Common mechanisms of monoacylglycerol and fatty acid uptake by human intestinal Caco-2 cells

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Ho, Shiu-Ying, and Judith Storch. Common mechanisms of monoacylglycerol and fatty acid uptake by human intestinal Caco-2 cells. Am J Physiol Cell Physiol 281:C1106–C1117, 2001.—Free fatty acids (FFA) and sn-2 monoacylglycerol (sn-2-MG), the two hydrolysis products of dietary triacylglycerol, are absorbed from the lumen into polarized enterocytes that line the small intestine. Intensive studies regarding FFA transport across the brush-border membrane of the enterocyte are available; however, little is known about sn-2-MG transport. We therefore studied the kinetics of sn-2-MG transport, compared with those of long-chain FFA (LCFA), by human intestinal Caco-2 cells. To mimic postprandial luminal and plasma environments, we examined the uptake of taurocholate-mixed lipids and albumin-bound lipids at the apical (AP) and basolateral (BL) surfaces of Caco-2 cells, respectively. The results demonstrate that the uptake of sn-2-monoolein at both the AP and BL membranes appears to be a saturable function of the monomer concentration of sn-2-monoolein. Furthermore, trypsin preincubation inhibits sn-2-monoolein uptake at both AP and BL poles of cells. These results suggest that sn-2-monoolein uptake may be a protein-mediated process. Competition studies also support a protein-mediated mechanism and indicate that LCFA and LCMG may compete through the same membrane protein(s) at the AP surface of Caco-2 cells. The plasma membrane fatty acid-binding protein (FABP<sub>pm</sub>) is known to be expressed in Caco-2, and here we demonstrate that fatty acid transport protein (FATP) is also expressed. These putative plasma membrane LCFA transporters may be involved in the uptake of sn-2-monoolein into Caco-2 cells.

Albumin; taurocholate; fatty acid transport protein

Small intestinal epithelium contains morphologically and functionally polarized enterocytes that play a central role in lipid absorption. The average Western diet is composed of ~100 g of lipids per day, and >90% of dietary fat is triacylglycerol (TG) (54). Free fatty acids (FFA) and sn-2-monoacylglycerol (sn-2-MG) are the major products of pancreatic lipase hydrolysis of dietary TG (4). After digestion, these lipolytic products are dispersed as vesicles and bile salt-mixed micelles and functionally polarized enterocytes that play a central role in lipid absorption. The average Western diet is composed of ~100 g of lipids per day, and >90% of dietary fat is triacylglycerol (TG) (54). Free fatty acids (FFA) and sn-2-monoacylglycerol (sn-2-MG) are the major products of pancreatic lipase hydrolysis of dietary TG (4). After digestion, these lipolytic products are dispersed as vesicles and bile salt-mixed micelles and taken up by human intestinal Caco-2 cells. To mimic postprandial luminal and plasma environments, we examined the uptake of taurocholate-mixed lipids and albumin-bound lipids at the apical (AP) and basolateral (BL) surfaces of Caco-2 cells, respectively. The results demonstrate that the uptake of sn-2-monoolein at both the AP and BL membranes appears to be a saturable function of the monomer concentration of sn-2-monoolein. Furthermore, trypsin preincubation inhibits sn-2-monoolein uptake at both AP and BL poles of cells. These results suggest that sn-2-monoolein uptake may be a protein-mediated process. Competition studies also support a protein-mediated mechanism and indicate that LCFA and LCMG may compete through the same membrane protein(s) at the AP surface of Caco-2 cells. The plasma membrane fatty acid-binding protein (FABP<sub>pm</sub>) is known to be expressed in Caco-2, and here we demonstrate that fatty acid transport protein (FATP) is also expressed. These putative plasma membrane LCFA transporters may be involved in the uptake of sn-2-monoolein into Caco-2 cells.

Albumin; taurocholate; fatty acid transport protein
ularly (10, 22, 52). However, Caco-2 cells have a num-
ber of traits that do not resemble those of intestinal
absorptive cells: Caco-2 cells synthesize primarily apo-
lipoprotein B (apoB)-100, while normal human intesti-
te produces apoB48 (20); Caco-2 cells accumulate glycogen; Caco-2 cells possess some characteristics of fetal cells and colonic crypt cells; Caco-2 cells express lower levels of monoacylglycerol acyltransferase (MGAT) activity than do jejunal cells (53); and Caco-2 cells express only 10% of the I-FABP protein level of rat enterocytes (10). Nevertheless, Caco-2 cells remain the most well-characterized human intestinal cell line with respect to lipid metabolism and, on balance, per-
form most of the functions associated with intestinal lipid absorption, transport, and metabolism (23). Be-
cause Caco-2 cells express low MGAT activity (53), sn-2-MGs may not be rapidly metabolized immediately after entering the cells, assuming there are no alter-
native pathways. Therefore, Caco-2 cells should be a good model in which to study the transmembrane trans-
port of sn-2-MG, where uptake and metabolism are more readily distinguished.

