Role of Bile Salt-Dependent Cholesteryl Ester Hydrolase in the Uptake of Micellar Cholesterol by Intestinal Cells†

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ABSTRACT: The bile salt-dependent cholesteryl ester hydrolase (CEH; EC 3.1.1.13) has been proposed to promote the intestinal absorption of both the free and esterified (FC, CE) forms of dietary cholesterol. For example, it was recently reported that in the human intestinal cell line CaCo2, addition of bovine CEH to the medium increased the uptake and intracellular esterification of micellar FC supplied at subphysiological concentrations [Lopez-Candales et al. (1993) *Biochemistry 32*, 12085−12089]. To test the ability of CEH to promote micellar cholesterol uptake in a CaCo2 system under more physiological conditions, an in vitro model was developed. Cells stably expressing rat CEH were created by DNA transfection (Tr cells), and the uptake of micellar FC and its intracellular esterification were measured using isotopic methods in Tr and control cells. Experimental parameters that were varied included micellar composition (monoolein or egg PC; FC, CE, or both), the final concentration of micellar cholesterol (1 nM to 50 μM), the origin of CEH (endogenously synthesized vs exogenously added), and the species source of enzyme (rat, pig, man). The uptake of cholesterol that was derived from micellar CE was significantly increased 5−10-fold ($p < 0.001$) in Tr vs control cells as a result of the hydrolysis of the CE by the CEH and subsequent uptake of the liberated free cholesterol. In contrast, the uptake of micellar FC was not increased by the presence of CEH, whether it was endogenous or exogenous. In addition, based on TLC analysis of extracted cellular lipids, there was no evidence that CEH promoted the esterification of the FC that was taken up. These results were independent of cholesterol concentration and the non-sterol composition of the micelles. Although in the presence and absence of CEH there was comparable uptake of cholesterol by cells after a 4 h incubation with a particular type of micelle, micelles containing egg PC were not as effective FC donors as those containing monoolein. Overall, the data support a role of CEH in modulating the absorption of CE present in the intestinal lumen by a mechanism involving the hydrolysis of CE by CEH, thereby increasing the FC concentration gradient between the micellar and plasma membrane pools and enhancing the passive cellular uptake of free cholesterol.

The relationship between high plasma cholesterol levels and the development of premature atherosclerosis is well established. While there are controversies surrounding mechanisms, there is a general consensus that the amount of dietary cholesterol can influence plasma low-density lipoprotein (LDL)[1] levels and atherogenesis [for a recent review, see Spady et al. (1993)]. Recent clinical studies have suggested that the amount of dietary cholesterol *per se* is an incomplete index of the contribution of exogenous cholesterol to the plasma low-density lipoprotein cholesterol (LDL-C) pool (Miettinen & Kesaniemi, 1989); at a given level of dietary cholesterol intake, the inter-individual variation in the percentage of cholesterol absorbed correlated positively with plasma levels of LDL-C. Therefore, factors affecting cholesterol absorption very likely also contribute to the regulation of LDL-C plasma levels. Current knowledge of cholesterol absorption has been reviewed recently (Wilson & Rudel, 1994). Dietary cholesterol (both free and esterified, FC and CE, respectively) becomes incorporated into mixed micelles from which it is absorbed in the free form by intestinal cells. The micellar CE is converted to FC by the action of pancreatic bile salt-dependent cholesteryl ester hydrolase (CEH; EC 3.1.1.13).

The absorption of FC is known to be a saturable process (Grundy et al., 1969), and the existence of regulatory proteins has been hypothesized [for example, see Thurnhofer and Hauser (1990)]. In addition to its luminal hydrolytic role, CEH has been proposed to be one such regulatory protein by acting as either a transport factor (Lopez-Candales et al., 1993) or an intracellular esterifying enzyme which would create a gradient for the net flux of FC across the enterocyte cell membrane (Gallo et al., 1984; Rudd et al., 1987). Evidence cited in support of the ability of CEH to promote cholesterol absorption comes from studies in both animals

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[1] Abbreviations: CE, cholesteryl ester; CEH, bile salt-dependent cholesteryl ester hydrolase; FBS, fetal bovine serum; FC, free cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; MEM, minimal essential medium; MO, glyceryl monooleate, monoolein; NaTc, sodium taurocholate; Neo, CaCo2 cells transfected with control DNA (neomycin-resistance plasmid); Neo+, control CaCo2 cells incubated with exogenous CEH; PC, phosphatidylcholine; Tr, CaCo2 cells transfected with CEH cDNA and control DNA (neomycin-resistance plasmid); Tr+, transfected CaCo2 cells incubated with exogenous CEH.

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(Gallo et al., 1984) and cell culture (Lopez-Candales et al., 1993). On the other hand, other studies in animals (Watt & Simmonds, 1981) and cells (Huang & Hui, 1990) have not supported this view.

The present studies focus on the controversial hypothesis that CEH promotes the uptake of micellar free cholesterol by intestinal cells. To test this, an in vitro model system based on the human intestinal cell line CaCo2 was developed by transfection with CEH cDNA. Overall, the results suggest an indirect role for CEH in dietary cholesterol absorption, limited to the hydrolysis of cholesteryl esters, liberating cholesterol molecules, which then enter the luminal pool of free cholesterol. This would result in an increase in the FC concentration gradient between the micellar and plasma membrane pools and the enhanced passive cellular uptake of free cholesterol.

MATERIALS AND METHODS

All reagents and isotopically labeled compounds were purchased from Sigma (St. Louis, MO) and DuPont/NEN (Boston, MA), respectively, unless indicated otherwise.

Cell Culture. The CaCo2 cell line, which resembles small intestinal enterocytes, was originally derived from a human adenocarcinoma of the colon (Pinto et al., 1983; Hughes et al., 1987) and was provided by the American Type Culture Collection (Rockville, MD). Cells were maintained in growth medium [minimal essential medium (MEM) supplemented with basal medium Eagle vitamins, 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 50 μg/mL gentamicin]. Medium and vitamins were purchased from Life Technologies (Gaithersburg, MD). The ambient conditions for cell culture were 37 °C and an atmosphere of 95% air and 5% CO_2.

