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Inactivation of Cholesteryl Ester Transfer Protein by Cysteine Modification

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The present studies examine the effects of various cysteine-modifying reagents on human recombinant cholesteryl ester transfer protein (CETP) activity. Dithiothreitol or other reducing agents had no effect on CETP transfer activity. Alkylating agents, including iodoacetamide and N-ethyl maleimide, also did not affect transfer activity. However, incubation of CETP with hydrophobic thiol-modifying reagents such as p-chloromercuriphenylsulfonic acid (IC_{50} = 0.02 μM), 4,4′-dithiodipyridine (IC_{50} = 0.5 μM), or 4,4′-dithiobis (phenyl azide) (IC_{50} = 0.5 μM) resulted in complete, time-dependent inactivation of both the cholesteryl ester and triglyceride transfer activities. Inactivation could be prevented by including dithiothreitol in the incubation. Long chain fatty acyl coenzyme A compounds were also found to be effective CETP inhibitors. The extent of inhibition was time-dependent, and proportional to the chain length of the fatty acyl portion of the molecule. These results suggest that CETP contains an essential free cysteine that resides in a hydrophobic environment within the protein.

Cholesteryl ester transfer protein (CETP)[2] is a plasma protein that mediates the movement of neutral lipids and phospholipids between lipoprotein particles (for reviews, see refs. 1–3). CETP-mediated exchange of cholesteryl ester (CE) and triglyceride (TG) is thought to be important in determining the plasma levels and compositions of LDL, VLDL and HDL. Since CETP activity has been associated with increased risk of atherosclerosis in human populations (4,5), an understanding of the molecular process whereby CETP moves lipids between lipoproteins could provide fundamental insights into the regulation of normal lipid metabolism and the development of atherosclerosis.

CETP contains a total of 476 amino acids, seven of which are cysteines (6). Since extensive physical evidence suggests that CETP exists as a monomer (7–9), CETP must contain at least one unpaired cysteine. Several reports have shown that CETP can be inhibited by treatment with certain cysteine-modifying reagents, particularly mercurial-type reagents such as p-chloromercuriphenyl sulfonate (10,11). Cholesteryl chlorobromide (12) and U-617, an organomercurial cholesterol derivative (13), have also been reported to inhibit CETP, possibly by modification of cysteine. The intention of the present report is to provide a structure-activity profile for potential cysteine-modifying reagents and highly purified recombinant human CETP. More than 30 cysteine-modifying compounds were tested for their effect on CETP-mediated lipid transfer. These studies showed that hydrophobic cysteine modification reagents, but not similar hydrophilic reagents, can inactivate CETP. This suggests that an essential cysteine resides in a hydrophobic pocket of CETP, possibly in or adjacent to the neutral lipid binding site.

METHODS

Production and purification of CETP. Human CETP was prepared from the serum-free conditioned medium of recombinant BHK cells expressing a cDNA gene and purified to homogeneity by immunoaffinity chromatography using M468 monoclonal immunoaffinity chromatography as described (9).

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[2] Abbreviations used: CETP, cholesteryl ester transfer protein; LDL, low density lipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester; TG, triglyceride; DTNB, 5,5′-dithio-*bis*-2-nitrobenzoic acid; pCMPS, p-chloromercuriphenylsulfonic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; CoA, coenzyme A.

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CE and TG transfer assay for CETP activity. Either single label [^{3}H]CE-HDL$_3$ or dual label [^{3}H]CE/[^{14}C]TG-HDL$_3$ were used as donor particles. LDL was used as the acceptor particle. CETP-mediated lipid transfer was measured as previously described (9,14). The conditions were such that the rates of [^{3}H]CE transfer and of [^{14}C]TG transfer from HDL to LDL were linear with respect to time and with respect to CETP concentration (9).

Inhibitor compounds purchased from Sigma Chemical Company (St. Louis, MO, USA) or Aldrich Chemical Company (Milwaukee, WI, USA) were dissolved as stock solutions in 100% DMSO, then further serial diluted into 16% (v/v) DMSO. Each dilution of inhibitor, or control DMSO solution lacking inhibitor, was then mixed 1:1 (v/v) with purified recombinant human CETP in 10 mM Tris, 1 mM EDTA, 0.14 M NaCl, pH 7.4 and incubated for 16 hours at 37°C. To determine transfer activity, the CETP-inhibitor mixtures were then diluted an additional 8-fold into the assay mixture described above. The final DMSO concentration in the lipid transfer assay was 1% in all wells, including control wells that contained CETP treated with DMSO without test inhibitor. Samples were assayed in triplicate at each concentration.