There are two mechanisms that have been proposed for LCFA transport across the AP membrane of the enterocyte: passive diffusion through the lipid bilayer (21, 32) and carrier-mediated transport (1, 2, 38, 43, 46). Arguments for facilitated LCFA transport include (21, 32) and carrier-mediated transport (1, 2, 38, 43,

2-MG, where uptake and metabolism

Materials and Methods

Materials. Polycarbonate Transwell filter inserts (6.5-mm
diameter, 0.4-μm pore) were purchased from Costar (Cam-
bridge, MA). Tritium-labeled triolein, oleate, and palmitate
(9,10-3H) were from NEN (Boston, MA). Unlabeled fatty
acids and monoacylglycerols were obtained from Nu-Chek
Prep (Elysian, MN) and Serdary Research Lab (Englewood
Cliffs, NJ), respectively. BSA (essentially FFA free), trypsin,
pronase, Triton X-100, and pancreatic lipase (type VI) were
obtained from Sigma (St. Louis, MO). Sodium taurocholate
(TC) was purchased from Calbiochem (La Jolla, CA). Polyvi-
nylidene difluoride (PVDF) Immobilon-P plotting membranes
were from Millipore (Bedford, MA). The YM2 ultrafiltration
membranes were purchased from Amicon (Beverly, MA).
Dulbecco’s modified Eagle’s medium (DMEM), nonessential
amino acids, fetal bovine serum (FBS), penicillin, streptom-
cin, trypsin-EDTA, and oligo(dT)18 were from Life Technolo-
gies (Grand Island, NY). The sodium borate (3%)-coated
preparative thin-layer chromatography (TLC) plates were
obtained from Analtech (Newark, DE). Human monoclonal
CD36 antibody was purchased from Alexis (San Diego, CA).
The simian virus (SV) total RNA isolation kit and avian
myeloblastosis virus (AMV) reverse transcriptase were ob-
tained from Promega (Madison, WI). Primers for RT-PCR
were made by Genosys (Woodlands, TX).

Cell culture. Caco-2 cell cultures were obtained from Amer-
ican Type Culture Collection and were grown in DMEM with
4.5 g/l glucose, 4 mM glutamine, 100 U/ml penicillin, 100
mg/ml streptomycin, 1% nonessential amino acids, and 20% FBS in a 95% air-5% CO2 atmosphere at 37°C, as described
previously (23). The medium was changed every other day.
Cells were plated at a density 1×10^6 cells/cm^2 in 75-cm^2 flasks
and split with 0.25% trypsin-1 mM EDTA when they reached
70–90% confluence, as previously described (51, 52). For
experiments, cells were plated at a density of 3×10^5 cells/
cm^2 onto 6.5-mm polycarbonate Transwell filters. Cells with
4×10^4 cells/cm^2 on 6.5-mm polycarbonate Transwell filters,
or at 10^4 cells/cm^2 onto 12-mm-diam-
eter glass coverslips, which were placed in 24-well tissue
culture plates. Cells were grown to 14–18 days postconflu-
ence for the experiments. Transepithelial resistance (TER)
measurements were made to ensure tight junction formation,
and only monolayers with TER 250 Ω-cm^2 were used for
experiments (52). Most experiments were done with cells
grown on the filters; however, a few AP uptake experiments
were done with cells grown on glass coverslips; the AP uptake
results from these two conditions were similar.

Preparation of radiolabeled sn-2-MG. Because there is no
commercially available radiolabeled sn-2-MG, and because
the isomerization of monoacylglycerides between sn-1 and
sn-2 positions is likely to occur by acid, alkali, or heat (28),
[3H]sn-2-monoolein was freshly prepared before each exper-
iment. [3H]triolein (specific activity: 28 Ci/mmol) that has 3H
labeled on the double bond of all acyl chains was used as
substrate. [3H]sn-2–18:1 was prepared by digestion of radio-
labeled triolein with pancreatic lipase (type VI) (Sigma) at
37°C for 2 h, followed by 3% sodium borate-coated prepara-
tive TLC separation (27). The [3H]sn-2-monoolein was pre-
pared freshly before each uptake experiment to minimize the
possibility of acyl group isomerization between sn-1 and
sn-2 positions and was always used within 2 wk. We found that
<10% of the [3H]sn-2-monoolein migrated as [3H]sn-1-mono-
olein after 1 mo of storage at −20°C.

Determination of critical micellar concentrations of sn-2-
monoolein. The critical micellar concentration (CMC) of
[3H]sn-2-monoolein in phosphate-buffered saline (PBS; 137
mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM
NaHPO4, pH 7.4) was determined at room temperature by
ultrafiltration with the use of a stirred ultrafiltration cell
(Amicon) with Amicon YM2 filters [exclusion limit: relative
molecular weight (Mr) of 1,000] (39, 50). Briefly, solutions
and dispersions of [3H]labeled lipids in PBS were filtered
through the Amicon ultrafiltration cell, and the radioactivi-
ties of the filtrate and of the dispersion retained on the filter
were determined. It is worth noting that the 1,000 Mz cut-off
filter would retain dimolecular as well as polymolecular mo-
noleoin aggregates; thus this method determines an appar-
ent CMC, which indicates the concentration at which mono-

Preparation of uptake media: TC-bound lipids. Radiola-
labeled oleic acid (18:1), palmitic acid (16:0), and sn-2-mono-
olein (sn-2–18:1) were dried under N2. The dried lipids were
then dissolved in ethanol (0.5% volume in final volume) and
subsequently dispersed in 10 mM TC (typical luminal bile
salt level) (9) in PBS to a concentration of 9 mM LCFA/MG and incubated for 1 h at 37°C with 90-rpm shaking to obtain an optically clear solution. Serial dilutions of TC-mixed lipids were then made by adding desired volumes of 10 mM TC in PBS to obtain total LCFA/MG concentrations of 25–8,000 μM. The specific activities of the uptake solutions were 0.5 μCi/nmol for each ligand. The monomer concentrations of LCFA in TC mixtures were determined using the fluorescent probe ADIFAB (Molecular Probes, Eugene, OR) (37) and the ultrafiltration method described by Tranchant et al. (50) and Schulthess et al. (39). The monomer concentrations of sn-2–18:1 were determined only by the ultrafiltration method because ADIFAB does not bind MG.