Cell Transfection. The rat pancreatic CEH cDNA in the pECE1 plasmid, a eukaryotic cell expression vector utilizing the SV40 early promotor, was kindly provided by Drs. Jang H. Han and William J. Rutter (University of California, San Francisco) and previously described by them (Han et al., 1987). Hereafter, this plasmid is referred to as pCEH. A plasmid (pMAMneo), that expresses resistance to neomycin and its analogue G418, was purchased from Clontech (Palo Alto, CA). Cells were cotransfected with pCEH and pMAMneo by the calcium phosphate precipitation technique (Sambrook et al., 1989), and clones viable in medium containing 100 μg/mL G418 and stably expressing CEH activity were selected as we described previously (Zolfaghari et al., 1993). For a control cell line, only the pMAMneo plasmid was used for the transfection. In addition to the assays of enzymatic activity described below, stable expression of CEH mRNA only in cells transfected with CEH cDNA was also confirmed by Northern blot analysis (Zolfaghari et al., 1993) of cell lines transfected with pCEH and pMAMneo or pMAMneo alone (data not shown).

Exogenous Cholesteryl Ester Hydrolases. In experiments in which exogenous CEH was added to cell culture medium, either porcine pancreatic or human breast milk CEH [which has the identical amino acid sequence to the pancreatic enzyme (Wang & Hartsuck, 1993)] was used. Porcine pancreatic CEH, 1.35 mg of protein/mL in 50 mM potassium phosphate, pH 6.0, was kindly provided by Dr. Howard L. Brockman (University of Minnesota, Austin, MN) and diluted in medium prior to use. Human breast milk CEH, lyophilized after dialysis against dH_2O, was kindly provided by Dr. Dominique Lombardo (INSERM U260, Marseille, France) and reconstituted and diluted in medium prior to use.

Preparation of Micelles. In experiments in which the final concentration of micellar cholesterol was either 50 or 5 μM, [4-^{14}C]cholesterol (supplied with a specific activity of 57.1 mCi/mmol) was used. In experiments in which the final concentration of micellar cholesterol in the medium was 1 nM or in experiments involving cholesteryl ester (alone or in combination with free cholesterol), [1,2-^{3}H(N)]cholesterol (supplied as 48.1 Ci/mmol) and [*cholesteryl*-4-^{14}C]cholesteryl oleate (supplied as 53.4 mCi/mmol) were used.

In the majority of the experiments, mixed micelles containing glyceryl monooleate (monoolein, MO), sodium taurocholate (NaTc), and cholesterol (free, ester, or both) were used. They were prepared as previously described (Johnson & Cain, 1988). Briefly, trace amounts of labeled cholesterol and/or cholesteryl ester were mixed with unlabeled cholesterol and/or cholesteryl ester, combined with MO in organic solvent solution, and evaporated to dryness under N_2 at 40 °C. The dried lipids were then suspended in 50 mL of MEM made 6 mM in NaTc so that concentrations of components were 2 mM MO, and either 2 nM, 10 μM, or 100 μM free cholesterol and 0, 10 μM, or 20 μM cholesteryl ester. In some experiments, egg phosphatidylcholine (PC, 2 mM) was substituted for MO. In this case, the dried lipids were suspended in MEM/NaTc by sonication for 1 h in a water bath at 37 °C. The final radioisotope concentration (as cpm/mL) of either FC or CE was between 1×10^5 and 3×10^5. Micellar solutions did not exhibit any turbidity. Before application to cells, these solutions were filtered through 0.45 μm filter units (Millipore, Bedford, MA) and gassed at 37 °C for 2 h in 95% air, 5% CO_2. For each well, 1 mL of suspended micelles was used.

Cell Association of Labeled Cholesterol. In this report, the terms cell association and uptake will be used interchangeably. CaCo2 cells transfected with pCEH and pMAMneo (Tr) or control cells transfected with pMAMneo (NEO) were grown in 75 cm^2 flasks in Eagle's MEM containing 10% FBS, 2 mM glutamine, and 0.1 mg of G418/mL. Cells were passaged upon reaching 80% confluency and, for experiments, seeded into six-well plates (35 mm diameter wells) at a density of 10^6 cells per well. In initial experiments, two different transfected lines were used (1H9, 4A2); since the results with either line were comparable (data not shown), only one (4A2) was used subsequently. Medium was changed twice a week, and 2 weeks after cells were confluent, at which time CaCo2 cells are maximally differentiated (Field et al., 1987; Reisher et al., 1993) and the typical cell protein was 1 mg/well, the uptake of labeled cholesterol derived from mixed micelles was studied as follows:

Medium was aspirated from each well, and cells were washed with MEM 3 times. One milliliter of MEM supplemented with 5% lipoprotein-deficient serum, 2 mM glutamine, and 6 mM NaTc (medium A) was added, and cells were incubated for 12 h at 37 °C to allow for the accumulation of CEH activity in the medium of Tr cells. An aliquot of 20 μL of conditioned medium was taken for the assay of CEH enzymatic activity (see below). One milliliter of suspended micelles containing either free cholesterol, or cholesteryl oleate, or both, was added. The composition of the micelles was varied to provide the concentrations of FC and CE indicated under Results. The final concentrations of the other micellar components were

kept at 6 mM (NaTC) and 1 mM (egg PC or MO). The cells were then incubated for an additional 4 h.

In experiments examining the effects of exogenously supplied CEH, cells were also studied 2 weeks after reaching confluency. After the cells were washed as described above, either the porcine or the human enzyme was added to medium A. Ten minutes were allowed for homogeneous dispersion into the medium, and 20 μL was then removed for enzyme assay. Next, 1 mL of mixed micelles was added, and the cells were incubated for 4 h. The final concentration of exogenous CEH was 100 nM.

