Inhibition of Acyl Coenzyme A:Cholesterol Acyltransferase Blocks Esterification But Not Uptake of Cholesterol in Caco-2 Cells

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The effects of cholesterol esterase (CEase) and acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitors on the uptake and esterification of cholesterol in Caco-2 cells were examined. CEase increased the uptake of [^{3}H]cholesterol from bile salt mixed-micelles by 2.5- to 3.0-fold and its esterification by greater than 25-fold. Inhibition of cellular ACAT activity with CL277082 or CP113818 had little or no effect on cholesterol uptake measured in the presence or absence of CEase. The subsequent esterification of [^{3}H]cholesterol was reduced greater than 90% by each ACAT inhibitor. Similar results were obtained in cells in which ACAT activity was induced by preincubation either with 25-hydroxycholesterol and mevalonic acid or with CEase and bile salt mixed-micelles containing 100 μmol/L cholesterol. Neither ACAT inhibitor had an effect on CEase-mediated synthesis or hydrolysis of cholesteryl oleate in vitro. Thus, the uptake of cholesterol from bile salt mixed-micelles in the presence or absence of CEase was not regulated by the level of cellular ACAT expression. The subsequent esterification of exogenous sterol was not due to CEase, but was completely dependent on ACAT activity. The dissociation of cholesterol uptake from ACAT activity suggests that the factors controlling the transfer of sterol from extracellular media to the cell are different from the factors regulating the cellular level of cholesterol esterification.

THE LEVEL OF SERUM low-density lipoprotein (LDL) cholesterol is an independent risk factor for the development of atherosclerotic vascular disease.[1] LDL levels are controlled in part by endogenous synthesis of cholesterol, by receptor-mediated catabolism of lipoproteins, and by the absorption of cholesterol in the small intestine. Of the 1,200 to 1,500 mg cholesterol presented to the small bowel each day, about one third is from the diet, with the remainder from endogenous sources, predominately the bile.[2] Cholesterol from each of these sources is believed to form a homogenous pool of sterol within the lumen of the intestine, and recent information suggests that endogenous and exogenous cholesterol are absorbed similarly.[2] Interest in the pharmacologic control of cholesterol absorption has increased with the realization that the level of LDL cholesterol is more tightly linked to the absorption of cholesterol than previously thought.[3]

Cholesterol absorption by the small intestine is a complex process that is dependent on bile salts, pancreatic secretion, other lipids, and physical processes within the intestine.[2,4] Unlike the absorption of other lipids, absorption of cholesterol is not complete. In humans, for example, only 40% to 50% of a cholesterol load is absorbed.[2] This observation, together with the known individual hyperresponsiveness and hyporesponsiveness of serum cholesterol to changes in dietary cholesterol in inbred strains of experimental animals and in humans,[5,6] suggests that differences in the response to dietary cholesterol have a genetic basis.

Despite the importance of the intestine in whole-body cholesterol homeostasis, the factors that control intestinal sterol absorption are not completely understood. Two enzymes, acyl coenzyme A:cholesterol acyltransferase ([ACAT] EC 2.3.1.26) and pancreatic cholesterol esterase ([CEase] EC 3.1.1.13), have been studied extensively in this regard.[7,8] Support for a role of ACAT in cholesterol absorption has come from the following observations. ACAT activity has been found in the small intestine in all species examined, including humans, and is induced by cholesterol feeding[7]; and inhibition of intestinal ACAT activity in vivo by a number of compounds reduced cholesterol transport in lymph[9-14] and blunted the increase in plasma cholesterol or the cholesterol content of the liver in response to feeding animals cholesterol-rich diets.[10,15-20] On the other hand, ACAT inhibitors have not been consistently effective at either reducing cholesterol absorption in animals fed different diets or reducing cholesterol absorption or serum cholesterol levels in human trials thus far.[21,22] Although ACAT probably provides esterified cholesterol for chylomicron formation, its capacity to regulate the transfer of unesterified cholesterol from the intestinal lumen to the enterocyte is less clear. Recent study suggests, moreover, that the hypocholesterolemic properties of certain classes of ACAT inhibitors may be related more to their effect on hepatic lipoprotein secretion than on cholesterol absorption by the small bowel.[10,16,23]

A role for pancreatic secretion in cholesterol absorption was demonstrated by Mueller[24] more than 80 years ago. The observation that serum cholesterol levels decline after pancreatectomy and rebound after administration of pancreatin or pancreatic juice has been confirmed in experimental animals[25-27] and in studies of pancreatic insufficiency in humans.[28,29] Although these conditions are associated with gross lipid malabsorption, cholesterol absorption appears to be affected more than that of other lipids.[29] CEase is the component of pancreatic juice responsible for hydrolysis of dietary cholesteryl ester within the intestinal lumen[30,31]; however, its role in cholesterol absorption under normal conditions has remained controversial.[31-33] CEase activity is correlated with an enhanced rate of esterification of exogenous cholesterol within the small bowel of experimental animals[33] and in isolated intestinal cells,[34] and was thus thought to contribute to cholesterol esterification in the gut. The enzyme has been localized within enterocytes of the small intestine by immunocytochemistry,[35] and immunodepletion of pancreatic juice with anti-CEase antibodies decreased cholesterol absorption in chow-fed rats.[33] Purified bovine pancreatic or human breast milk CEase has been shown in some studies[34,36,37] but not others[38] to promote the uptake and esterification of cholesterol in intestinal cells in

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culture. Although these experiments support a role for CEase in cholesterol absorption, CEase knockout mice appear to absorb unesterified cholesterol normally,[31] demonstrating that CEase is not required for cholesterol absorption in vivo. Thus, the relative contribution of CEase and ACAT to the overall uptake and esterification of exogenous cholesterol is not yet clear.