Preparation of uptake media: BSA-bound lipids. Radiolabeled 18:1, 16:0, and sn-2–18:1 were dried under N₂. The dried lipids were dissolved in 0.5% (vol/vol) ethanol relative to BSA in PBS to a concentration of 9 mM BSA (typical plasma level) (3) in PBS, pH 7.4. Media were incubated for 1 h at 37°C with 90-rpm shaking to obtain an optically clear solution. Serial dilutions of BSA-bound lipids were made by adding desired volumes of 100 μM BSA in PBS to obtain total LCFA/MG concentrations of 25–1,800 μM. The specific activities of the uptake solutions were 0.5 μCi/nmol for each ligand. The monomer concentrations of LCFA bound to BSA were calculated from the equilibrium binding constants as reported by Spector et al. (44) as well as those reported by Richieri and Kleinfeld (35), and the monomer concentration of sn-2–18:1 bound to BSA was calculated from the binding constant determined by Thumser et al. (49).

LCFA and MG uptake assay. Initial rates of uptake of TC-mixed lipids at the AP membrane as well as the initial rates of uptake of BSA-bound lipids at the BL membrane were determined as previously described (51). Briefly, the cells were washed with PBS twice, and then the uptake medium for either side was added to the cells. After designated times of incubation, the uptake medium was rapidly aspirated off, and the filter insert was immediately dipped into an ice-cold 0.5% BSA solution (“stop” solution) to stop cellular uptake and remove surface-bound lipid. The cells were then washed with ice-cold stop solution two more times, followed by washing with ice-cold PBS three times, and then were homogenized into 0.05% Triton X-100 solution (51). Protein content (6) and radioactivity were determined after sonication of cells for 15 s with a Branson sonifier equipped with a microtip (Danbury, CT). Initial rates of uptake were determined over a range of ligand concentrations to obtain the apparent Michaelis-Menten constant (Kₘ) and maximum velocity (Vₘₕₘₚₙₖ) of uptake, as described previously (51). Because the levels of “donor” lipid are far exceeded by levels of “acceptor” (i.e., cell phospholipid), it was assumed that unidirectional uptake was being monitored, rather than exchange.

Competition studies. For competition studies, excess unlabeled lipids were added to radiolabeled BSA-bound lipids, and the uptake assays were performed as described above. The monomer concentration for each concentration of lipid used in these studies was calculated as described above and was always below the corresponding CMC value (36).

Protease studies. The effect of trypsin on sn-2–18:1, 18:1, and 16:0 uptake in Caco-2 cells was determined. As previously found, the AP surface remained intact at higher protease concentrations than did the BL surface (51); therefore, the protease studies were performed with 15-min preincubation with trypsin at 0.5 mg/ml at the AP surface and 0.05 mg/ml at the BL surface. The cells were then washed, the TER was measured to ensure the integrity of the tight junctions, and the uptake experiments were performed within 5 min.

mRNA determination. RT-PCR was used to evaluate the presence of mRNA transcripts for the membrane transporters fatty acid translocase (FAT/CD36) and fatty acid transport protein (FATP) in Caco-2. All determinations were done in triplicate, at minimum. RNA from rat adipose tissue was used as a positive control for both transcripts. RNA was prepared from Caco-2 cells grown in 75-cm² flasks at designated times postconfluence, as indicated. Total RNA was extracted from cells by using the SV total RNA isolation kit from Promega. The first-strand DNA synthesis was performed at 42°C for 1 h, followed by 94°C for 10 min for enzyme inactivation. PCR was then carried out by using primers designed on the basis of a human intestinal form of FAT, hsFATP₄ (13), as follows: 5’ GTGTC'TGTCTCCACAGTGC' TTACG '3’ (nt 31–60) and 5’ AGATAACACCAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The primers for FAT/CD36 were designed using the cDNA of human CD36 (55) as follows: 5’ CCAAGAGTGTCAAGAACAGGCTTCTCTCAATAGCCG ‘3’ (nt 31–60) and 5’ AGATAGGCTTCTCTCAATAGCCG ‘3’ (nt –1849 to –1820). The pr
Results

Determination of CMC values for sn-2-monoolein. The CMC of $[^3]$H-sn-2-monoolein was determined using ultrafiltration (39, 50). When the sn-2-monoolein concentration was below its CMC, the radioactivity ratio of the filtrate and the solution in the filtration cell was nearly 1:1. After the concentration of $[^3]$H-labeled lipid in PBS reached its CMC, the radioactivity ratio was dramatically decreased. The midpoint of this linear break was estimated as its CMC, as shown in Fig. 1. The average CMC value obtained for sn-2-monoolein was 4.2 ± 0.5 μM. The CMC values for oleic acid (6.0 μM) and palmitic acid (4.0 μM) were obtained by assuming that the $K_m$ of the radioactive ligand was unchanged upon addition of competitive ligands.