At the conclusion of either type of experiment, conditioned medium was removed, and the cells were rinsed once with MEM and twice with phosphate buffer solution. Cellular lipids were extracted as previously described (Zolfaghari et al., 1993). Briefly, cells were incubated at room temperature with 2 mL of 2-propanol, and lipids were recovered by aspirating the 2-propanol. Wells were washed with 2-propanol once and the aspirate and wash combined. These 2-propanol extracts were then evaporated to dryness under N_2 at 40 °C and resuspended in 1 mL of chloroform. A 100 μL aliquot was taken for scintillation counting and the remainder stored at −20 °C until used for the analysis of cholesterol and cholesteryl ester content (see below). Cell association of labeled cholesterol was calculated as the percent of total cpm added to the medium that was recovered in the extract. At the end of each experiment, the overall recovery of labeled material (cell extract plus medium) was typically 70−90% of the radioactivity added at time zero. Each experiment included a well with no cells to control for nonspecific adhesion of labeled material to plastic. For each condition, at least three wells of cells were used within each experiment, and each entire experiment was repeated at least twice.

At the conclusion of experiments with micelles containing CE, lipids were also extracted from conditioned medium by the method of Bligh and Dyer (1959). The chloroform phase was kept at −20 °C until used for the analysis of cholesterol and cholesteryl ester content.

To determine the contents of labeled cholesterol and cholesteryl ester in the extracts of cells or media, chloroform was evaporated under N_2, and the extracted lipids were redissolved in 25 μL of chloroform/methanol (1:1). To 10 μL of that solution were added 100 μg of FC and 10 μg of CE (to serve as carrier), and lipids were separated on Instant Thin Layer Chromatography sheets (ITLC SA; Gelman Sciences, Ann Arbor, MI) using petroleum ether/toluene (65:35). Plates were dried and stained with iodine, and spots of FC and CE were excised and put into vials containing Scintiverse (Fisher Scientific, Pittsburgh, PA). The radioactivity in each vial was then determined by scintillation counting. The mass of cholesterol derived from the mixed micelles and its distribution as free or esterified were calculated on the basis of the combined mass of labeled and unlabeled cholesterol provided by the micelles, the percent uptake of label, and the distribution of label as FC and CE determined by the ITLC analysis.

To determine cell protein mass/well, after the 2-propanol was removed from a well, 2 mL of Lowry A solution was added to solubilize protein, and analysis was done using a modified Lowry procedure (Markwell et al., 1978).

CEH Activity Assay. The radiometric assay of CEH activity has been described previously (Harrison et al., 1979, 1988). Briefly, the reaction mixture (final volume of 0.19 mL) contained 0.05 M Tris−maleate, pH 7.0, 40 mM sodium cholate, and appropriately diluted medium, purified enzyme, or cell lysate (cells homogenized in 0.25 M sucrose). The reaction was initiated by adding 0.01 mL (2 nmol) of cholesteryl [1-^{14}C]oleate (supplied with specific activity 53 mCi/mmol; Amersham, Arlington Heights, IL). Tubes were incubated in a 37 °C water bath for 1 h. The released [^{14}C]-oleate was extracted and quantitated by scintillation counting. A unit of CEH activity was defined as 1 nmol of [^{14}C]oleate released per hour. Activity determinations were done in duplicate, and the coefficient of variability was less than 10%.

The cell lysate and conditioned medium of CaCo2 cells transfected with pMAMneo as well as heat-inactivated FBS contained no significant CEH activity. The specific activities of the porcine and human enzymes were 8.5×10^5 and 1.5×10^5 units/mg of protein, respectively, comparable to literature values (Rudd et al., 1987; Swan et al., 1992).

Statistical Analysis. Comparisons for significant differences were performed either by Student's t test for comparison of two groups or by ANOVA for comparison of four groups, using standard computer software (InStat, GraphPAD Inc., San Diego, CA).

RESULTS

Secretion of Catalytically Active CEH by Transfected CaCo2 Cells. The CaCo2 cell line is an established model for the study of human enterocyte metabolism (Pinto et al., 1983; Hughes et al., 1987). These cells lack CEH mRNA and have no other significant bile salt-dependent hydrolytic activity against CE (R. Zolfaghari, unpublished observations). By transfecting CaCo2 cells with CEH cDNA or a control plasmid (pMAMneo), four in vitro systems were available for study: (1) cells with no endogenous or exogenous CEH activity (Neo); (2) transfected cells secreting CEH activity (Tr); (3 and 4) Neo cells or Tr cells with exogenous activity added to medium (Neo+, Tr+). These models represent the range of in vivo possibilities implied by the presence of CEH mRNA in the enterocytes of at least one species [rabbit (Zolfaghari et al., 1992; confirmed by Rea et al., 1993)] and not in another [rat (Kissel et al., 1989; Zolfaghari et al., 1992)]. Until the status of human enterocytes with regard to CEH mRNA is established, either possibility must be considered in man.

Similar to the transfected rat hepatoma cells we previously studied (Zolfaghari et al., 1993), transfected CaCo2 cells secreted CEH with curvilinear accumulation over time (Figure 1). There was no detectable activity at time 0 (sample taken immediately after fresh medium applied), and at 0.5, 12, and 24 h, accumulated activity in the medium was 0.81 ± 0.1, 21.2 ± 4.4, and 77 ± 5.3 units/mg of cell protein (mean ± SD, $n \geq 3$). At 24 h, the cell lysate CEH activity was 17.4% ± 1.5 of the accumulated activity in the medium. On the basis of the specific activity of purified rat CEH (Camulli et al., 1989), the activity in the conditioned medium at the end of 12 h corresponded to an estimated enzyme concentration of 700 nM.