To assess the relative role of ACAT and CEase in regulating the uptake and esterification of exogenous cholesterol by intestinal cells, two structurally distinct ACAT inhibitors and purified CEase have been examined for their ability to modulate these processes in human intestine–derived Caco-2 cells.

MATERIALS AND METHODS

Materials

The human Caco-2 cell line and dimethylsulfoxide (DMSO) were obtained from the American Type Culture Collection (Rockville, MD). [9,10-^{3}H(*N*)]oleic acid (10.0 Ci/mmol), [oleate-1-^{14}C]cholesteryl oleate (59.5 mCi/mmol), and [26-^{14}C]cholesterol (52.6 mCi/mmol) were purchased from NEN-Dupont (Wilmington, DE). [1-^{14}C]oleic acid (55 mCi/mmol) and [1,2-^{3}H(*N*)]cholesterol (53.8 Ci/mmol) were obtained from American Radiolabeled Chemical (St Louis, MO). [1α,2α(*n*)-^{3}H]cholesteryl oleate (49 Ci/mmol) was purchased from Amersham (Arlington, Heights, IL). CP113818 was a generous gift from Pfizer Central Research Division (Groton, CT). CL277082 was synthesized at the Department of Chemistry of CV Therapeutics as previously described.[17] Cholesterol was purchased from Steraloids (Wilton, NH). All tissue culture supplies were obtained from GIBCO-BRL (Gaithersburg, MD). The Mycoplasma PCR Primer Set was purchased from Stratagene (La Jolla, CA). CEase was purified to homogeneity from bovine pancreas and human breast milk as described.[39] All other chemicals were obtained from Sigma Chemical (St Louis, MO).

Cell Culture

Caco-2 cells were cultured in medium A (Eagle's minimal essential medium [MEM] containing 20% fetal bovine serum, 292 μg glutamine/mL, 100 U penicillin/mL, and 100 μg streptomycin/mL) at 37°C under a humidified atmosphere of 95% air and 5% CO_2. The cells were seeded at a density of 1.0×10^5/cm^2 (day 0) and were used for experiments on day 4 to 6, by which time monolayers of approximately 2.0×10^5 cells/cm^2 and 53 μg cell protein/cm^2 had formed. Cultures were routinely screened for mycoplasma contamination using the Mycoplasma PCR Primer Set, and were consistently negative. Cell protein content was measured after solubilization with 1.0 mol/L NaOH and neutralization with HCl using the BioRad (Richmond, CA) protein assay kit with bovine serum albumin as a standard. Statistical analyses were performed using the computer program InStat 2.01 (Sigma Chemical, St Louis, MO) and either a paired or unpaired two-tailed *t* test or the Mann-Whitney test.

Cholesterol Uptake and Esterification

Vesicles of [^{3}H]cholesterol and egg phosphatidylcholine were prepared by mixing 60 μCi [^{3}H]cholesterol with 50 mg egg phosphatidylcholine in a final volume of 10.0 mL 0.1 mol/L Tris hydrochloride, pH 7.2. The mixture was sonicated with a Branson (Danbury, CT) sonicator on setting 3 for 45 to 60 minutes at 4°C. The vesicles were centrifuged for 30 minutes at 9,500 × *g* at 4°C, and the supernatant fraction was removed and stored at 4°C for up to 4 weeks. Cell monolayers were washed once with 2.0 mL ice-cold Earle's balanced salt solution (EBSS) containing 0.01 mol/L HEPES, pH 7.4, and each dish received 1.5 mL medium B (MEM containing 0.01 mol/L HEPES, pH 7.4, 4.0 mmol/L sodium taurocholate, and 1.0% bovine serum albumin) or medium D (MEM containing 0.01 mol/L HEPES, pH 7.4, 2.0 mmol/L sodium taurochenodeoxycholate, and 1.0% bovine serum albumin) containing the indicated amount of CEase dissolved in medium C (0.2 mol/L NaCl and 0.025 mol/L sodium citrate, pH 5.1) or medium C alone, 0.1 μCi [^{3}H]cholesterol/egg phosphatidylcholine vesicles, and the indicated amount of drug or carrier alone. After the indicated time at 37°C, the media were removed and the cells were washed twice with phosphate-buffered saline (PBS) containing bovine serum albumin 1.0 mg/mL, and then twice with PBS alone. The cells were scraped from the dishes, centrifuged for 10 minutes at 1,000 rpm at 4°C, and resuspended in PBS. A sample was removed for determination of cell protein, and the remainder was extracted for total lipids by the addition of chloroform/methanol (2:1 vol/vol) as previously described.[37] A sample of [^{14}C]cholesterol (12,000 dpm) and [^{14}C]cholesteryl ester (2,500 dpm) was added before lipid extraction to quantify recoveries. The lipid extracts were dried under N_2, spotted onto silica gel G plates, and developed using hexane/diethyl ether/acetic acid (86:16:1 vol/vol/vol) as solvent. The lipid-containing areas were visualized with I_2 vapor, and were scraped into scintillation vials for quantification of radioactivity by scintillation counting. In some instances, the data are expressed as CEase-facilitated uptake or esterification of exogenous [^{3}H]cholesterol, which represents the difference between incubation in the presence (total uptake or esterification) and absence (nonfacilitated uptake or esterification) of CEase.