Determination of initial rates of lipid uptake by Caco-2. Initial rates of uptake of AP TC-bound lipids as well as the initial rates of BL BSA-bound lipid uptake were determined as described in Materials and Methods. The AP uptake of TC-mixed lipids as well as BL uptake of BSA-bound lipids was found to be a linear function of time within 20 s (Fig. 2, A and B). Therefore, the kinetic parameters of uptake were determined using the 10-s point for various concentrations of lipids, to ensure initial rate conditions.

Kinetic studies of TC-mixed lipid uptake at the AP surface. To mimic the postprandial intestine, we used bile salt micellar solutions for AP uptake studies. The monomer concentrations of 18:1, 16:0, and sn-2–18:1 in TC-mixed micelles were obtained by using the ADIFAB probe method and/or the filtration method, as described in Materials and Methods. Because ADIFAB is very sensitive to even trace amounts of ethanol (53), the unbound concentrations of LCFA in TC micelles were measured in an ethanol-free environment, as reported (53). The unbound concentrations of LCFA obtained from ADIFAB and from the filtration method were consistently similar (data not shown).

AP uptakes of TC-mixed 18:1, 16:0, and sn-2–18:1 AP uptake were plotted as a function of unbound (monomer) concentration of lipid (Fig. 3A). The results show that AP uptake of TC-mixed sn-2–18:1, 18:1, and 16:0 appears to be a saturable function of monomer lipid concentration. This finding suggests that uptake of both sn-2–18:1 and LCFA at the AP surface of the Caco-2 occurs by a facilitated transport process. Woolf plots, which display the lipid monomer concentration [S] on the x-axis and [S]/V (uptake velocity) on the y-axis, were constructed from the data to determine the apparent $K_m$ and $V_{\text{max}}$ values, as described previously (51). The results in Table 1 demonstrate that for AP uptake of TC-mixed lipids, 18:1 has the highest $V_{\text{max}}$ (18,276 ± 3,764 pmol·mg⁻¹·10 s⁻¹) followed by 16:0 (9,854 ± 2,955 pmol·mg⁻¹·10 s⁻¹) and then sn-2–18:1 (7,319 ± 2,897 pmol·mg⁻¹·10 s⁻¹). The $K_m$ for 18:1 (0.15 ± 0.06 μM) was slightly higher than that for sn-2–18:1 (0.03 ± 0.01 μM), suggesting a somewhat higher apparent affinity for sn-2–18:1 relative to 18:1. The average $V_{\text{max}}$ of sn-2–18:1 at the AP surface was approximately one-half that of 18:1, which, interestingly, fits the stoichiometric ratio of FFA vs. MG after TG hydrolysis by pancreatic TG lipase in the lumen of the intestine. In all experiments, the $V_{\text{max}}$ of 18:1 uptake was greater than that of 16:0 ($P < 0.01$).

Kinetic studies of BSA-bound lipid uptake at BL and AP surfaces. To model plasma LCFA and MG uptake by enterocytes, we used BSA-bound lipids for BL surface uptake studies. Uptake of BSA-bound lipids across the BL surface was determined, and the results were
and 18:1 were more than threefold higher than those at the BL surface, suggesting that, indeed, the AP membrane of Caco-2 has a higher intrinsic capacity for LCFA uptake than the BL membrane (Table 1), although clearly not orders of magnitude different. In contrast to values for LCFA, the similar \( V_{\text{max}} \) and \( K_m \) values for BSA-bound sn-2–18:1 at both the AP and BL membranes suggest a similar uptake capacity for sn-2–18:1 at both poles of the cell.

The very large increase in the net uptake of TC-mixed lipids relative to BSA-bound lipids was further addressed. Because bile salts act as detergents and may have deleterious effects on cell membranes when the concentration exceeds their CMC, we measured the initial rate of uptake of LCFA with or without preincubation with 10 mM TC for 10 s, and the uptake rates were not affected (data not shown). Furthermore, we measured the TER of Caco-2 monolayers after incubation with 10 mM TC for 10 s as a further control for monolayer integrity. The results demonstrated that the TER of Caco-2 remained above 250 \( \Omega \cdot \text{cm}^2 \), indicating that the tight junctions remained intact. Indeed, others have reported no cell loss and minimal leakage (<1.5%), as checked by a low-molecular-weight probe, after 5 h of incubation with a 12 mM TC micellar solution in Caco-2 (33). Therefore, the dramatic increase in \( V_{\text{max}} \) for lipid uptake from a 10 mM TC micellar solution relative to BSA-bound lipids does not appear to be due to damage of the plasma membrane or of monolayer integrity by high concentrations of TC.

In recent years, Kleinfeld and coworkers have developed a new approach for determining unbound FFA concentrations, and a different set of LCFA binding affinities for BSA has emerged from the use of this method (35). The ADIFAB method is a sensitive fluorescence-based assay that relies on an emission wavelength shift between ligand-free and ligand-bound forms of the ADIFAB probe. The major difference between use of the older BSA \( K_d \) values and the newer, lower \( K_d \) values obtained with ADIFAB is that the calculated unbound concentrations of LCFA fall into the micromolar range for the former and the nanomolar range for the latter when the same total concentrations of BSA and LCFA are used. Thus when we reconstructed the uptake data using the \( K_d \) values obtained with ADIFAB (34), saturable kinetics appeared to occur when the unbound concentration of LCFA was below 15 nM. At higher concentrations, a linear relationship between uptake velocity and unbound LCFA concentration was observed (Fig. 4). With the use of such unbound concentrations of LCFA, the calculated \( K_m \) and \( V_{\text{max}} \) values obtained for the saturable portion were 3.95 \( \pm \) 0.45 nM and 20.0 \( \pm \) 3.28 pmol-mg\(^{-1}\)-10 s\(^{-1}\) for 18:1 and 6.38 \( \pm \) 0.23 nM and 90.19 \( \pm \) 1.04 pmol-mg\(^{-1}\)-10 s\(^{-1}\) for 16:0. These observations suggest that both carrier-mediated and passive diffusion mechanisms for LCFA uptake by Caco-2 cells coexist.