Effects of CEH on the Uptake by Transfected Cells of Cholesterol Derived from Micelles Containing Cholesteryl Ester. The role of CEH in the uptake of dietary cholesterol by enterocytes has been traditionally thought to be limited to the hydrolysis of cholesteryl esters [reviewed in Wilson and Rudel (1994)]. To confirm that the Tr cells would duplicate this role, CE (radiolabeled in the cholesterol moiety

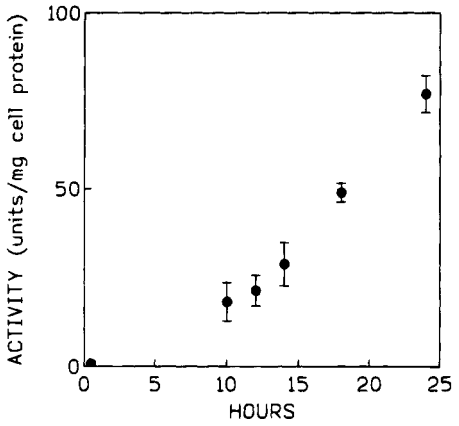

FIGURE 1: CEH enzymatic activity in conditioned medium of Tr cells. Enzymatic activity was measured in the conditioned medium of well-differentiated CaCo2 cells transfected with pCEH (Tr cells) at 0.5−24 h after adding fresh medium at time zero. Results are displayed as units per milligram of cell protein, mean ± SD, $n \geq 3$.

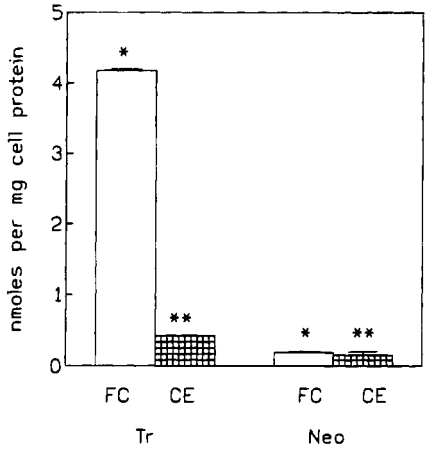

FIGURE 2: Distribution of micellar-derived cholesterol in Tr and Neo cells after incubation with mixed micelles providing 10 μM CE. Tr and Neo cells were incubated for 12 h to allow enzyme accumulation in Tr cell medium. Mixed micelles containing labeled CE (as cholesteryl oleate) were then added to the conditioned media of Tr and Neo cells to provide a final CE concentration of 10 μM. After a 4 h incubation, uptake of labeled cholesterol from the micelles was determined by scintillation counting of extracted cellular lipids. The distribution of cellular cholesterol between FC and CE in the extracts was assayed by ITLC, and the mass of each species was calculated (Materials and Methods). The results for each cell type were normalized to cell protein and are displayed as mean (±SD), $n = 6$. There was significantly more FC and CE in Tr cells (*$p < 0.001$, **$p < 0.0001$, respectively).

and incorporated into micelles) was presented to Neo and Tr cells at a final concentration of 10 μM. This concentration was selected because CE is typically about 20% of the cholesterol in the Western diet and we were utilizing 50 μM FC in other experiments (see below). In addition, micelles containing higher concentrations of CE were unstable. After 4 h of incubation, the percentage of label associated with Tr cells was 21.94% ± 4.18 compared to 2.85% ± 0.19 with control cells ($p = 0.002$, $n = 6$). When the separate labeled species (FC or CE) were measured in the lipid extracts of cells, nearly all of the cholesterol taken up by the Tr cells was found in the FC fraction (Figure 2). That this cholesterol had arisen from CEH-mediated hydrolysis of the CE provided by the micelle was supported by finding in the conditioned medium of Tr cells that 99.6% ± 0.04 ($n = 3$) of the labeled cholesterol was FC.

Table 1: Percent Uptake and Distribution of Micellar-Derived Cholesterol in Tr and Neo Cells Incubated with Mixed Micelles Providing Different Concentrations of FC[a]

	FC concentration		
	50 μM	5 μM	1 nM
	Tr Cells		
% uptake	22.4 ± 1.6	21.1 ± 2.3	16.2 ± 1.7
free cholesterol	20.7 ± 0.13	2.6 ± 0.03	0.16 ± 0.005
cholesteryl ester	1.6 ± 0.04	0.028 ± 0.003	0.003 ± 0.001
	Neo Cells		
% uptake	21.4 ± 1.5	21.6 ± 2.0	17.4 ± 1.2
free cholesterol	19.8 ± 0.12	2.8 ± 0.02	0.17 ± 0.002
cholesteryl ester	1.6 ± 0.04	0.14 ± 0.02	0.005 ± 0.001

[a] Tr and Neo cells were incubated for 12 h to allow enzyme accumulation in Tr cell medium. Mixed micelles containing labeled FC were then added to the conditioned media of Tr and Neo cells to provide a final cholesterol concentration of 1 nM, 5 μM, or 50 μM. After a 4 h incubation, uptake of labeled cholesterol from the micelles was determined by scintillation counting of extracted cellular lipids. The distribution of cellular cholesterol between FC and CE in the extracts was assayed by ITLC, and the molar quantity of each species was calculated (Materials and Methods). The percentage of micellar cholesterol taken up by the cells is given as mean ± SD, normalized to cell protein ($n \geq 6$). The calculated masses for FC and CE for the 50 μM and 5 μM conditions are given in nanomoles per milligram of cell protein. The calculated masses for the 1 nM condition are given in picomoles per milligram of cell protein. The mass results for each cell type are displayed as mean (±SD), $n \geq 3$.

Effects of CEH on the Cell Association of Micellar-Derived Free Cholesterol. The role of CEH in the uptake of FC from the intestinal lumen was investigated by using preparations of synthetic micelles containing different concentrations of FC. Although data on the effects of CEH in a CaCo2 system have already been reported for micelles that provided nanomolar concentrations of FC (Huang & Hui, 1990; Lopez-Candales et al., 1993), in addition to this concentration, we also wished to examine the effects using higher concentrations, since human intestinal fluid typically contains FC at micromolar levels in the micellar phase (Hernell et al., 1990).

