# Ezetimibe interferes with cholesterol trafficking from the plasma membrane to the endoplasmic reticulum in CaCo-2 cells

**F. Jeffrey Field,[1] Kim Watt, and Satya N. Mathur**

Department of Internal Medicine, University of Iowa, Iowa City, IA 52242

**Abstract** **Niemann-Pick C1-like 1 protein (NPC1L1) is the putative intestinal sterol transporter and the molecular target of ezetimibe, a potent inhibitor of cholesterol absorption. To address the role of NPC1L1 in cholesterol trafficking in intestine, the regulation of cholesterol trafficking by ezetimibe was studied in the human intestinal cell line, CaCo-2. Ezetimibe caused only a modest decrease in the uptake of micellar cholesterol, but markedly prevented its esterification. Cholesterol trafficking from the plasma membrane to the endoplasmic reticulum was profoundly disrupted by ezetimibe without altering the trafficking of cholesterol from the endoplasmic reticulum to the plasma membrane. Cholesterol oxidase-accessible cholesterol at the apical membrane was increased by ezetimibe. Cholesterol synthesis was modestly increased. Although the amount of cholesteryl esters secreted at the basolateral membrane was markedly decreased by ezetimibe, the transport of lipids and the number of lipoprotein particles secreted were not altered. NPC1L1 gene and protein expression were decreased by sterol influx, whereas cholesterol depletion enhanced NPC1L1 gene and protein expression. These results suggest that NPC1L1 plays a role in cholesterol uptake and cholesterol trafficking from the plasma membrane to the endoplasmic reticulum. Interfering with its function will profoundly decrease the amount of cholesterol transported into lymph.**—Field, F. J., K. Watt, and S. N. Mathur. **Ezetimibe interferes with cholesterol trafficking from the plasma membrane to the endoplasmic reticulum in CaCo-2 cells.** ***J. Lipid Res.*** **2007.** 48: **1735–1745.**

**Supplementary key words** Niemann-Pick type C1 • Niemann-Pick C1-like 1 • lipoproteins

Niemann-Pick C1-like 1 (NPC1L1) protein is critical in facilitating the absorption of cholesterol (and phytosterols) by the small intestine (1–3). Although expressed within several tissues within the body, the NPC1L1 gene is expressed predominately in liver and small intestine (1, 3). In rodents, NPC1L1 is highly expressed on the surface of jejunal absorptive cells (1). In mice lacking NPC1L1, cholesterol absorption is significantly reduced, and in response to cholesterol feeding, the normally observed increase in plasma and hepatic cholesterol levels is prevented (1, 2). The seminal discovery of NPC1L1 emanated from an intense effort to understand the mechanism of action of a potent inhibitor of cholesterol absorption, ezetimibe (4–7). It was correctly surmised that ezetimibe was interfering with a specific intestinal sterol transporter located on the apical membrane of an absorptive cell. Recent elegant studies have clearly demonstrated that NPC1L1 is the molecular target of ezetimibe (8).

It is predicted that NPC1L1, like its homolog, Niemann-Pick C1 (NPC1), facilitates cholesterol trafficking within cells (3, 9). Defective synthesis of normal NPC1 disrupts the movement of cholesterol from an endosomal/lysosomal compartment resulting in Niemann-Pick disease type C1, a fatal cholesterol storage disease characterized by cholesterol accumulation in liver, spleen, and brain (10, 11). Therefore, it is likely that intestinal NPC1L1 is also involved in cholesterol trafficking. In support of this, both NPC1 and NPC1L1 have 13 membrane-spanning domains and a conserved N-terminal domain. Both are predicted to be highly glycosylated; and importantly, both proteins contain a sterol-sensing domain, a domain that is conserved in a number of different proteins, all involved in regulating various aspects of cholesterol metabolism (1, 9, 10, 12, 13).

Although it is now very clear that NPC1L1 is critical in the absorption of cholesterol by the small intestine, it is not clear how NPC1L1 facilitates sterol absorption at the cellular or molecular level. Therefore, the present study was performed to investigate the role of NPC1L1 in cho-

Abbreviations: ABC, ATP-binding cassette; apoB, apolipoprotein B; BB, brush-border membranes; DMEM, Dulbecco's Modified Eagle's Medium; LDH, lactate dehydrogenase; NBBM, nonbrush-border membranes; NPC1, Niemann-Pick type C1; NPC1L1, Niemann-Pick C1-like 1 protein.