RESULTS AND DISCUSSION

Effect of cysteine-modifying reagents on CETP activity. A dual-label lipid transfer assay was used to assess the effect of cysteine-modifying reagents on CETP-mediated transfer of CE or TG from HDL to LDL. Since modification of cysteine could be time-dependent, compounds were either tested following direct addition into the transfer assay, or following a 16 hour pre-incubation with CETP. Table I summarizes the IC_{50} values for inhibition of CE transfer following the 16 hour pre-incubation with CETP. None of the compounds shown in Table I were inhibitory when added immediately prior to the transfer assay. In all cases where inhibition was observed, the IC_{50} values for TG transfer were within 2-fold of the values for inhibition of CE transfer. The mercurial agent pCMPS was the most potent inhibitor with an $IC_{50} = 0.02$ μM. This concentration is only slightly higher than the concentration of CETP the transfer assay, 0.016 μM, thus the reaction with CETP was essentially stoichiometric. The next most potent group of inhibitors includes, 4,4′-dithiodipyridine, and 2,2′-dithiodipyridine, 4,4′-dithiobis(phenyl azide), all with IC_{50} values of 0.5 μM. These small aromatic compounds are hydrophobic and are devoid of charged groups. Introduction of hydrophilic or charged groups onto the compounds generally reduced the inhibitory potency. For example, 6,6′-dithiodinicotinic acid had an IC_{50} that was 100-fold lower than the non-charged analog 2,2′-dithiodipyridine. Other similar charged compounds including 2,2′-dithiodibenzoic acid ($IC_{50} = 800$ μM) and 5,5′-dithiobis(2-nitrobenzoic acid) ($IC_{50} = 800$ μM) were even less potent.

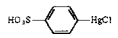
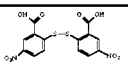
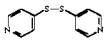
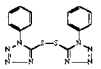
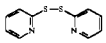
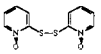
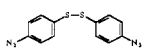
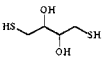
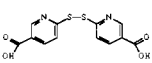
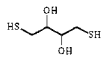
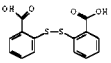
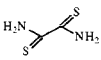
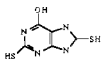
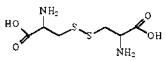
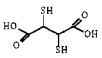
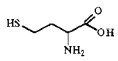
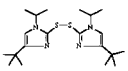
Alkylating agents were also tested as CETP inhibitors. Iodoacetamide and N-ethyl maleimide did not inhibit at concentrations as high as 1,250 μM. Iodoacetic acid had a measurable, but very high IC_{50}, 800 μM.

Many of the compounds shown in Table I, including dithiothreitol, cysteine, homocysteine or glutathione, are agents that can reduce disulfide bonds. However, none of the reducing agents had substantial effects on activity at concentrations as high as 1,250 μM. Dithiodiglycolic acid and oxidized or reduced thioctic acid had IC_{50} values of about 800 μM. Thus, reducing agents, in general, had minimal effect on CETP activity.

Inhibition of CETP-mediated CE and TG transfer activity by p-CMPS and 4,4′-dithiodipyridine. The dose-response curves for the two most potent CETP inhibitors, p-CMPS and 4,4′-dithiodipyridine, are shown in Fig. 1A and Fig. 1B, respectively. The IC_{50} value for inhibition of [^{3}H]CE transfer by pCMPS was 0.02 μM and was 0.5 μM for 4,4′-dithiodipyridine. The inhibition of CE transfer relative to TG transfer was approximately equal for both compounds. Neither compound was inhibitory when added to CETP immediately before the 2 hour transfer assay, but both compounds were very effective inhibitors when preincubated with CETP overnight prior to the 2 hour transfer assay. Thus inhibition was time-dependent. The time-dependence for inhibition is consistent with a covalent modification of the protein.