Cells (Tr or Neo) were incubated 12 h to allow enzyme accumulation in medium containing 5% lipoprotein-deficient serum and 6 mM NaTc. Then, an equal volume of micelles was added to provide a final FC concentration of 50 μM and the incubation continued for 4 h, a time chosen because by then significant micellar cholesterol has been taken up by intestinal cells both in vitro (CaCo2: Field et al., 1987; Lopez-Candales et al., 1993; rabbit brush border: Thurnhofer & Hauser, 1990) and in vivo. For example, Simmonds has reported that by 3 h in man and 4 h in rat, 37% and 20%, respectively, of a test dose of labeled cholesterol given as a micellar solution was absorbed [Simmonds et al., 1967; Watt & Simmonds, 1981; see also Green and Green (1983) and Wilson and Rudel (1994)].

The cell association (as mean percent uptake ± SD, normalized for cell protein) of FC was similar ($p > 0.5$, $n = 6$) between the Tr (22.4 ± 1.6) and Neo cells (21.4 ± 1.5). The lack of the effects of CEH was also observed with incubation times shorter (1 h) and longer (18 h) than 4 h (data not shown). The possible concentration dependence of these results was next examined by using micelles providing final concentrations of FC of 1 nM and 5 μM. The uptake of FC was measured at the end of the 4 h incubation period. As shown in Table 1, there was no significant difference in the percent uptake of micellar

cholesterol between Tr and Neo cells. In addition, the distribution of cellular cholesterol (FC vs CE) was predominantly (>90%) in the free form with all three types of micelles (1 nM, 5 μM, 50 μM). Overall, in these experiments there was no evidence for a significant effect of CEH on the uptake of cholesterol from micelles containing FC. Furthermore, as in transfected hepatoma cells (Zolfaghari et al., 1993), there was no evidence that CEH was mediating any quantitatively significant esterification of cholesterol.

Effects of the Animal Species and Source (Endogenous vs Exogenous) of CEH on the Uptake of Cholesterol. Previous studies have established species differences in the tissue expression of CEH (Zolfaghari et al., 1992). For example, in the rat, there is only one source of intestinal CEH activity, namely, the enzyme secreted by the pancreas. In contrast, in the rabbit there are two potential sources of intestinal CEH activity; in addition to the exogenous source (the pancreas), there may be an endogenous source (the enterocytes). Besides possible differential effects of the source of enzyme on the association of free cholesterol and the production of intracellular CE, there may also be effects related to the species of origin of CEH. The porcine, human, rat, and bovine enzymes, though broadly similar in their catalytic activities and amino acid sequences, differ in their molecular weights and degree of glycosylation (Wang & Hartsuck, 1993). Also to be noted is that the previous CaCo2-CEH studies (Huang & Hui, 1990; Lopez-Candales et al., 1993) have been in heterologous systems (human cells−nonhuman enzyme).

To test whether these factors influenced our results, additional experiments were performed to test the effects of the animal species and source (endogenous vs exogenous) of enzyme. It has been reported that the concentration of CEH in intestinal fluid is 100 nM (Lopez-Candales et al., 1993). Thus, in the first experiment, a final concentration of 100 nM pancreatic porcine CEH was added to Tr and Neo cells (Tr+, Neo+). After a 10 min period to allow thorough mixing, micelles were added (final concentration of FC = 1 nM), and the incubation was continued for 4 h. By using ANOVA to compare the results to those obtained in parallel experiments in which no exogenous enzyme was added, it was found that the percent of cholesterol associated with either Tr or Neo cells was not significantly affected by the addition of porcine CEH (Figure 3). Interestingly, if only the extremes of CEH activity in the medium were compared (Tr+ vs Neo), there was an indication that the percent cell association was less in the Tr+ group (13.2% ± 0.9 vs 17.0% ± 1.0; $p < 0.05$, $n = 5$). Similar results were obtained by adding human CEH (Figure 4); cell association in the maximal group (Tr+, 11.8% ± 0.8) relative to the minimal group (Neo, 17.4% ± 0.3) was also significantly decreased ($p < 0.05$, $n = 3$).

When the distribution of labeled cholesterol between FC and CE in the cellular lipid extracts was examined, the same pattern was seen for both porcine and human CEH; i.e., most of the cholesterol was in the free form (Figure 5). Along with the differences in percent cell association between the Tr+ and the Neo group shown in Figure 4, there were also differences ($p < 0.05$, $n = 5$) in the absolute amounts (picomoles per milligram of cell protein) of FC and CE in the extracts from these two groups [FC: 0.12 ± 0.0006 (Tr+) vs 0.17 ± 0.0001 (Neo); CE: 0.002 ± 0.0004 (Tr+) vs 0.005 ± 0.0001 (Neo)]. The same trends in the cell association of cholesterol (Tr+ < Neo) persisted when human CEH was

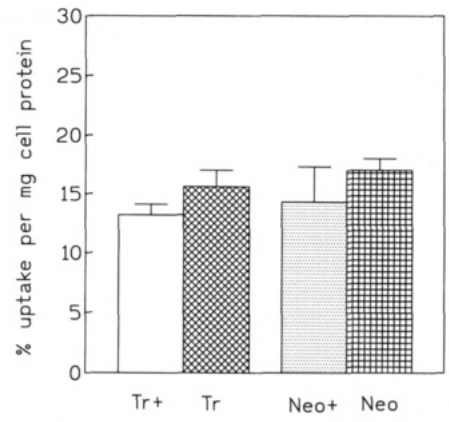

FIGURE 3: Effects of added porcine CEH on uptake by Tr and Neo cells of cholesterol from mixed micelles providing 1 nM FC. A final concentration of 100 nM porcine CEH was added to the media of Tr and Neo cells (Tr+, Neo+). Mixed micelles providing a final FC concentration of 1 nM were then added to the media of Tr+ and Neo+ cells, as well as to the media of Tr and Neo cells not exposed to the porcine CEH. After a 4 h incubation, uptake of labeled cholesterol from the micelles was determined by scintillation counting of extracted cellular lipids. The results for each treatment condition were normalized to cell protein and are expressed as mean percent uptake (±SD), n =5.