[1] To whom correspondence should be addressed.
e-mail: f-jeffrey-field@uiowa.edu

*Manuscript received 18 January 2007 and in revised form 12 April 2007.*
*Published, JLR Papers in Press, May 1, 2007.*
*DOI 10.1194/jlr.M700029-JLR200*

lesterol trafficking within an intestinal cell. The results suggest that NPC1L1 does facilitate the uptake of cholesterol from the intestinal lumen, albeit modestly. The predominate role of NPC1L1 is in facilitating trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum, which in turn, results in its eventual esterification and incorporation into triacylglycerol-rich lipoproteins.

## EXPERIMENTAL PROCEDURES

### Materials

[$^3$H] oleic acid (5 Ci/mmol), [$^3$H] cholesterol (48.3 Ci/mmol), [$^3$H] cholesteryl oleate (75.3 Ci/mmol), [1-$^{14}$C] oleoyl CoA ($53.5 \times 10^{-5}$ Ci/mmol), and [$^{14}$C] acetate (.0565 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA). Delipidated fetal calf serum was from Intracel (Issaquah, WA). Protease inhibitor cocktail, sodium taurocholate, Tri Reagent, cholesterol oxidase (Streptomyces species), sodium pyruvate, β-NADH and oleic acid were from Sigma Chemicals (St. Louis, MO). Rabbit polyclonal antibody anti-human NPC1L1 were from Cayman Chemicals (Ann Arbor, MI). Rabbit polyclonal antibody anti-human β actin was purchased from ABCAM (Cambridge, MA). The bicinchoninic protein assay kit, goat anti-rabbit HRP conjugated antibody and superSignal west femto maximum sensitivity substrate chemiluminescent detection kit were from Pierce Biotechnology, Inc. (Rockford, IL). ACAT inhibitor, PD128042 was a generous gift from Brian Krause, Parke-Davis Pharmaceutical Division, Warner-Lambert Co. (Pfizer Inc., Ann Arbor, MI). Alamar Blue was purchased from Invitrogen.

### Cell culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in Dulbecco's Minimum Essential Medium (DMEM) (GIBCO, Grand Island, NY) with 4.5 g/l glucose, and supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. Once the flasks reached 80% confluency, the cells were split and seeded at a density of $0.2 \times 10^5$ cells/well onto polycarbonate micropore membranes (0.4 μm pore size, 24 mm diameter) inserted into transwells (Costar, Cambridge, MA). Cells were fed every other day and were used 14 days after seeding.

### Cell viability/proliferation

Cell viability and proliferation were monitored throughout the studies. Lactate dehydrogenase (LDH) activity released into the apical medium and in cell homogenates was estimated as described in the information provided by the manufacturer (L-Lactic Dehydrogenase assay kit, Catalog # 340-LD, Sigma). In this procedure, the loss of β-NADH (reduced form) via absorbance at 340 nm in a linear range over time was detected using a 96-well microplate spectrophotometer μquant from BioTek Instruments, (Winooski, Vermont). Alamar Blue reduction (Invitrogen), an assay that monitors the reducing environment of proliferating cells, was performed exactly as described by the manufacturer. After 24 h of incubation with the treatments, the percent release of LDH activity into the apical medium or Alamar Blue reduction was unaltered. Total protein recovered per filter also did not change with any of the overnight treatments (~0.75 mg ± 0.03 per filter). Thus, the data presented in the figures are depicted per dish.

### Micelle preparation

Appropriate volumes of ethanol stock solutions containing the individual lipids were evaporated under nitrogen and the dried lipids were dissolved in DMEM. The resulting solution was stirred vigorously at 37°C until clear.

### Cholesterol uptake assay

CaCo-2 cells were incubated at 37°C for 10 h in 1.5 ml of apical medium containing 0.5% FBS and 0, 0.005, 0.01, 0.025, or 0.05 mM ezetimibe in DMEM. Ezetimibe was added to the apical medium in ethanol (0.04%). DMEM was added in the basal chamber. At 1, 3, and 6 h before the end of the incubation, a 15 × concentrated micellar solution was added to the apical medium to obtain a final concentration of 5 mM taurocholate, 0.25 mM oleic acid, 0.05 mM cholesterol, and 7.5 μC [$^3$H] cholesterol/dish. At the end of the incubation, the unincorporated radiolabeled cholesterol was removed by washing the cells four times with 1.5 ml of cold DMEM. Cell lipids were extracted with 1.5 ml of hexane/isopropyl alcohol/water (3:2:0.1, v/v/v). Unlabeled cholesterol and cholesteryl ester mass were added as carriers. A portion of the lipid extract was taken and counted in a Packard liquid scintillation counter to determine total cholesterol taken up by the cells. The remaining lipid extract was separated by thin-layer chromatography on a silica gel G plate (Uniplate, 250 microns, Analtech, Inc. Newark, DE), using a solvent system containing hexanes/diethyl ether/acetic acid, (90/10/1, v/v/v). Cholesterol and cholesteryl ester bands were localized by authentic standards, scraped from the plate and counted.