[^{3}H]Oleate Incorporation Into Cellular Lipids

Incorporation of [^{3}H]oleate into cellular lipids was determined as described previously.[40] In preliminary studies, [^{3}H]oleate incorporation into cellular cholesteryl ester was found to be linear to 2.5 hours of incubation at 37°C. [^{3}H]oleate incorporation into cellular cholesterol ester reached a maximum in the presence of 100 nmol/L pancreatic CEase at a micellar cholesterol concentration of 100 μmol/L within 2.5 hours of incubation at 37°C. In other studies, inhibition of [^{3}H]oleate incorporation into cellular cholesteryl ester by 50 μmol/L CL277082 and 0.11 μmol/L CP113818 was maximal within 2.5 hours at 37°C.

Preparation of Microsomal Homogenates and Measurement of ACAT Activity and Cholesteryl Oleate Synthesis and Hydrolysis In Vitro

ACAT activity was measured in microsomal membranes from Caco-2 cells.[41] The incubations were limited to 10 minutes, since we found a significant departure from linearity after this time. The incubation mixtures contained 150 μg microsomal protein, 10 μmol/L [^{14}C]oleoyl CoA (~0.14 μCi) in a final volume of 250 μL 0.1 mol/L potassium phosphate, pH 7.4, bovine serum albumin 1.0 mg/mL, 0.05 mol/L KCl, 0.03 mmol/L EDTA, 0.25 mol/L sucrose, and 5.0 mmol/L dithiothreitol. Studies of activity as a function of microsomal protein and substrate concentration showed that product formation was linear to 250 μg protein and that maximal activity was obtained at 10.0 μmol/L oleoyl CoA. The preparation of vesicles and measurement of cholesteryl oleate synthesis and hydrolysis were as described.[37,39]

RESULTS

A role for CEase and ACAT in regulating the uptake and esterification of exogenous cholesterol was assessed using homogenous CEase and specific inhibitors of ACAT. The ACAT inhibitors used were the trisubstituted urea CL277082[17] and the structurally distinct 2-hexythiodecanamide derivative CP113818.[42] To examine ACAT inhibition by these agents, Caco-2 cells were incubated in the presence or absence of either compound, after which [^{3}H]oleate incorporation into cholesteryl ester was determined. Both CL277082 and CP113818 produced a dose-dependent inhibition of [^{3}H]oleate incorporation into cholesteryl ester, with IC_{50} values of approximately 2.0

and 0.01 μmol/L, respectively, similar to previous reports.[17,42] These data confirmed previous findings that CL277082 and CP113818 are potent ACAT inhibitors in Caco-2 cells.

The sterol ester synthetic activity of CEase is activated at low bile salt concentrations,[43] suggesting that CEase may be responsible for a portion of cholesterol esterification in the intestine. To assess whether CL277082 or CP113818 had effects on CEase activity, the ACAT inhibitors were incubated with bovine pancreatic and human breast milk CEase at low and high bile salt concentrations in vitro. Neither CL277082 nor CP113818 over a wide range of concentrations (1.0 to 1,000.0 μmol/L and 0.001 to 10.0 μmol/L, respectively) inhibited the in vitro synthesis (0.3 mmol/L bile salt) or hydrolysis (4.0 mmol/L bile salt) of cholesterol-[^{14}C]oleate by bovine or human CEase (data not shown). These data thus demonstrate that CL277082 and CP113818 have little or no effect on CEase synthetic or hydrolytic activities in vitro, and they may thereby be used to differentiate between ACAT and CEase cholesteryl ester synthetic activities.

Incubation of Caco-2 cells with either bovine or human CEase for 6.0 hours at 37°C increased the uptake and esterification of [^{3}H]cholesterol in a concentration-dependent manner (Fig 1 A and B), similar to a previous report.[37] Maximal increases in the uptake of [^{3}H]cholesterol were achieved for bovine and human CEase at approximately 100 and 800 nmol/L, respectively. It should be noted that the CEase-mediated increase in cellular [^{3}H]cholesterol did not necessarily reflect net sterol uptake. Since cell cholesterol mass was not measured in these studies, accretion of radiolabel in the cells may represent micellar cholesterol that has exchanged with cell membrane sterol. Little or no esterification of [^{3}H]cholesterol was observed during incubations in the absence of CEase. In the presence of CEase, these rates were greatly increased (Fig 1B). Differences in cholesterol uptake and esterification between the bovine and human enzymes were maintained when the data were normalized to the cholesteryl oleate hydrolytic or synthetic activity of each enzyme. CEase activity in the duodenal juice of healthy normal adults was measured by Fredrikzon and Olivecrona.[44] They reported CEase activities of 3.9 to 29.5 *p*-nitrophenol U/mL duodenal content. Based on the molecular weight of human CEase of 120,000,[39] the assay values of Fredrikzon and Olivecrona,[44] and the specific activity of our purified enzyme (40 *p*-nitrophenol U/mg protein),[39] the concentration of CEase in human duodenal contents can be estimated at 0.83 to 6.25 $\times$ 10^{-6} mol/L. This estimate may be high due to *p*-nitrophenylacetate hydrolysis by other lipases present in the duodenal contents. However, the human pancreas secretes between 6 and 20 g enzyme per day,[45] and from data in the rat, about 2.4% of this is CEase.[46] If this amount is similar to that in humans, then between 0.14 and 0.48 g CEase will be distributed into about 2,000 mL duodenal contents during a meal.[47] This would produce a range of CEase concentrations in the duodenum from 0.6 to 2.0 $\times$ 10^{-6} mol/L, similar to the estimate above. Thus, the concentrations of human CEase used in the present study are at the low end of the physiological range. Similar calculations for the bovine enzyme could not be made, since (to the best of our knowledge) the CEase content of cow duodenal juice has not been reported. It was previously estimated that the physiological CEase concentration was 100 nmol/L[37]; however, it is unclear how this estimate was made, since no details or references were provided in the report. Bovine pancreatic CEase was used in the studies described herein at 100 nmol/L, a concentration that produced a level of cholesterol uptake similar to that of 800 nmol/L human CEase.