Effect of DTT on inhibition by 4,4′-dithiodipyridine. The mechanism of inhibition of CETP by 4,4′-dithiodipyridine is likely to involve disulfide exchange of a thiopyridine with a free cysteine on CETP, resulting in the formation of a mixed disulfide between CETP and thiopyridine. If this

Table I
IC_{50} Values for Inhibition of CETP-Mediated CE Transfer

Compound	Structure	IC_{50}	Compound	Structure	IC_{50}
p-chloromercuriphenyl sulfonic acid		0.02	5,5'-dithiobis(2-nitrobenzoic acid)		> 1,250
4,4'-dithiodipyridine		0.5	5,5'-ditiobis(1-phenyl-1*H*-tetrazole)		> 1250
2,2'-dithiodipyridine		0.5	2,2'-dithiobis (pyridine-N-oxide)		> 1250
4,4'-dithiobis (phenyl azide)		0.5	dithiothreitol		> 1,250
6,6'-dithiodinicotinic acid		50	dithioerythritol		> 1,250
2,4-dithio-5-methyl pyrimidine		80	iodoacetamide		> 1,250
2,4'-dithiopyrimidine		160	N-ethyl maleimide		> 1,250
2,2'-dithiodibenzoic acid		800	dithiooxamide		> 1,250
2,8-dithio-6-oxypurine		800	cysteine		> 1,250
2,6-dithiopurine		800	cystine		> 1,250
dithiodiglycolic acid		800	homocysteine		> 1,250
iodoacetic acid		800	glutathione (oxidized)	$(GluCysGly)_2$	> 1,250
2,2'-dithiobis(4-tert-butyl-1-isopropyl-imidazole)		> 1250	glutathione (reduced)	GluCysGly	> 1,250

mechanism is correct, then the exchange reaction could not occur in the presence of disulfide reducing agents. Figure 2 shows an experiment that is consistent with this model. Inhibition of CETP by 4,4′-dithiodipyridine was completely prevented by the reducing agent DTT.

Inhibition of CETP activity by fatty acyl CoA's. Fatty acyl coenzyme A compounds were tested for inhibition of CETP activity. Figure 3 shows that palmitoyl CoA (IC_{50} = 0.3 μM) was a potent CETP inhibitor, but that the shorter chain octanoyl CoA, propionyl CoA, and acetyl CoA compounds were not inhibitory. Triglyceride transfer was inhibited to an equal extent as cholesteryl ester transfer by palmitoyl CoA. In contrast to the inhibitors described above, palmitoyl CoA was also inhibitory when added directly into the transfer assay without prior preincubation with CETP, although the IC_{50} was increased to about 1 μM under those conditions. Oleoyl CoA was also tested as a CETP inhibitor without preincubation, and was found to have an IC_{50} = 2 μM. The time-dependence of inhibition by palmitoyl CoA suggests that cysteine modification may be involved in the inactivation process, and could inactivate CETP either by acylation or by disulfide exchange.

Conclusions. Treatment of CETP with the hydrophobic thiol-reactive reagents pCMPS, 2,2′-

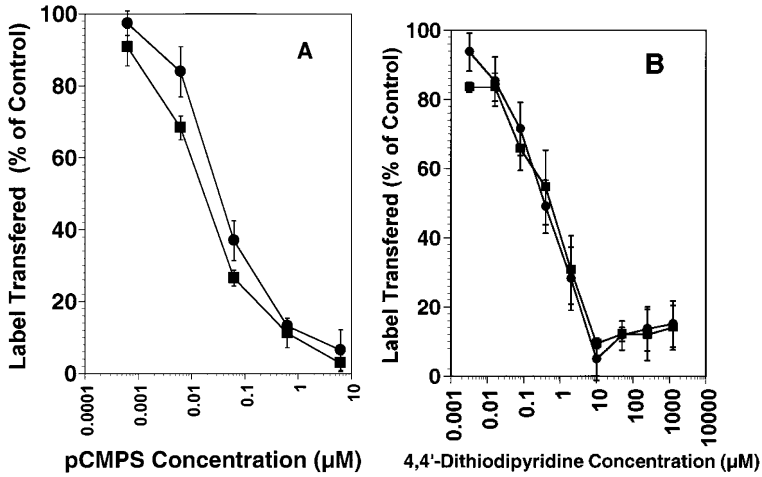


FIG. 1. Inhibition of CETP-mediated [^{3}H]CE and [^{14}C]TG transfer by pCMPS and 4,4′-dithiodipyridine. CETP was incubated for 16 hours at 37°C with the indicated concentrations of pCMPS (A) or 4,4′-dithiodipyridine (B). The dual label transfer assay was used to measure the CETP-mediated rate of transfer of [^{3}H]CE (■) and [^{14}C]TG (●) from HDL to LDL. The concentrations indicated for the inhibitors are the final concentrations in the assay, and reflect an 8-fold dilution from the preincubation concentrations. The final CETP concentration in the assay was 220 ng/ml (16 nM).