**Protease studies.** To determine whether protease-sensitive membrane proteins were involved in the saturable kinetics of uptake of LCFA and MG, we preincubated Caco-2 cells with trypsin and performed...
uptake studies within 5 min after incubation. The results show that preincubation with trypsin at the AP surface and at the BL surface inhibits the uptake of TC-mixed 18:1, 16:0, and sn-2–18:1 by 20–40% compared with control cells (Table 2). Trypsin preincubation also inhibited BSA-bound sn-2–18:1 uptake at both AP and BL membranes by 11% and 30%, respectively. These results support the hypothesis that membrane proteins may be involved in the uptake of LCFA and sn-2–18:1 across the AP and BL surfaces of the Caco-2 cell.

**Competitive inhibition studies.** To further explore the saturable nature of LCFA and MG transport in Caco-2 cells, we conducted studies to determine whether competitive inhibition of uptake occurred. The AP uptake of radioactive 18:1 or sn-2–18:1 (monomer concentrations of 0.01 and 0.02 μM, respectively) was measured in the presence of increasing concentrations of unlabeled 18:1 and MG, with total concentrations always below their CMC. Figure 5A shows that the uptake of radiolabeled 18:1 was competitively inhibited by excess unlabeled 18:1, as well as by LCMG [sn-2–18:1 and sn-2-monopalmitin (sn-2–16:0)] but not by medium-chain MG [sn-2-caprin (sn-2–10:0)], in a dose-dependent manner. Similarly, Fig. 5B shows that the uptake of [3H]sn-2-monoolein was competitively
inhibited by the addition of excess unlabeled LCFA (18:1) and MG (sn-1–18:1, sn-2–18:1, and sn-2–16:0) but not by medium-chain FFA [capric acid (10:0)] or medium-chain MG [sn-2-caprin (sn-2–10:0)]. These competition studies further suggest that LCFA and LCMG uptake is, at least in part, protein mediated. Furthermore, they imply that LCFA and LCMG may compete for the same protein(s) on the AP membrane of Caco-2. Figure 6 shows the experimental (solid line) and theoretical (dashed line) competitive inhibition curves for sn-2–18:1. To obtain a measure of relative inhibition of uptake, we assumed that the $K_m$ of BSA-bound $[3H]$sn-2–18:1 was unchanged upon addition of competitive ligands. The theoretical values, which were calculated on the basis of competitive kinetics, assuming the apparent $K_m$ equals the $K_i$ (40), are very similar to those obtained experimentally. In this case, the calculated $K_i$ values for sn-1–18:1, sn-2–16:0, and 18:1 were similar, in the range of 0.2 μM. In addition, the calculated $K_i$ values for sn-2–18:1 and sn-2–16:0 inhibition of $[3H]18:1$ uptake were 0.16 ± 0.04 and 0.17 ± 0.02 μM, respectively (Table 3). The $K_i$ values obtained for these ligands suggest similar competitive kinetics for LCFA and sn-2–18:1.

Transcript and protein expression of candidate membrane transport proteins. We previously reported that Caco-2 cells express the 43-kDa plasma membrane fatty acid binding protein (FABPpm), though preincubation with anti-FABPpm antibodies did not inhibit LCFA uptake (51). At that time, FABPpm was the only putative transmembrane transporter known to be present in the intestine. It is now thought that several membrane transporters, which may also participate in LCFA uptake, may coexist in Caco-2 cells. Therefore, blocking only one membrane transporter may not substantially inhibit LCFA uptake. FATP and FAT/CD36 are putative LCFA transporters that are expressed in various tissues, including intestine (13, 31). In this

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ligand</th>
<th>TC (AP)</th>
<th>BSA (BL)</th>
<th>BSA (AP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$, pmol·mg$^{-1}·10^9$ s$^{-1}$</td>
<td>sn-2–18:1</td>
<td>7,319 ± 2,897</td>
<td>21.8 ± 5.8*</td>
<td>17.4 ± 5.8*</td>
</tr>
<tr>
<td>18:1</td>
<td>18,276 ± 7,665‡</td>
<td>29.3 ± 9.2*</td>
<td>100.7 ± 31.9††</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>9,854 ± 2,955§</td>
<td>85.6 ± 7.4*‡§</td>
<td>298.4 ± 40.6*‡§</td>
<td></td>
</tr>
<tr>
<td>$K_m$, μM</td>
<td>sn-2–18:1</td>
<td>0.03 ± 0.01</td>
<td>0.35 ± 0.11*</td>
<td>0.33 ± 0.14*</td>
</tr>
<tr>
<td>18:1</td>
<td>0.15 ± 0.06§</td>
<td>0.23 ± 0.13</td>
<td>0.58 ± 0.05*‡</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>0.06 ± 0.01</td>
<td>0.21 ± 0.04*</td>
<td>0.48 ± 0.03†</td>
<td></td>
</tr>
</tbody>
</table>

