ~15%. These results mirror those obtained in transfer assays involving different lipoprotein classes as donor and acceptor (27). Like that seen with CETP, an increase in the negative charge of LDL protein did not alter the effects of oleate on LTIP activity. Whether added to assays containing unmodified donor and acceptor LDL or to those containing acetylated donor and acceptor LDL (REM = 2.1), oleate caused nearly identical suppression of LTIP activity (Fig. 4B).

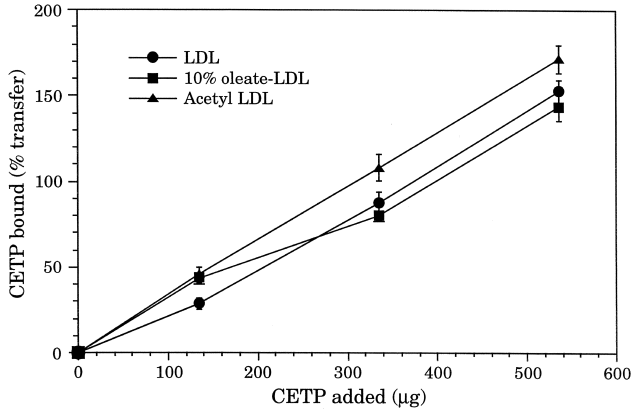

Fig. 3. Steady-state binding of CETP to native and modified LDL. Partially purified CETP at the indicated dose was mixed continuously with Sepharose-bound LDL, 10% oleate LDL, or acetyl LDL (relative electrophoretic mobilities, 1, 2.4, and 3.5, respectively) for 3 h. After the solid phase was rapidly pelleted, unbound CETP was measured by CETP activity assay. Bound CETP was calculated as described in Experimental Procedures. The specific activity of CETP averaged 1.2% CE transfer/μg protein. Results are the mean ± SD (n = 3). These results are representative of two experiments.

Our previous studies strongly suggest that LTIP functions by binding to lipoproteins and displacing CETP (11). Consequently, we measured the stable binding of LTIP to native and oleate-modified LDL to determine whether the suppression of LTIP activity by high-level oleate results from disruption of LTIP binding to the lipoprotein surface. Native and oleate-enriched LDLs (10 wt%) were bound to Sepharose, and 1 ml columns were prepared. LTIP was applied at the same LTIP/lipoprotein ratio used in transfer assays, and fractions were collected and examined for LTIP content. Following elution of LTIP, the column matrix was assayed for lipoprotein cholesterol and fatty acid content. Although the fatty acid content of the LDL matrix was reduced by the extensive elution protocol, the oleate content of LDL after the experiment was still sufficient to suppress LTIP activity by >80%. LTIP that initially bound to the LDL matrix remained tightly associated and did not dissociate even after being washed with 10 column volumes of buffer containing 150 mM NaCl (**Fig. 5A**). Eluant buffer containing 10 mM NaCl, which rapidly dissociates CETP from LDL (Fig. 2), was equally ineffective in dissociating LTIP (data not shown). Further, the presence of elevated oleate in LDL had no measurable affect on LTIP binding, despite greatly suppressing its activity (Fig. 5B).

DISCUSSION

In this study, we report the influence of LDL negative charge on CETP and LTIP activities. We have focused on LDL charge properties because this lipoprotein is affected most by increased free fatty acid levels (27), is a major target for oxidative modification events that derivatize apolipoproteins and increase surface charge (49), and is the primary substrate for LTIP (17). In distinction to previous studies, here we have compared the effects of protein- and lipid-phase modifications on these activities using a lipid transfer assay that employs only one lipoprotein class as donor and acceptor. This approach eliminated concerns that charge modifications might alter the rate of competing reactions, such as those between two donor particles, and thus influence the measured transfer rate between the donor and acceptor. We have observed that both CETP and LTIP are acutely sensitive to the negative charge density of the lipid phase, whereas equivalent charge modification of the protein phase induces only modest effects. Further, as shown by concomitant modification by acetylation and oleate addition, there is no cross-talk between the charge modifications of lipid and protein domains with respect to their effects on LTIP and CETP activity. The failure of LDL acetylation to inhibit CETP activity in LDL-only transfer assays contrasts with the inhibition observed here and reported by others (22) in HDL-to-LDL transfer assays. This inhibition has been attributed to excessive interaction of CETP with acetylated LDL (22). However, our data clearly show that acetylation of