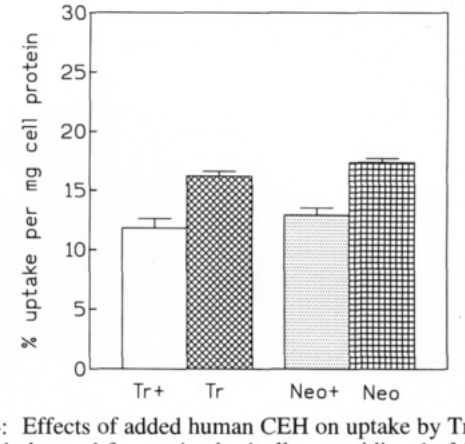

FIGURE 4: Effects of added human CEH on uptake by Tr and Neo cells of cholesterol from mixed micelles providing 1 nM FC. The same experiment as in Figure 3 was performed, substituting human CEH for the porcine enzyme (n = 3).

used in experiments in which the micelles provided FC at a more physiologic concentration (50 μM), though these trends were no longer statistically significant (Figure 6). Overall, our observation that CEH did not promote the cell association of micellar cholesterol persisted, regardless of the species source, mode of addition, or the concentration of the enzyme.

Effects of Changes in the Non-Cholesterol Components of Micelles on the Cell Association of Cholesterol. Because the properties of micelles may vary depending on their composition, we substituted egg PC for MO (Materials and Methods), creating a micelle similar to that employed in another CaCo2 study (Lopez-Candales et al., 1993). When human CEH (100 nM, final concentration) and micelles containing egg PC (1 mM) and FC (1 nM) were added as in the previous section to the medium of Neo cells, the cell-associated CE values were (picomoles per milligram of cell protein) 0.001 ± 0.0002 (Neo+) and 0.00043 ± 0.00001 (Neo). Thus, similar to the previous report (Lopez-Candales et al., 1993), the addition of enzyme increased the cell-associated CE approximately 2-fold in 4 h ($p < 0.01$, n = 3). In contrast, cell-associated FC was essentially identical (picomoles per milligram of cell protein: 0.11 ± 0.0002, Neo+; and 0.11 ± 0.0003, Neo). Given that the FC values

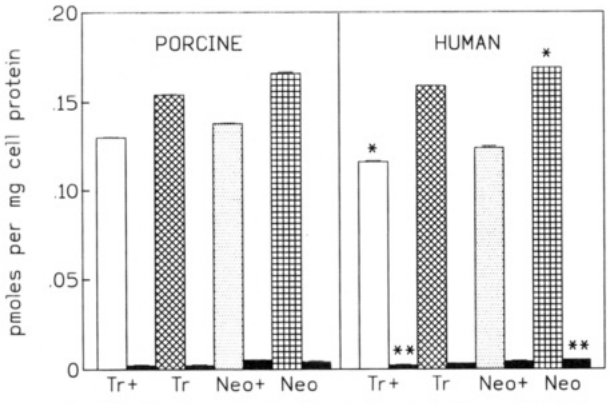

FIGURE 5: Distribution of micellar-derived cholesterol in Tr and Neo cells after incubation with added CEH and mixed micelles providing 1 nM FC. Experiments as in Figures 3 and 4 were performed, with subsequent analysis of the extracted cellular lipids done as in Figure 2. The results were normalized to cell protein and are displayed as mean (±SD), $n = 5$. Open and patterned bars, FC; solid bars, CE. Statistical differences ($p < 0.05$) in the amounts of FC (*) and CE (**) were observed between Tr+ and Neo when 100 nM human CEH was added.

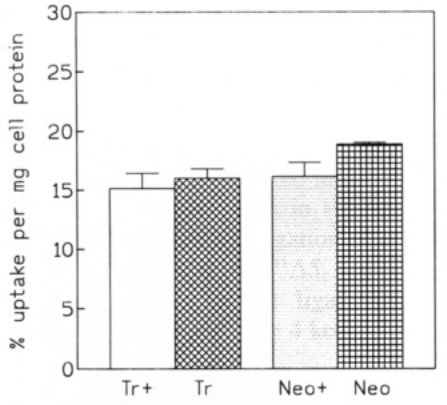

FIGURE 6: Effects of added human CEH on the uptake by Tr and Neo cells of cholesterol from mixed micelles providing 50 μM FC. Experiments as in Figure 4 were performed except that the final concentration of micellar FC was 50 μM ($n = 3$).

were at least 100 times greater than those for CE, the uptake of total cholesterol (FC + CE) by the cells was therefore similar and independent of the presence of CEH.

Note that by comparing results for Neo cells incubated with egg PC micelles providing 1 nM FC (total cell-associated cholesterol of 0.11 pmol/mg of cell protein) to the corresponding data in Table 1 for Neo cells incubated with MO micelles providing 1 nM FC (0.175 pmol/mg of cell protein), there is the suggestion that the latter type of micelles was more effective in donating cholesterol to the cells. The greater effectiveness of MO micelles became even more apparent in experiments using micelles providing FC concentrations more physiologic (50 μM) than 1 nM. As shown in Figure 7, independent of the addition of human CEH, there was significantly more ($p < 0.001$, $n = 3$) cell-associated FC when MO micelles were used. In addition, the statistically significant, though quantitatively negligible, increase (0.00043 vs 0.001 pmol/mg of cell protein) in CE previously observed when control cells were incubated in the presence of 1 nM FC and 100 nM human CEH was no longer observed at a 50 μM concentration of FC, independent of MO and egg PC.

Effects of CEH on the Cell Association of Cholesterol from Micelles Containing both FC and CE. Since in the Western

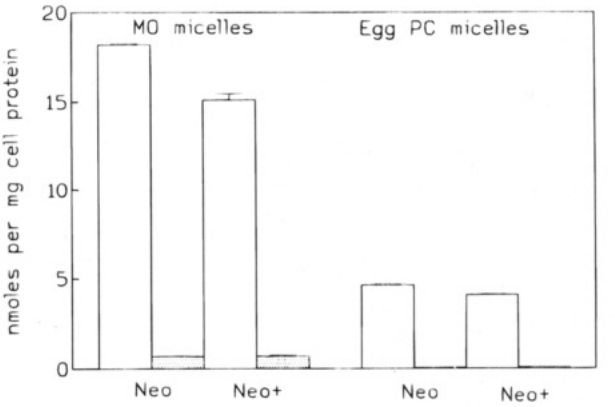

FIGURE 7: Effects of the non-cholesterol composition of mixed micelles on the distribution in Neo and Neo+ cells of micellar-derived cholesterol. Neo and Neo+ cells were treated as in Figure 3, except that the mixed micelles provided 50 μM FC and contained either the MO previously used or egg PC. The subsequent analysis of the extracted cellular lipids was done as in Figure 2. Results are displayed as mean (±SD), $n = 3$. Open bars, FC; dotted bars, CE.