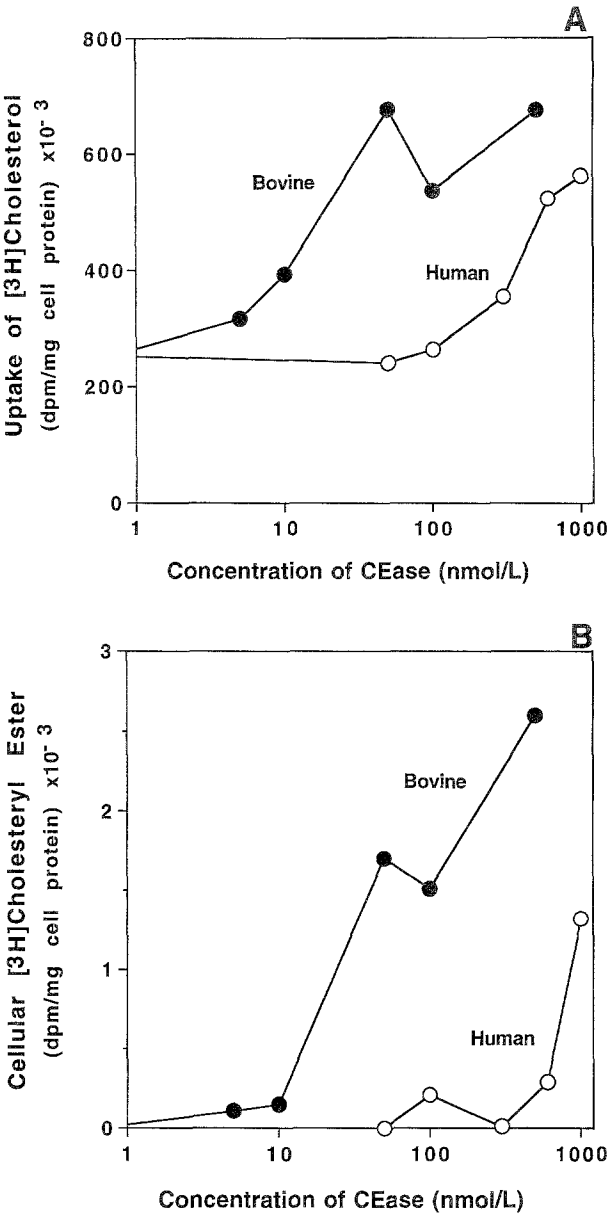

Fig 1. Concentration-dependent increase in [^{3}H]cholesterol uptake and esterification by bovine and human CEase. Each monolayer was incubated with 1.5 mL medium B containing [^{3}H]cholesterol/egg phosphatidylcholine vesicles (0.34 μCi [^{3}H]cholesterol) and the indicated concentration of bovine pancreatic (●) or human breast milk (○) CEase. After 6.0 hours at 37°C, the uptake (A) and esterification (B) of [^{3}H]cholesterol were quantified. Each point represents data from a single dish of cells and is representative of 2 separate experiments.

To examine the effects of ACAT blockade on cholesterol uptake, Caco-2 cells were preincubated for 18 to 24 hours with inhibitory concentrations of CP113818 or CL277082. The uptake and esterification of micellar [^{3}H]cholesterol was subsequently measured by incubation for various times at 37°C.

Relative to incubation with carrier alone, 50.0 μmol/L CL277082 had little or no effect on the uptake of [^{3}H]cholesterol in either the presence or absence of CEase (Fig 2A). Despite this, the accumulation of radiolabeled cholesteryl ester in the presence of CEase was decreased more than 90% by 50.0 μmol/L CL277082 (Fig 2B). Similar effects were observed with 0.11 μmol/L CP113818 (data not shown). In addition, neither 50.0 μmol/L CL277082 nor 0.11 μmol/L CP113818 reduced human CEase-facilitated uptake of [^{3}H]cholesterol. Both agents, however, prevented the accumulation of cellular [^{3}H]cholesteryl ester (data not shown).