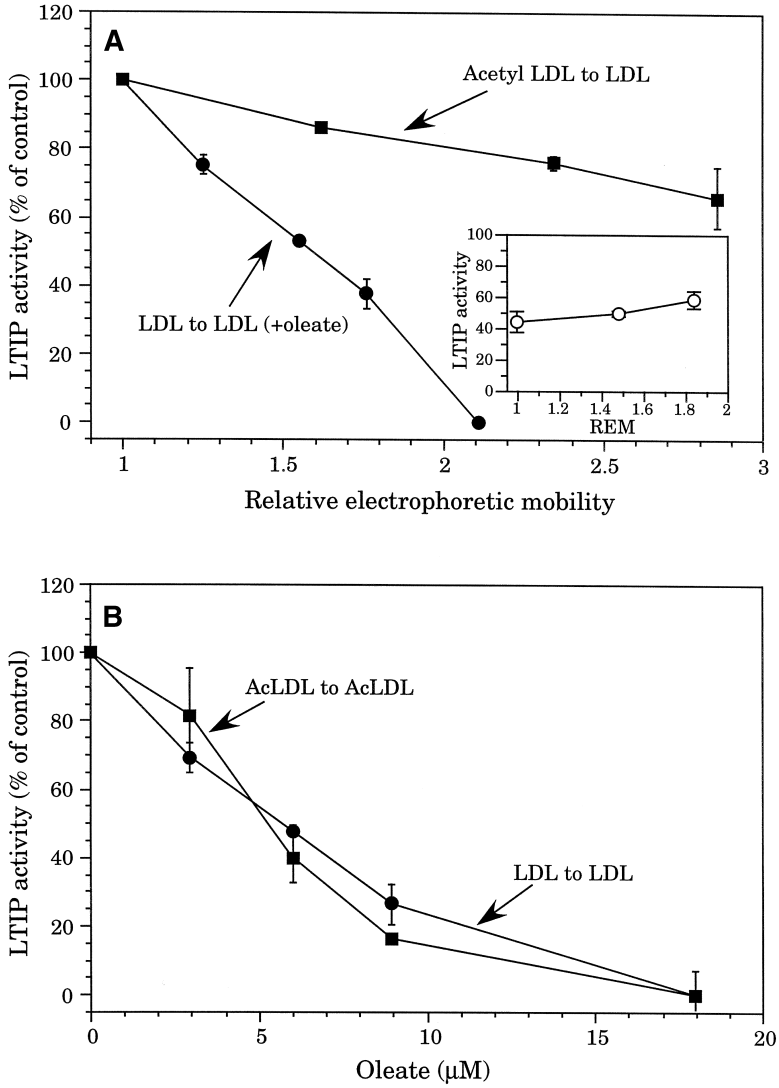

Fig. 4. Effect of lipid- and protein-phase charge modification on lipid transfer inhibitor protein (LTIP) activity. The influence of oleate enrichment or acetylation on LTIP activity was measured as described in Experimental Procedures. Assays contained 10 μg cholesterol each of the indicated donor and acceptor, 10.6 μg CETP, ± 3.1 μg LTIP in a total volume of 0.5 ml. A: Influence of charge modification on LTIP activity. The effect of oleate on LTIP activity between native labeled LDL and biotinylated LDL, or the influence of acetylation on LTIP activity between radiolabeled, acetylated LDL and biotinylated LDL is shown relative to the electrophoretic mobility change induced by these modifications. Inset: Effect of LDL acetylation on LTIP activity in HDL-to-LDL transfer assays. B: Lack of additivity of acetylation and oleate on modulating LTIP activity. Oleate was added at the indicated final concentration to lipid transfer assays containing either radiolabeled, native LDL and biotinylated LDL acceptor, or radiolabeled, acetylated LDL and biotinylated, acetylated LDL acceptor. The acetylated donor and acceptor LDL used in this study had 40% of its lysyl groups acetylated, and an REM of 2.15. LTIP activity in the absence of oleate was 48.9% and 31.4% for lipid transfer reactions between LDL and biotinylated LDL versus between acetyl LDL and biotinylated acetyl LDL, respectively. All data are the mean ± SD (n = 2). The results are typical of three similar experiments.

the donor LDL, or of both donor and acceptor LDL, has little affect on CETP activity, indicating that this inhibition must be due to other mechanisms, such as alterations in the rate of competing transfer reactions or an influence of acetylated LDL on the CETP substrate properties of HDL.

CETP binding to lipoproteins is most commonly measured by non-steady-state methods, such as gel filtration (12, 19, 22) or column chromatography with immobilized lipoproteins (11), which measure the formation of isolatable CETP-lipoprotein complexes. Multiple studies have reported the increased formation of CETP-lipoprotein complexes when lipoprotein electronegativity is increased. It is generally assumed that this increased association leads to increased CETP activity (19, 20); however, excessive binding of CETP may lead to decreased activity (22). Fully understanding this relationship has been complicated by the use of multiple approaches to altering lipoprotein charge and limited assessment of the correlation between charge modification, CETP binding, and CETP activity.