Table 2 : Distribution of Micellar-Derived Cholesterol in Tr and Neo Cells after Incubation with Mixed Micelles Containing both FC and CE[a]

	Tr	Neo
Cell Association of Cholesterol from Micellar FC		
^{3}H-FC	7.53 ± 0.06(*)	9.84 ± 0.03(*)
^{3}H-CE	0.37 ± 0.09	0.56 ± 0.17
Cell Association of Cholesterol from Micellar CE		
^{14}C-FC	1.07 ± 0.007	0.16 ± 0.004
^{14}C-CE	0.07 ± 0.006	0.1 ± 0.004

[a] Tr and Neo cells were incubated for 12 h to allow enzyme accumulation in Tr cell medium. Mixed micelles containing both FC and CE were then added to the conditioned media of Tr and Neo cells to provide a final FC and CE concentration of 50 μM and 5 μM, respectively. The micelles contained unlabeled cholesteryl oleate, [*cholesteryl*-4-14C]cholesteryl oleate, unlabeled FC, and [1,2-3H(N)]-cholesterol. After a 4 h incubation, uptake of labeled cholesterol from the micelles was determined by scintillation counting of extracted cellular lipids. The distribution of cellular cholesterol between FC and CE in the extracts was assayed by ITLC, and the mass of each species was calculated (Materials and Methods). The results for each cell type are given as nanomoles per milligram of cell protein and displayed as mean (±SD). For each cell line, the distribution of cell-associated cholesterol (as FC or CE) is presented for sterol originating from FC (3H-labeled) or from CE (14C-labeled). All comparisons between Tr and Neo cells are significant at $p < 0.0001$, except for (*), where the significance is at a level of $p < 0.005$, $n = 6$.

diet, dietary cholesterol contains both the free (approximately 80−90%) and esterified forms, we tested whether the presence of both forms in the same micelle affected the cell association results. As earlier, Tr and Neo cells were maintained for 12 h to allow enzyme accumulation and then incubated for 4 h with micelles. Sufficient micelles were added so that the final concentrations of their components were 50 μM FC, 5 μM CE (cholesteryl oleate), 1 mM MO, and 6 mM NaTc. As shown in Table 2, total cell-associated cholesterol was not increased in the Tr cells [in fact, there was a small ($p < 0.001$, $n = 6$) decrease, 9.04 ± 0.98 nmol/mg of cell protein vs 10.66 ± 0.33 nmol/mg of cell protein].. As in the earlier results with CE-containing micelles (Figure 2), CEH was associated with an increase in the amount of the cell-associated cholesterol derived from micellar CE, with the majority present in the FC fraction. Also, as before, the hydrolysis of micellar CE by CEH was supported by finding in the conditioned medium of Tr cells that 99.4% ± 0.07 (*n*

= 3) of the ^{14}C-label originating from [*cholesteryl*-4-^{14}C]-cholesteryl oleate was now present in the FC fraction.

DISCUSSION

The major result from this study is that in a human intestinal model cell culture system, CEH played a significant role in the uptake of micellar cholesterol that was initially in the ester, but not free, form. The results indicated that this effect was a consequence of conversion of the CE to FC and free fatty acids by the enzymatic activity either endogenously (Figure 1) or exogenously supplied to the medium, with subsequent uptake of the liberated FC. The inability to promote the cellular uptake of micellar cholesterol already in the free form was independent of FC concentration [from subphysiologic (nonomolar) to micromolar range], the non-sterol composition of the micelles (MO or PC), the species of CEH (rat, pig, man), the source of CEH (endogenously or exogenously supplied), or the amount of CEH in the incubation medium (transfected or transfected supplemented with exogenous CEH).

Comparisons to Other Studies. To our knowledge, there are only three other papers that have used intestinal cell culture systems to investigate the potential for CEH to promote the uptake of FC—one using primary rat enterocytes (Gallo et al., 1977) and two using CaCo2 cells (Huang & Hui, 1990; Lopez-Candales et al., 1993). In Gallo et al. (1977), the ability of rat pancreatic CEH to promote the uptake of micellar FC by freshly isolated rat enterocytes was examined. Final concentrations of FC (250 μM; labeled and unlabeled) and bile salt (5 mM) in the physiological range were used, and cholesterol uptake was determined in the presence or absence of enzyme added to the medium. The added CEH did not increase the uptake of labeled cholesterol. No variations in micellar composition similar to those we made were reported. In the first CaCo2 study, Huang and Hui (1990) added porcine pancreatic CEH to the medium and measured the uptake of labeled cholesterol from micelles containing FC and CE. In agreement with our results, there was an increase in the uptake of labeled cholesterol derived from the micellar CE and not from FC. Relative to our protocols, however, the conditions used by Huang and Hui were unphysiologic: (1) The final concentrations of micellar bile salt and FC were approximately 0.1 mM and 10 nM, respectively, far below the concentrations of bile salt (approximately 10 mM) and micellar FC (in excess of 100 μM) in human intestinal fluid (Hernell et al., 1990). (2) The final concentration of CE was approximately 10 μM, in great molar excess to the FC content. As noted earlier, in intestinal fluid the concentration of FC is significantly greater than that of CE (Hernell et al., 1990). (3) The CaCo2 cells were used shortly after achieving confluency. The differentiation state of CaCo2 cells varies by day in culture, and they do not achieve the maximum intestinal cell phenotype until 10–15 days after confluence (Field et al., 1987; Reisher et al., 1993).