When [^{3}H]cholesterol uptake and esterification were examined over a wide range of concentrations of CL277082 or CP113818, neither compound had significant effects on CEase-mediated accumulation of [^{3}H]cholesterol in Caco-2 cells (Fig 3). The esterification of CEase-derived [^{3}H]cholesterol, in contrast, was reduced in a concentration-dependent manner by each compound, with IC_{50} values of approximately 12 nmol/L and 500 nmol/L for CP113818 and CL277082, respectively (Fig 3). The concentration-dependent inhibition of CEase-facilitated [^{3}H]cholesteryl ester formation was positively correlated (r^2 = .91 and .96 for CL277082 and CP113818, respectively) with the inhibition of [^{3}H]oleate incorporation into esterified cholesterol already described, suggesting that cholesteryl ester accumulation was mediated by ACAT and not CEase. Thus, at concentrations that inhibit ACAT activity, neither CL277082 nor CP113818 had any significant effects on radiolabeled cholesterol uptake in the presence or absence of CEase.

In these studies, ACAT inhibitors had no significant effect on cholesterol uptake in Caco-2 cells. In contrast, inhibition of ACAT has been reported in some studies[10,11,13,18,20] to reduce cholesterol absorption in experimental animals fed cholesterol-rich diets. Under these conditions, ACAT activity in the intestine is increased severalfold.[7] Therefore, ACAT activity was induced in Caco-2 cells by incubation for 20 hours at 37°C with a mixture of 25-hydroxycholesterol 7.5 μg/mL and 0.1 mmol/L mevalonolactone, conditions known to induce ACAT activity in intestinal cells.[45] This procedure increased [^{3}H]oleate incorporation into cellular cholesteryl ester by more than 8.0-fold, from 1.6 ± 0.3 to 13.5 ± 1.3 × 10^{-4} dpm/mg cell protein (mean ± SD, $P < .005$, n = 6 dishes of cells for each incubation), indicating that ACAT activity was induced in these cells. Cholesterol uptake, esterification, and their potential blockade by ACAT inhibition were then studied in the ACAT-induced Caco-2 cells.

Incubation with CEase increased the uptake and esterification of [^{3}H]cholesterol (Table 1, medium 1). Addition of 0.11 μmol/L CP113818 had no effect on uptake but reduced the esterification of exogenous [^{3}H]cholesterol nearly 70% (medium 1 *v* 2). Comparable results were obtained for ACAT-induced cells (medium 1 *v* 3). In these cells, basal esterification of [^{3}H]cholesterol in the absence of CEase was increased greater than 100-fold, and this level was increased another twofold in the presence of CEase (medium 1 *v* 3). Incubation of ACAT-induced cells with 0.11 μmol/L CP113818 had no significant effects on the uptake of [^{3}H]cholesterol in the absence or presence of CEase, whereas esterification of exogenous [^{3}H]cholesterol was reduced greater than 85% (Table 1, medium 4).