In this study, we demonstrate that although increased electronegativity does markedly stabilize CETP-LDL complex formation in the presence of a low-ionic-strength buffer, there is little correlation between binding and CETP activity. For example, with acetylated LDL, a 2-fold apparent increase in affinity resulted in only a 10% increase in CETP activity, and an 8-fold increased affinity yielded a 30% stimulation of transfer activity. Similarly, a 2-fold increase in binding induced by oleate stimulated CETP activity by 50%, but a 4-fold increase in binding resulted in marked inhibition of CETP. It has been suggested that CETP activity depends on the presence of an optimum surface charge (22, 24). However, the data presented here clearly show that modified lipoproteins of similar surface charge, as measured by electrophoretic mobility, have different capacities to bind CETP and support CETP activity. This finding is consistent with the observation that CETP ineffectively forms isolatable complexes with native LDL but forms very stable associations with HDL (12, 22, 50), yet both LDL and HDL, when added on an equal lipid surface (phospholipid) basis, support comparable lipid transfer activities (18). Furthermore, while establishing conditions for this binding protocol, we observed that CETP binding to native LDL and

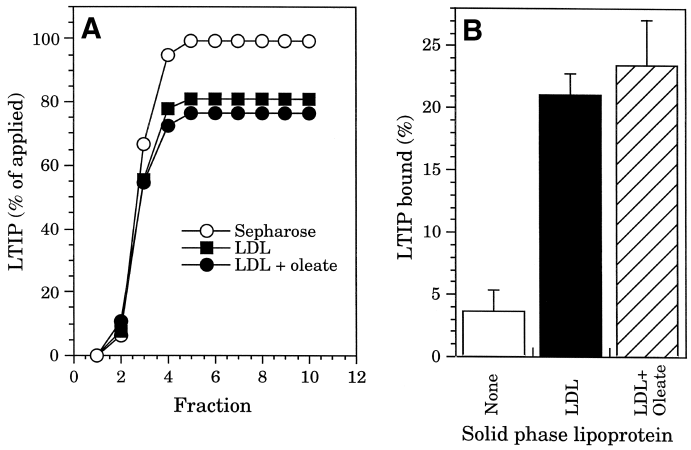

Fig. 5. Stable binding of LTIP to native and oleate-modified LDL. LDL or LDL containing 10% oleate (oleate-phospholipid, wt/wt) was bound to CNBr-Sepharose as described in Experimental Procedures. One milliliter columns containing 1 mg LDL cholesterol were prepared and LTIP (1 mg protein) applied at a flow rate of 8 ml/hr. Columns were subsequently eluted with 0.25% EDTA and 10 mM NaCl (pH 7.4). Fractions were then assayed for LTIP content. A: Elution profile of LTIP applied to columns containing Sepharose alone, LDL-Sepharose, or oleate-enriched LDL-Sepharose. Data points are the average of duplicate determinations. B: Binding of LTIP to native and oleate-modified LDL. Results are the mean ± SD of calculated binding values determined in multiple experiments similar to that shown in A (Sepharose alone, n = 2; LDL-Sepharose, n = 5; oleate/LDL-Sepharose, n = 3).

HDL appears to be driven primarily by hydrophobic interactions, because binding to these lipoproteins was high in the presence of 150 mM NaCl and decreased in a salt-dependent fashion to nearly zero in the absence of NaCl. Similar findings have been reported by Nishida, Arai, and Nishida (22). This contrasts with the increased binding of CETP to modified LDLs as a function of their electronegativity, showing that this interaction is primarily ionic in nature and distinct from the interaction that occurs with native lipoproteins. Overall, these findings indicate that the capacity of a native or modified lipoprotein to form a stable complex with CETP provides little information on how that lipoprotein will function in the lipid transfer process. Consistent with this conclusion, despite the marked difference in CETP binding as a function of NaCl concentration (noted above), CETP activity varied only 25% when assayed in buffers containing 0 or 150 NaCl (22, 44).