Cells were grown either on glass coverslips or on polycarbonate filters for uptake experiments as described. Monomer concentrations of ligand in taurocholate (TC)-mixed lipids were measured by ADIFAB or filtration, as described in MATERIALS AND METHODS. Monomer concentrations of BSA-bound long-chain fatty acids (LCFA) were derived from dissociation constants ($K_d$) reported by Spector et al. (44). Monomer concentrations of BSA-bound monoacylglycerols (MG) were derived from $K_d$ values reported by Thumser et al. (49). Maximum velocity ($V_{max}$) and Michaelis-Menten constant ($K_m$) values were obtained from Woolf plots as described previously (51). LCMG, long-chain monoacylglycerols; AP, apical surface; BL, basolateral surface. Data are expressed as means ± SD from at least 5 separate experiments. *P < 0.05 vs. TC (AP). †P < 0.05 vs. BSA (BL). ‡P < 0.05 vs. sn-2–18:1. §P < 0.05 vs. 18:1.

Fig. 4. Apical uptake of BSA-bound-LCFA by Caco-2 cells as a function of monomer concentration using $K_d$ values reported by Richieri and Kleinfeld (35). The calculated apparent Michaelis-Menten constant ($K_m$) values for 18:1 (●) and 16:0 (○) are 3.95 ± 0.45 and 6.38 ± 0.23 nM, respectively. Results are means ± SD ($n = 3$) from a representative of 3 experiments. Inset: uptake data for unbound LCFA concentrations of ≤17 nM.
study, we determined the mRNA expression of FATP and FAT/CD36 in Caco-2 cells using RT-PCR. The results show that Caco-2 cells do express the human intestinal FATP (hsFATP4) (Fig. 7); however, no detectable FAT/CD36 was found at either the mRNA or protein level (data not shown).

**DISCUSSION**

The enterocytes of the small intestine are responsible for uptake of the hydrolysis products of dietary TG, FFA, and sn-2-MG. In the present studies, the kinetics of sn-2-MG as well as LCFA at the both AP and BL surfaces were evaluated using human intestinal Caco-2 cells as an enterocyte model. To ensure that all uptake studies were performed using monomeric lipid, we measured the CMC of sn-2–18:1, since it has not been previously reported (Fig. 1). The result obtained for sn-2–18:1 (4.2 ± 0.5 μM) is in the same range as that reported for 1-O-hexadecylglycerol (2.8 ± 0.3 μM) by Schulthess et al. (39) and those reported for 18:1 (6

Table 2. Effect of trypsin on sn-2-monoolein, oleic acid, and palmitic acid uptake by filter-grown Caco-2 cells

<table>
<thead>
<tr>
<th>Ligands</th>
<th>AP, %control</th>
<th>BL, %control</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-sn-2–18:1</td>
<td>88.7 ± 11.1</td>
<td>70.3 ± 5.7a</td>
</tr>
<tr>
<td>TC-sn-2–18:1</td>
<td>56.7 ± 5.1†</td>
<td>61.0 ± 10.0a</td>
</tr>
<tr>
<td>TC–18:1</td>
<td>77.4 ± 2.6a</td>
<td>74.4 ± 5.7a</td>
</tr>
<tr>
<td>TC–16:0</td>
<td>82.0 ± 7.3a</td>
<td>81.4 ± 3.4a</td>
</tr>
</tbody>
</table>

Filter-grown cells were pretreated with trypsin (0.5 mg/ml AP and 0.05 mg/ml BL) for 15 min under conditions in which the transepithelial resistance (TER) was not affected, as described in MATERIALS AND METHODS. TC-mixed or BSA-bound lipid uptake was subsequently determined within 5 min at an sn-2–18:1-to-BSA ratio of 1:1 or a lipid-to-TC ratio of 1:5. sn-2–18:1, sn-2-Monoolein; 18:1, oleic acid; 16:0, palmitic acid. Data are expressed as %uptake compared with that of control groups that were not pretreated with trypsin. Values are means ± SD from 3 separate experiments. *P < 0.05 vs. control (100%). †P < 0.05 vs. AP (BSA-sn-2–18:1).

A

![Graph A](https://example.com/graphA.png)

**A**: radioactive 18:1 uptake: competition by unlabeled free fatty acids (FFA) and monoacylglycerols (MG). sn-2–16:0, sn-2-Monopalmitin; sn-2–10:0, sn-2-monocaprin; 1× cold competitors = 0.02 μM unbound lipid concentration.

B

![Graph B](https://example.com/graphB.png)

**B**: radioactive sn-2–18:1 uptake: competition by unlabeled FFA and MG. 1× cold competitors = 0.03 μM unbound lipid concentration. Results are means ± SD (n = 3) from a representative of 3 separate experiments.