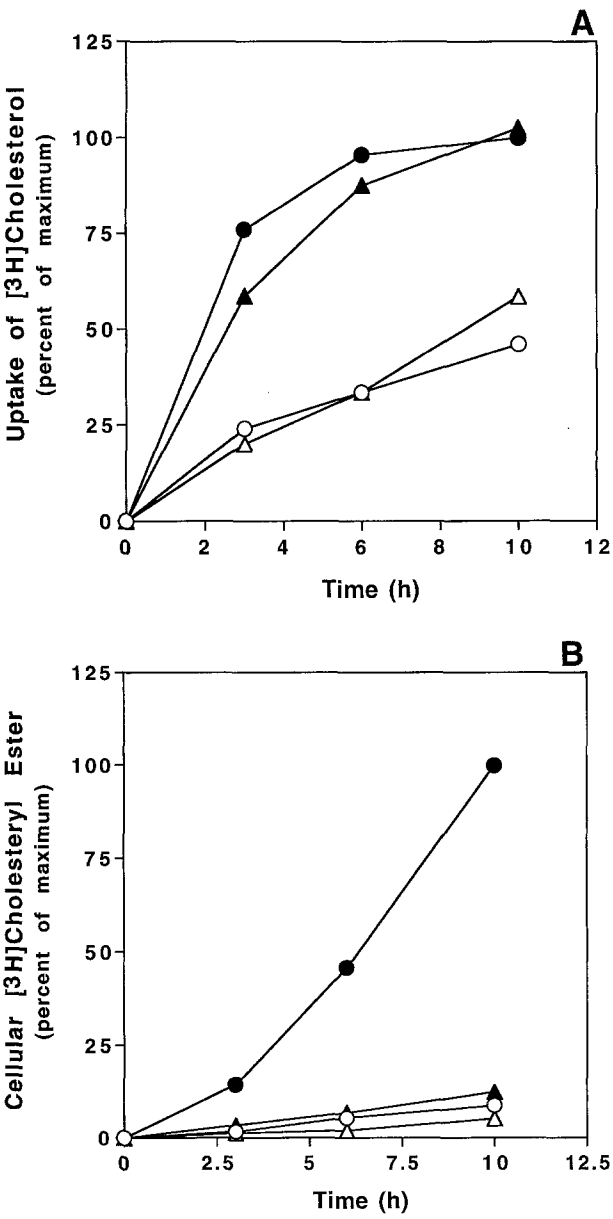

Fig 2. Effects of CL277082 on bovine pancreatic CEase-facilitated cholesterol uptake and esterification in Caco-2 cells. Each dish of cells received 2.0 mL fresh medium A containing either carrier alone (●, ○) or 50.0 μmol/L CL277082 (▲, △) dissolved in ethanol (final concentration, 0.1%). After 18 to 20 hours at 37°C, the media were removed, the cells were washed, and each dish of cells received 1.5 mL medium B containing fresh additions of either carrier alone or CL277082, [^{3}H]cholesterol/egg phosphatidylcholine vesicles (0.34 μCi [^{3}H]cholesterol) with (●, ▲) or without (○, △) 100 nmol/L bovine pancreatic CEase. After the indicated time at 37°C, the uptake (A) and esterification (B) of [^{3}H]cholesterol was quantified. Each point represents the mean value of 2 dishes of cells from 2 separate experiments. All data points in A and B were normalized to the 10.0-hour uptake or esterification time point, respectively, in the presence of CEase, which was set at 100%. For uptake, this value was 263.7 × 10^3 dpm/mg cell protein, and for esterification it was 10.3 × 10^3 dpm/mg cell protein.

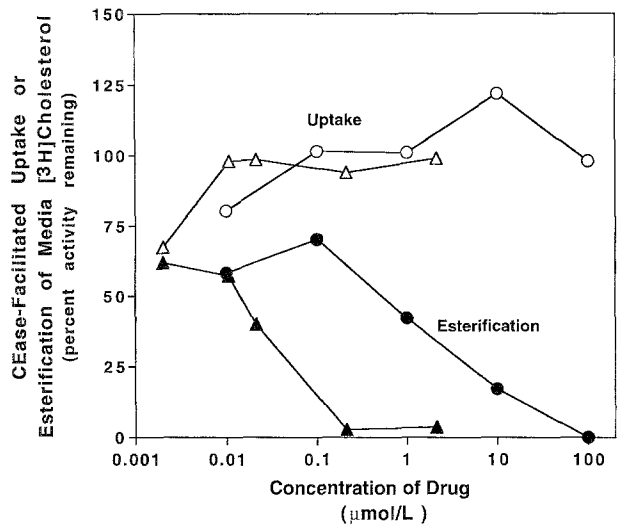

Fig 3. Esterification of CEase-derived cholesterol is inhibited by CL277082 or CP113818 in Caco-2 cells. Cells were incubated with the indicated concentration of CL277082 (●, ○), CP113818 (▲, △), or carrier alone for 18 to 20 hours at 37°C. The uptake (○, △) and esterification (●, ▲) of [^{3}H]cholesterol were subsequently measured during 6.0-hour incubations at 37°C in the presence of fresh drug or carrier as described in Fig 2. Each point represents the mean value of 2 dishes of cells from 2 separate experiments. The 100% values for facilitated uptake and esterification (mean ± SD, n = 4 dishes of cells) were 320.4 ± 75.4 × 10^3 dpm/mg cell protein and 1.62 ± 0.79 × 10^3 dpm/mg cell protein, respectively.

Table 1. Effect of CP113818 on Cholesterol Uptake and Esterification in Caco-2 Cells Preincubated With 25-Hydroxycholesterol and Mevalonolactone

Medium	CEase	[^{3}H]Cholesterol Metabolism (× 10^{-3} dpm/mg cell protein) Uptake	Esterification
1. Control	None	199.0 ± 18.9	0.01 ± 0.02
	100 nmol/L	387.3 ± 56.5*	0.97 ± 0.22*
2. +CP113818	None	193.3 ± 22.4	0.003 ± 0.005
	100 nmol/L	347.8 ± 32.8*	0.35 ± 0.15*†
3. 25-Hydroxycholesterol	None	193.7 ± 30.0	1.45 ± 0.33
+DL-mevalonolactone	100 nmol/L	369.0 ± 93.7*	3.10 ± 0.35*
4. 25-Hydroxycholesterol +DL-mevalonolactone	None	182.2 ± 15.7	0.05 ± 0.09†
+CP113818	100 nmol/L	360.3 ± 39.3*	0.43 ± 0.27*†

NOTE. Each dish of cells received 2.0 mL fresh medium A containing either carrier alone (final ethanol concentration, 0.15%) or 25-hydroxycholesterol 7.5 μg/mL and mevalonolactone 13.0 μg/mL dissolved in water. After 18 to 20 hours at 37°C, each dish received DMSO (final concentration, 0.25%) or 0.11 μmol/L CP113818. After 3.0 hours at 37°C, the media were removed, the cell monolayers were washed, and cholesterol uptake was measured by incubating each dish of cells for 5.0 hours at 37°C with 1.5 mL medium B containing fresh additions of carrier, 25-hydroxycholesterol and mevalonolactone, and/or CP113818 [^{3}H]cholesterol/egg phosphatidylcholine vesicles in the presence and absence of 100 nmol/L bovine CEase. Each value represents the mean ± SEM of 3 separate experiments of duplicate dishes of cells.

*$P < .05$ *v* no CEase.

†$P < .03$ *v* no CP113818.