In marked contrast to non-steady-state binding properties, under steady-state conditions, the capacity of LDL to bind CETP remained rather constant over a narrow CETP dose range, even though lipid transfer activities were either stimulated or inhibited by charge modification. These results indicate that the balance of association and dissociation steps, as occur in the steady-state environment of a lipid transfer reaction, are relatively unaffected by changes in the electronegative potential of LDL. Studies with mutant recombinant CETP have demonstrated that CETP binding to the lipoprotein surface is essential for the lipid transfer process (51). Similar studies have shown that recombinant CETP mutated in the carboxy-terminal region of the protein binds lipoproteins normally but does not facilitate lipid transfer (8). These findings are consistent with our previous studies showing that varying the CE content of the phospholipid surface of donor particles significantly alters CE transfer rates without influencing CETP binding to the donor particle (14, 15). Thus, binding of CETP to the lipoprotein surface is necessary but not sufficient for transfer. These observations may explain the lack of correlation between CETP steady-state binding and CETP activity, suggesting that charge modification of LDL influences CETP activity by mechanisms independent of its binding. These mechanisms may include perturbation of CETP's structure once bound to the charge-modified lipoprotein surface, which prevents it from attaining an optimum conformation, or modification of the lipoprotein structure, which alters the concentration of CE available to the active site of CETP.

Unlike that observed for CETP, LTIP activity was not stimulated by increased negative charge of the lipid phase but, rather, was strongly suppressed. As previously reported, LTIP activity is completely blocked by nonesterifed fatty acid concentrations that either stimulate or only modestly suppress CETP activity (27). We have previously reported that steady-state binding of LTIP to lipoproteins is not altered by levels of oleate that suppress its activity (27). Here we further demonstrate that the stable binding of LTIP to LDL, which may be more relevant, given the proposed mechanism of inhibition by LTIP (7–10), is also unaltered by oleate concentrations sufficient to completely block its activity. Consistent with our earlier findings (32), this indicates that the suppression of LTIP activity by oleate is best explained by its ability to perturb the lipoprotein surface and not by the disruption of LTIP binding. Similar to CETP, LTIP was relatively unaffected by modification of LDL protein; however, the influence of charge modification of this domain was mildly inhibitory to LTIP activity, whereas it stimulated CETP activity. The minor inhibition of LTIP activity by protein acetylation may reflect enhanced ionic interaction of LTIP with apolipoproteins, thus reducing the concentration of LTIP available to bind to the lipid surface, where it is active.

In summary, we report that CETP and LTIP activities are strongly influenced by the charge density of LDL. However, modification of the negative charge of protein and lipid domains elicited distinct effects, with increase in the electronegativity of the lipid phase a far more potent modulator of both activities. At lower levels, oleate stimulated CETP and inhibited LTIP activity. However, because isolated LDL contains residual LTIP (16), we suggest that part or all of the apparent stimulation of CETP observed here and previously (22, 27, 48) actually arises from the suppression of endogenous LTIP by low-level oleate. This is consistent with fluorescence studies in which an increase in the negative charge of synthetic substrates (i.e.,

LTIP-free) by oleate or phosphatidylserine did not stimulate CETP (25). If this suggestion is correct, then the overall effect of oleate on LTIP and CETP activities may be the same (i.e., inhibitory), differing only in the sensitivity of these two proteins to the degree of charge modification of the lipid domain. The formation of CETP-lipoprotein complexes is strongly influenced by the negative charge of LDL, but this association, which appears primarily ionic in nature, does not correlate with CETP activity. Under steady-state conditions, CETP binding is not affected by enhanced surface charge density of either the protein or the lipid domain. This indicates that whereas CETP binding is essential for lipid transfer, it is not rate limiting to the transfer process under these assay conditions. Likewise, LTIP activity was strongly suppressed by oleate under conditions in which the interaction of LTIP with LDL was not altered. This supports our previous hypothesis that oleate modulates LTIP by perturbing the structure of the surface lipids (32). Overall, the data strongly show that the influence of these charge modifications on CETP and LTIP binding properties is a poor predictor of their functional consequences.

These studies illustrate that CETP and LTIP activities are uniquely sensitive to changes in lipoprotein surface lipid charge. Elevated free fatty acid levels occur under numerous conditions, such as acutely during postprandial lipemia (19, 20) and following aerobic exercise (52), or chronically in diabetes, obesity, and nephrotic syndrome (26, 53), and in coronary heart disease and normal ageing (54). Given the differential sensitivity of these factors to free fatty acid levels, the impact on lipoprotein metabolism will be complex. At moderately elevated fatty acid levels, the selective suppression of LTIP may lead to the formation of small, dense LDL (27) and impede LCAT activity (18, 55), whereas high levels may cause functional CETP deficiency. LDL oxidation plays an important role in atherogenesis (56). Our data suggest that oxidative derivatization of LDL protein lysyl groups is unlikely to significantly impact CETP or LTIP activities; however, it is possible that lipid oxidation products, such as lysophospholipids, aldehydes, oxysterols, etc., may be potent modulators of these surface-active transfer factors.

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