Fig. 5. Competitive uptake of lipids in Caco-2 cells. Uptake media were prepared by adding excess unlabeled lipids (below their CMC) to radiolabeled BSA-bound lipids, and uptake assays were performed as described in MATERIALS AND METHODS. A: radioactive 18:1 uptake: competition by unlabeled free fatty acids (FFA) and monoacylglycerols (MG). sn-2–16:0, sn-2-Monopalmitin; sn-2–10:0, sn-2-monocaprin; 1× cold competitors = 0.02 μM unbound lipid concentration. B: radioactive sn-2–18:1 uptake: competition by unlabeled FFA and MG. 10:0, Capric acid; 1× cold competitors = 0.03 μM unbound lipid concentration. Results are means ± SD (n = 3) from a representative of 3 separate experiments.
tive ligands, as described in MATERIALS AND METHODS. The inhibitory constants ($K_i$) of competitive ligands were obtained by assuming that $K_m$ was unchanged upon the addition of competitive ligands, as described in MATERIALS AND METHODS. $K_m$ values used for 18:1 and sn-2–18:1 were from the BSA-bound lipid uptake at the AP surface and were 0.58 and 0.33 μM, respectively. sn-2–16:0, sn-2-Monopalmitin. Results are means ± SD from 3 separate experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_m$ μM</th>
<th>$[^{3}H]18:1$ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>sn-2–18:1</td>
<td>0.21 ± 0.17</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>sn-1–18:1</td>
<td>0.27 ± 0.17</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>16:0</td>
<td>0.19 ± 0.07</td>
<td></td>
</tr>
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The inhibitory constants ($K_i$) of competitive ligands were obtained by assuming that $K_m$ was unchanged upon the addition of competitive ligands, as described in MATERIALS AND METHODS. $K_m$ values used for 18:1 and sn-2–18:1 were from the BSA-bound lipid uptake at the AP surface and were 0.58 and 0.33 μM, respectively. sn-2–16:0, sn-2-Monopalmitin. Results are means ± SD from 3 separate experiments.

Fig. 6. Competitive inhibition of $[^{3}H]sn$-2–18:1 uptake by unlabeled sn-2–18:1 at the apical membrane of Caco-2 cells. To a fixed concentration of BSA (100 μM), labeled sn-2–18:1 was added to give a constant unbound concentration of 0.03 μM, and increasing amounts of unlabeled sn-2–18:1 were added to give a range of unbound sn-2–18:1 concentrations. The solid line represents experimental values, and the dashed line represents theoretical values based on competitive kinetics and calculated as described in MATERIALS AND METHODS. Results are means ± SD ($n = 3$) from a representative of 3 separate experiments.

Fig. 7. Expression of hsFATP1, a human intestinal form of fatty acid transport protein (FATP) in Caco-2 cells. The mRNA of the putative membrane LCFA transporter hsFATP1 was evaluated by RT-PCR as described in MATERIALS AND METHODS. Lane 1, day 21 postconfluence (D21); lane 2, day 14 postconfluence (D14); lane 3, day 7 postconfluence (D7); lane 4, Caco-2 cells at confluence (D0); lane 5, rat jejunal mucosa (JEJ); lane 6, rat adipose tissue (AT). The corresponding band for hsFATP1 is 1,800 bp. The positive controls are human liver fatty acid-binding protein (L-FABP; 400 bp) and β-actin (300 bp).

Table 3. $K_i$ values of competitive ligands calculated from competition studies

<table>
<thead>
<tr>
<th>Ligand</th>
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The inhibitory constants ($K_i$) of competitive ligands were obtained by assuming that $K_m$ was unchanged upon the addition of competitive ligands, as described in MATERIALS AND METHODS. $K_m$ values used for 18:1 and sn-2–18:1 were from the BSA-bound lipid uptake at the AP surface and were 0.58 and 0.33 μM, respectively. sn-2–16:0, sn-2-Monopalmitin. Results are means ± SD from 3 separate experiments.

Fig. 6. Competitive inhibition of $[^{3}H]sn$-2–18:1 uptake by unlabeled sn-2–18:1 at the apical membrane of Caco-2 cells. To a fixed concentration of BSA (100 μM), labeled sn-2–18:1 was added to give a constant unbound concentration of 0.03 μM, and increasing amounts of unlabeled sn-2–18:1 were added to give a range of unbound sn-2–18:1 concentrations. The solid line represents experimental values, and the dashed line represents theoretical values based on competitive kinetics and calculated as described in MATERIALS AND METHODS. Results are means ± SD ($n = 3$) from a representative of 3 separate experiments.
norm. The protein-mediated pathways may serve to direct fatty acids and MG to specific intracellular targets when lipid levels are low, while the diffusive uptake may represent a largely unregulated "overflow" pathway that could nevertheless effect uptake of large quantities of lipid.

In agreement with others (22, 33, 51), TC-mixed LCFA uptake had dramatically increased $V_{\text{max}}$ values compared with those of BSA-bound LCFA. This difference was also evident for MG. We ensured that the differential AP vs. BL uptake capacities were not simply a result of micellar disruption of the AP plasma membrane during the TC incubations. Preincubation of Caco-2 monolayers with identical micellar solutions did not have any effect on subsequent uptake (data not shown), indicating that damage to the membrane during the micelle incubation did not occur. Hofmann (19) estimated that the product of the concentration gradient and the aqueous diffusion coefficient was 150 times higher for micellar aggregates compared with a monomer solution. Therefore, it may be reasonable to get similarly higher $V_{\text{max}}$ values for micellar TC-mixed LCFA uptake than for BSA-bound LCFA. At present, however, the precise role of TC in the uptake kinetics requires further exploration.