dithiodipyridine, 4,4′-dithiodipyridine and 4,4′-dithiobis(phenyl azide) resulted in complete inactivation of CETP. In general, the introduction of charged groups onto the inhibitory compounds reduced the inhibitory potency. However, pCMPS was a highly potent inhibitor (IC_{50} = 0.05 μM), in spite of the presence of a sulfonate group on the phenyl ring of this compound. The inhibition of CETP by many of the compounds studied here, including pCMPS, required overnight incubation with CETP. The slow kinetics for CETP inhibition by pCMPS is surprising given the extremely high affinity and very rapid reaction kinetics that pCMPS has for thiol groups on accessible cysteines (16). This suggests that the essential cysteine on CETP is buried in a hydrophobic pocket,

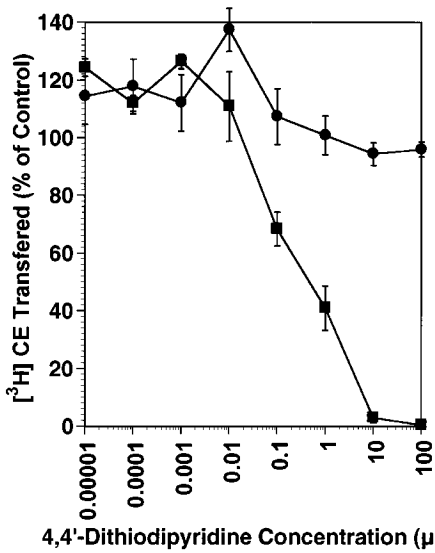


FIG. 2. Inhibition of CETP-mediated [^{3}H]CE transfer by 4,4′-dithiodipyridine in the presence or absence of DTT. CETP was treated with 4,4′-dithiodipyridine, as described in the legend to Fig. 1, but in the presence (●) or absence (■) of 1 mM DTT. Transfer of [^{3}H]CE from HDL to LDL by 220 ng/ml (16 nM) CETP was measured as described in Methods.

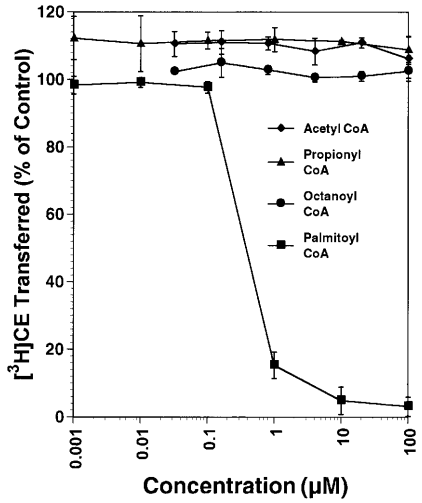


FIG. 3. Inhibition of CETP-mediated [^{3}H]CE transfer by fatty acyl CoA. CETP was treated with the indicated fatty acyl CoA compounds for 16 hours, as described in the legend to Fig. 1. The dual label transfer assay was used to measure the CETP-mediated rate of transfer of [^{3}H]CE and [^{14}C]TG from HDL to LDL. The data shown are for inhibition of [^{3}H]CE transfer, but the data for inhibition of [^{14}C]TG transfer were identical.

and that diffusion into the hydrophobic site is a rate-limiting step for inhibition by this compound. In contrast, the long chain fatty acyl compounds were inhibitory when added immediately before the assay, suggesting that these compounds may contain sufficient structural similarity to the natural substrates for CETP to allow rapid access to the lipid binding site. Longer incubation with the fatty acyl CoA compounds enhanced their potency, suggesting that covalent modification by acylation or disulfide exchange occurred after binding to the CETP site.

Previous reports have suggested that modification of partially purified human plasma CETP with mercurial type sulfhydryl reagents resulted in selective inhibition of TG transfer relative to CE transfer (10,15). In contrast, U-617, an organomercurial derivative of cholesterol, has recently been reported to preferentially inhibit CE transfer relative to TG transfer by recombinant monkey CETP, possibly by a two-step mechanism involving thiol modification (13). All of the inhibitory compounds. In the present study, including the mercurial type inhibitors, blocked both CE and TG transfer to equal extents. The reason for the different results in these three studies it is not clear, but the most obvious difference amongst the studies is the source of CETP.

In summary, CETP can be inactivated using hydrophobic cysteine modification reagents, suggesting that an essential cysteine may be near the lipid binding site.

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