In the second CaCo2 paper, from the laboratory of Louis Lange (Lopez-Candales et al., 1993), the authors report that bovine pancreatic CEH added to the medium promoted the uptake (by a factor of 3) of micellar FC. While the final concentration of bile salt was physiological (8 mM), other components of their micellar system were not: (1) The final concentration of FC was typically 1 nM. (2) The CaCo2 cells were used shortly after becoming confluent. Although we made attempts to replicate some of the conditions of Lopez-Candales et al. (using a final concentration of micellar cholesterol of 1 nM; substituting egg PC for MO, and adding an exogenous source of CEH to the medium), we were still unable to reproduce the promotion of the uptake of free cholesterol. This may have been due either to the relatively undifferentiated state of their cells or to clonal variations among the CaCo2 lines we, Huang and Hui, and Lopez-Candales et al. studied. Related to this is the recent abstract by Bosner (in collaboration with Lange's laboratory; Bosner et al., 1994) reporting a partial purification from CaCo2 cells of a proteoglycan, presumably on the plasma membrane, that binds CEH. This protein, perhaps transiently expressed in their CaCo2 clone prior to differentiation, may have contributed to their results. Alternatively, the discrepancy may involve a species-specific property of CEH. Both Huang and Hui and we used porcine CEH, and we also had available the rat and human enzymes, whereas Lopez-Candales et al. reported results with only the bovine enzyme. There are many variations in both the coding sequences and the degree of posttranslational modifications among the mammalian CEH's (Wang & Hartsuck, 1993), and these may have functional consequences.

Broadly, then, there are two major features that clearly distinguish the present from the other cell culture studies: (1) the use of more physiologically relevant conditions and/or extensive variation of experimental parameters, and (2) the reporting of results obtained with the system most relevant to human metabolism—human cells with human enzyme. Despite these strengths, comparison of our results in vitro to those obtained in more intact models would provide a valuable context for interpretation. To our knowledge, unfortunately, there are no relevant human clinical studies, but there are reports of animal experiments on the role of CEH in cholesterol absorption. In an early study from Vahouny and colleagues (Borja et al., 1964), rats were surgically treated to divert pancreatic, biliary, or both types of secretions, and free cholesterol absorption was measured after intestinal infusions containing pancreatic juice, bile, or both. Appreciable absorption of cholesterol was shown in the absence of pancreatic juice, provided bile salts were present. This did not support a direct role for the CEH in pancreatic juice in cholesterol absorption. In a more recent study (Gallo et al., 1984), pancreatic juice was surgically diverted, and juice immunodepleted of CEH or normal juice was infused. The free cholesterol absorption in the CEH-depleted group was less: in the CEH-depleted group, the micromoles of FC + CE in intestinal lymph after a test meal was approximately 31% of control (their Table 4: 2.8 vs 9.0). The intestinal lymph flow during the experiment, however, was also significantly reduced in the CEH-depleted group (8.2 mL vs 23.2 mL, or 35% of control), indicating that the immunodepleted infusate may have had nonspecific effects on intestinal function. Watt and Simmonds (1981) have also studied the role of CEH in free cholesterol absorption in surgically diverted rats. Using animals having intestinal lymph fistulae with either biliary or pancreaticobiliary fistulae, cholesterol absorption was independent of pancreatic flow. Similar to Borja et al. (1964), they concluded that the primary determinant of cholesterol absorption was the bile salt supply. Overall, we interpret the bulk of the animal data to be consistent with our results in vitro; i.e., CEH did not significantly promote the uptake of free cholesterol.

Concluding Remarks. A simple model for the role of CEH in the absorption of dietary cholesterol is that it catalyzes CE hydrolysis, increasing the free cholesterol concentration gradient between the micellar and plasma membrane pools and enhancing the passive cellular uptake of free cholesterol. More complex models hypothesize additional ways in which cholesterol flux across the enterocyte can be promoted by CEH, for example, by its functioning either as a carrier for free and/or esterified cholesterol (for example, Hui & Huang, 1989; Lopez-Candales et al., 1993) or as an intracellular esterifying enzyme (Gallo et al., 1984; Rudd & Brockman, 1984). Our results do not support the carrier hypothesis. For example, the uptake of micellar FC was unaffected by the presence of CEH, in spite of numerous variations in the experimental parameters. When data from experiments employing micelles containing either FC or CE are compared, there was also no evidence to support a carrier role for the direct uptake of CE. By interpolation (Table 1), with a micelle providing a final concentration of 10 μM FC, the uptake of total cholesterol would be about 4.8 nmol (mg of cell protein)$^{-1}$ (4 h)$^{-1}$. From micelles providing a final concentration of 10 μM CE (Figure 2), the actual measured cholesterol uptake by Tr cells was 4.6 nmol (mg of cell protein)$^{-1}$ (4 h)$^{-1}$. Given the >99% conversion of the labeled CE to FC observed in Tr cell medium, consistent with the presence of abundant CEH activity (at least 21.2 units/mg of cell protein, Figure 1), the uptake of cholesterol from the CE-containing micelle could be completely explained by the hydrolytic release of free cholesterol and its subsequent uptake by passive diffusion down its concentration gradient into the cell. Our results also did not support the second hypothetical mechanism (intracellular esterification). In general, there were no quantitatively significant differences in the fraction of the cholesterol taken up that was esterified between cells with and without CEH.

In summary, the role in dietary cholesterol absorption of CEH best supported by our data is its ability to hydrolyze CE in the intestinal lumen. This is consistent with the observations by Shiratori and Goodman (1964) that in the intestinal lumen virtually complete hydrolysis of cholesteryl esters occurs before cholesterol absorption and by Vahouny and Treadwell (1958) that the absorptions of several species of cholesteryl esters were related to their relative rates of hydrolysis by CEH. Because of inherent limitations of both the cell culture and animal systems used to date, there may well be other effects of CEH on cholesterol absorption, but additional research, perhaps utilizing new experimental models, will be needed to evaluate this possibility.

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