When the AP and BL uptake of LCFA from BSA solutions was compared, it was found that AP uptake had a greater $V_{\text{max}}$ than did BL uptake (Table 1). This could be related to differences in the plasma membrane composition between the AP and BL domains of the enterocytes (7). It has been shown that the AP membrane of the enterocyte has more intramembrane particles, higher protein/lipid and cholesterol/phospholipid ratios, and lower fluidity than the BL membrane. In addition, we previously showed that increased AP uptake of LCFA is not due to greater AP plasma membrane surface area in Caco-2 cells but, rather, is likely to reflect enterocyte-specific FFA transport and metabolism (52). In contrast, the AP and BL uptake of BSA-bound LCMG shows similar $V_{\text{max}}$ and $K_m$ values (Table 1). However, as we previously determined the ratio of the surface area of AP and BL membranes of Caco-2 to be 1:3 (52), this implies more effective uptake of MG at the AP surface, similar to LCFA.

The observation that AP uptake of TC-mixed 18:1 has a higher $V_{\text{max}}$ than TC-mixed 16:0, whereas BSA-bound 18:1 has a lower $V_{\text{max}}$ compared with BSA-bound 16:0, was very consistent over more than seven separate experiments (Table 1). Similarly, in 1968, Simmonds et al. (41) showed that 18:1 in TC solutions has a higher absorption rate than 16:0, using the intestinal perfusion technique in rats. In addition, Ockner et al. (30) showed that 16:0 in TC micelles has a lower rate of absorption compared with linoleic acid. This finding was ascribed to the slower esterification rate of 16:0 in the rat intestine (30). The present studies were performed at 10-s time periods, and we found that >90% of radioactive LCFA remained unesterified in Caco-2 cells at this time interval (Ref. 51 and data not shown). Therefore, the lower $V_{\text{max}}$ value obtained for TC-mixed 16:0 was not likely related to a slower esterification rate. It is possible that a somewhat lower $K_d$ of L-FABP for 18:1 than that for 16:0 (37) may result in binding or transferring more 18:1 relative to 16:0 away from the plasma membranes by L-FABP.

As proposed by Sorrentino et al. (43), the monomer concentration of LCFA in BSA-bound LCFA solution is the major factor that determines the uptake velocity. In such a case, the solubility of lipid in the aqueous phase may play an important role. On the basis of the free energy of transfer from hydrocarbon to water, $sn$-2–18:1 has the highest aqueous solubility, followed by 18:1 and then 16:0 (42). Therefore, the adsorption of lipids to the plasma membranes is presumably highest for 16:0, followed by 18:1 and then $sn$-2–18:1. Indeed, studies have shown that rabbit BBM bound more 16:0 than 18:1 (32), suggesting a higher membrane solubility for 16:0 relative to 18:1. Thus the higher $V_{\text{max}}$ values obtained for BSA-bound 16:0 relative to 18:1, at both the AP and BL surfaces of the Caco-2 cells, may reflect the intrinsic binding of monomeric lipids by the membrane.

In contrast to BSA-bound lipids, it has been proposed that TC-mixed lipids may be taken up through a collisional transfer mechanism (48). In such a mechanism, the rate and number of effective collisions between micelles and the plasma membrane, as well as the rate of ligand dissociation from the micelle into the membranes, must be taken into consideration. If the size of the micelle is smaller, the micelle numbers would be greater at a fixed concentration of LCFA and TC. This would result in more effective collisions between micelles and the plasma membrane. Therefore, the $V_{\text{max}}$ would, presumably, be higher. In addition, the $V_{\text{max}}$ could also be higher if the rate of ligand dissociation from the micelle to the membrane were greater. Studies of fluorescent FFA transfer in micelles indicate that unsaturated FFA dissociation occurs more rapidly than saturated FFA dissociation and that collisional transfer of lipid occurs at micelle concentrations similar to those used in the present studies (45). Thus a higher dissociation rate for 18:1 may contribute to the observed higher $V_{\text{max}}$ in TC uptake studies, compared with that of 16:0. In addition, a collision-based transfer mechanism could account, in part, for the higher uptake rates observed with TC micelles compared with BSA-bound lipids and, perhaps, for the different apparent $K_m$ values for TC-mixed lipids relative to BSA-bound lipids.

There are at least three membrane proteins that have been proposed to participate in LCFA transport in the small intestine: FABPpm (46), FAT/CD36 (1), and FATP (38). Previously, we demonstrated the expression of FABPpm in Caco-2 cells (51); here we studied additional intestinal membrane protein(s) that may also contribute to total LCFA uptake. We report, for the first time, that Caco-2 cells do express the transcript of FATP (Fig. 7). In contrast, mRNA as well as protein expression of FAT/CD36 was not detectable in Caco-2 cells (data not shown). Thus both FABPpm and FATP in Caco-2 cells may contribute to the saturable
component of MG and FFA uptake observed in this study. Trypsin preincubation inhibited the uptake of sn-2–18:1 at both the AP and BL membranes by up to 40% (Table 2), further supporting the hypothesis that membrane protein(s) may be involved in facilitating MG and FFA uptake in Caco-2. In addition, the competition studies suggest that sn-2–18:1 and 18:1 could compete through the same putative carrier(s) in Caco-2 cells. Indeed, Stremmel (46) has shown that both FFA and MG interact with FABPpm. No such data are presently available for FATP.

In the present studies, we examined the kinetics and mechanism of sn-2-monolein uptake at both the AP and BL plasma membrane of Caco-2 cells, using substrate incubations that resemble normal physiological conditions. The kinetic data obtained are consistent with the presence of saturable uptake mechanisms at both the AP and BL plasma membranes. The observation of competitive sn-2-monolein uptake by LCFA and by LCMG suggests that the same membrane protein(s) may be involved in the absorption of these ligands.

We thank Dr. Alfred Thumser for helpful discussions.

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