Similar results were obtained when ACAT was induced in Caco-2 cells by incubation with CEase and bile salt/phospholipid micelles containing 100 μmol/L cholesterol. At this concentration of cholesterol and in the absence of CEase, [^{3}H]oleate incorporation into cholesteryl ester was at background levels. This increased 184% in the presence of 100 nmol/L CEase (Table 2). Addition of 0.11 μmol/L CP113818 reduced [^{3}H]oleate incorporation into cellular cholesteryl ester in the presence of CEase to background levels (Table 2), indicating that ACAT was inhibited under these conditions. No consistent effects of CP113818 on [^{3}H]oleate incorporation were seen in the absence of CEase. In parallel studies, the uptake of [^{3}H]cholesterol was increased more than threefold, as compared with buffer alone, by incubation of Caco-2 cells with 100 nmol/L CEase and bile salt/phospholipid micelles containing physiological ratios of bile salt, phospholipid, and cholesterol[2] (Fig 4). In contrast to the inhibition of ACAT activity by 0.11 μmol/L CP113818 under these conditions, the drug had no effect on the uptake of [^{3}H]cholesterol in either the presence or absence of CEase (Fig 4). The amount of [^{3}H]cholesteryl ester formed in these experiments was below the limit of detection due to the low specific activity of the micellar cholesterol. Thus, under conditions in which ACAT activity in Caco-2 cells was increased, inhibition of ACAT by CP113818 had no effect on cholesterol uptake in the presence or absence of CEase.

DISCUSSION

Cholesterol absorption is a complex event that is dependent on a number of physical processes and enzymatic activities within both the lumen of the small bowel and the cells of the small intestine.[2,4] Although the luminal factors that participate in cholesterol absorption are not completely defined, the present results with Caco-2 cells suggest that CEase may participate in this process by facilitating cholesterol uptake from bile salt mixed-micelles. The lack of effect of ACAT inhibitors on the cholesterol oleate synthetic activity of CEase demonstrated further that CEase did not play a major role in esterifying cholesterol taken up from the extracellular medium. Esterification of the absorbed cholesterol was dependent on ACAT activity, but ACAT did not appear to regulate the level of

Table 2. Induction of ACAT Activity in Caco-2 Cells by Incubation With Bile Salt/Phospholipid Micelles Containing 100 μmol/L Cholesterol

Addition	CEase	[^{3}H]Oleate Incorporation Into Cholesteryl Ester (×10^{-4} dpm/mg cell protein)
DMSO	None	2.04 ± 0.34
	100 nmol/L	3.76 ± 0.28*
CP113818 (0.11 μmol/L)	None	1.08 ± 0.5
	100 nmol/L	2.02 ± 0.25*†

NOTE. Each dish of cells was incubated as described in Fig 4 except that the last incubation contained bile salt/egg phosphatidylcholine micelles and unlabeled cholesterol. The cells were washed, each monolayer was incubated with 0.25 mL medium A containing 0.01 mL [^{3}H]oleate/albumin complexes (~2.0 μCi) at 37°C for 2.0 hours, and [^{3}H]oleate incorporation into cell cholesteryl ester was measured. Each value represents the mean ± SD of triplicate dishes of cells and is representative of 2 separate experiments.

*$P < .05$ *v* no CEase.

†$P < .05$ *v* CEase in the absence of CP113818.

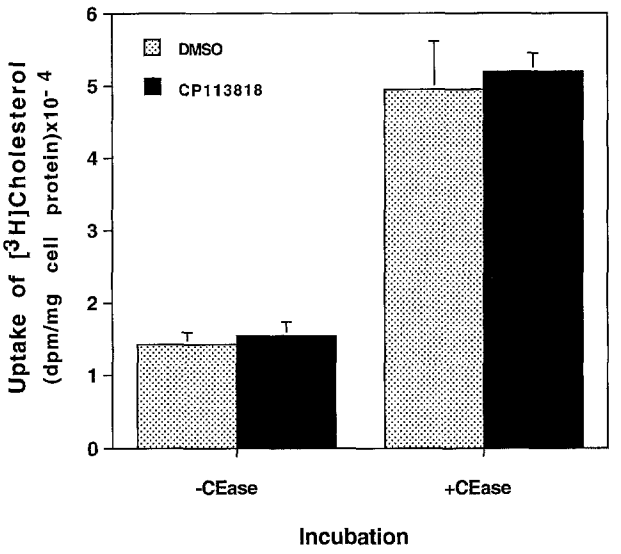

Fig 4. Effect of CP113818 on CEase-facilitated uptake of [^{3}H]cholesterol in Caco-2 cells incubated with 100 μmol/L cholesterol. Each dish of cells received 0.25 mL medium D with bile salt/phospholipid micelles containing 100 μmol/L cholesterol and either buffer alone or 100 nmol/L bovine pancreatic CEase. After 2.5 hours at 37°C, the media were removed and each dish received medium with fresh additions and either DMSO (final concentration, 0.68%) or 0.11 μmol/L CP113818. After 3.0 hours at 37°C, the media were removed, the cell monolayers were washed, and [^{3}H]cholesterol uptake was measured by incubating each dish of cells of 3.0 hours at 37°C with 0.25 mL medium D containing fresh additions and [^{3}H]cholesterol/egg phosphatidylcholine vesicles. Each value represents the mean ± SD of triplicate dishes of cells and is representative of 2 separate experiments.

cholesterol uptake by Caco-2 cells in either the presence or absence of CEase. These results suggest that CEase and ACAT can act sequentially to facilitate the uptake and the esterification of cholesterol, respectively, in intestinal mucosal cells.

While factors such as bile salts and pancreatic secretion are essential for the efficient absorption of cholesterol,[2,4] the importance of CEase and ACAT in sterol transport within the intestine has remained controversial. Using the same cell system, some studies[36,37] have shown an effect of CEase on cholesterol uptake, but others[38,48] have not. The absence of an effect of CEase on unesterified cholesterol uptake in Caco-2 cells reported by Huang and Hui[48] was most likely due to the low concentration of bile salt (63 μmol/L taurocholate) used in their study.[37] Shamir et al[38] also found no effect of CEase on unesterified cholesterol absorption in Caco-2 cells. In that study with phospholipid/bile salt/cholesterol micelles, the concentration of human CEase used was 100 nmol/L. This concentration of human CEase was too low to facilitate cholesterol uptake and is probably well below the physiological range in humans (Fig 1). We recently found that CEase-facilitated cholesterol uptake from mixed-micelles containing phosphatidylcholine, cholesterol, and bile salt to Caco-2 cells was dependent on the phospholipase A1 activity of CEase.[49] Phosphatidylcholine appeared to limit cholesterol uptake, as shown previously in humans,[50] rats,[51-53] and Caco-2 cells.[48] This inhibition was relieved by CEase through hydrolysis of micellar phosphatidylcholine, producing lysophosphatidylcholine and free fatty acid.[49] Addition of these lipids to the bile salt micelles increased background cholesterol uptake to levels seen in the presence of CEase. This suggested that the lack of effect of CEase on cholesterol uptake reported by others[32,35,48] could be explained by the use of bile salt micelles containing the emulsifying agent monoolein and/or free fatty acid rather than phospholipid.

Although our studies show that CEase can facilitate cholesterol uptake in the Caco-2 system, the role of CEase in lipid absorption in vivo remains unclear. Hydrolysis of dietary steryl or vitamin esters appears to be dependent on CEase activity[31,8]; however, the wide substrate specificity of the enzyme[8] suggests that CEase might play a more general role in lipid absorption as part of a redundant lipolytic enzyme mechanism. In the newborn, for example, the enzyme appears to be important for digestion and absorption of milk triacylglycerols and cholesterol.[8] In addition, CEase knockout mice appeared to absorb unesterified cholesterol normally,[31] suggesting that other pancreatic proteins such as phospholipase A2[49] and/or triglyceride lipase might regulate cholesterol absorption. As shown for the phospholipase A1 activity of CEase,[49] phospholipase A2 could play such a role during digestion of luminal phospholipid by generating lysophospholipid and free fatty acid. Triglyceride lipase-colipase might also act indirectly by generating free fatty acids and 2-monoglyceride.[54,55] These amphiphilic lipolytic products[56] enhance cholesterol absorption probably by increasing cholesterol solubility in bile salt micelles through alterations in micelle size or in cholesterol partitioning within the bile salt mixed-micelle.[51,57-60]

A CEase-catalyzed hydrolysis of micellar phospholipid and production of free fatty acid may also explain the increase in fractional esterification of exogenous cholesterol observed in the present study and previous reports.[36,37] Free fatty acids are known to activate ACAT,[7] and micellar phospholipid is completely hydrolyzed to lysophospholipid and free fatty acid within 1.5 hours at 37°C in our system.[49] Although other explanations cannot be ruled out, the low background levels of cholesterol esterification in Caco-2 cells coupled with fatty acid induction of ACAT activity could explain the differences seen in the level of CEase-facilitated cholesterol uptake (twofold to threefold) and esterification (>25-fold). Similarly, the CEase-facilitated increase in fractional esterification of cholesterol reported by Gallo et al[35] could also be explained by CEase-mediated hydrolysis of micellar monoglyceride. Monoolein is a substrate for CEase,[8] although the enzyme has a relatively low reactivity with this substrate, particularly with the sn-2 versus the sn-1 ester.[8] The form of monoolein used in their study[35] was not mentioned, but hydrolysis of either form under the conditions used could have provided additional free fatty acid that further increased cholesterol esterification through the ACAT pathway.

The observation that CL277082 and CP113818 inhibited cholesterol esterification but failed to limit cholesterol uptake suggested that sterol transfer from the extracellular medium to the cell and cellular cholesterol esterification are regulated differently. These data are consistent with a recent study reported by Burrier et al[61] in cholesterol-fed hamsters. Using a short-term model of cholesterol absorption, they showed that

the ACAT inhibitor PD128042, also known as CI976, blocked cholesteryl ester output by the intestine but had no effect on the transfer of cholesterol from the lumen to the enterocytes. Similar results were also reported by Field et al[62] in Caco-2 cells, where PD128042 blocked esterification but had little or no effect on cholesterol uptake from taurocholate-monoolein micelles. These results predict that ACAT inhibitors should block the output of esterified cholesterol by the intestine, an observation that has been demonstrated repeatedly.[9-14] These results further suggest that ACAT plays a minor role, if any, in regulating the transfer of sterol to the mucosal cell.

Whether the proteins described herein or others are necessary or sufficient for cholesterol absorption in the small bowel in vivo is not yet proven. It is also unclear whether other factors[63] acting within the small intestinal cell facilitate the transport of cholesterol from the plasma membrane to the ACAT substrate compartment or to the pool of cholesterol destined for chylomicron synthesis and secretion. Identifying these regulatory factors should produce novel strategies to interrupt cholesterol absorption and thereby alter cholesterol homeostasis. The recent observation that cholesterol absorption and LDL cholesterol in hypercholesterolemic humans can be reduced nearly 80% and 40%, respectively, by combined treatment with neomycin and sitostanol[3] strongly suggests that pharmacologic control of cholesterol absorption is an effective means to decrease serum LDL cholesterol.

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