### Trafficking of newly synthesized cholesterol to the plasma membrane

Trafficking of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane was estimated by susceptibility of newly synthesized cholesterol to cholesterol oxidase in cells fixed with glutaraldehyde as described earlier (14). Briefly, CaCo-2 cells were preincubated for 1 h with 5 mM taurocholate micelles containing 0, 0.01, or 0.025 mM of ezetimibe. To label newly synthesized cholesterol, 0.15 ml of DMEM containing labeled [$^{14}$C] acetate was added to the apical medium containing the treatments and the incubation was continued for another 2, 4, or 6 h. The final concentration of the acetate in the apical medium was 60 μM with specific activity of 130 dpm/pmol. At the end of this incubation, the cells were washed two times with ice cold DMEM to remove unincorporated radiolabel. After washing two more times with 10 mM sodium phosphate buffer, pH 7.4, cells were incubated for 10 min at 4°C with water. The water was removed and the cells were fixed using 1% glutaraldehyde for 10 min at 4°C. The glutaraldehyde solution was then removed and cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, containing 0.310 M sucrose at 37°C. They were then incubated in this buffer for 15 min at 37°C. Cholesterol oxidase (5 units/ml) was then added to this buffer and the incubation was continued for 60 min at 37°C. The cells were again washed twice with cold phosphate buffered saline and harvested in 2 ml of 80% methanol containing 0.5 M NaOH. The lipids were saponified at 100°C for 1 h, followed by addition of 2 ml water. The nonsaponifiable lipids were extracted twice with 2 ml hexane. The combined hexane extract was washed once with water to remove alkali and residual fatty acids. An aliquot was taken and counted to determine total sterol synthesis. The remaining lipid extract was used to separate cholesterol and cholestenone on a silica gel thin-layer chromatography plate using hexane:diethylether:acetic acid (80/20/1, v/v). The radioactivity in the two sterol fractions was determined using TLC scanner from Bioscan, Inc., Washington, DC.

### Trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

CaCo-2 cells were incubated with 1 ml of DMEM containing 1% delipidated fetal calf serum and 2.5 μCi [$^3$H] cholesterol for 2 h at 4°C to label plasma membranes. Radiolabeled cholesterol was added to this apical medium in ethanol (1.2%, final concentration).The basal medium was replaced with 2.5 ml of cold DMEM. To remove unincorporated labeled cholesterol, cells were washed twice with cold DMEM. They were then incubated at 37°C in 1.5 ml of DMEM containing 5 mM TC ± 0.25 mM oleate, ± 0.01 mM ezetimibe for 8 h. The basal medium was replaced with 2.5 ml of DMEM. After the incubation, cells were washed with cold DMEM, and the lipids were extracted twice directly from the cells on the filter by adding 1 ml hexanes-isopropanol-water, 3:2:0.1 (v/v). Unlabeled cholesterol and cholesteryl ester mass were added as carriers. The lipids were separated by thin-layer chromatography and cholesterol and cholesteryl ester bands were localized by authentic standards, scraped from the plate, and counted.

### ACAT assay

CaCo-2 cells grown on T-75 flasks were incubated for 18 h with medium alone, 0.01 or 0.025 mM ezetimibe or 0.025 mM PD128042. The final concentration of the ethanol vehicle was 0.2% in all the media. At the end of the incubation, the cells were washed twice with DMEM and then harvested in a buffer containing 0.3 M sucrose, 0.05 M KCl, 0.04 M $KH_2PO_4$ and 0.03 M EDTA, pH 7.4 (ACAT buffer). The cells were homogenized by passing 10 times through a 22-gauge needle. The homogenate was spun at 81000 *g* for 30 min to isolate total membranes. From another set of T-75 flasks, the cells were harvested without any treatment and total membranes were isolated for use in experiments where ezetimibe or PD128042 was added in ethanol directly to the ACAT assay reaction.

To correct for possible differences in the amount of cholesterol substrate in total membrane preparations, membranes were preincubated for 10 min on ice with exogenous cholesterol in bile salt/cholesterol/PC mixed micelles according to the method of Chang et al. (15). Each reaction contained 100 or 200 μg protein, 100 nmoles taurocholate, 10 nmoles phosphatidylcholine, 50 nmoles exogenous cholesterol, 10.3 nmoles [1-$^{14}$C] oleoyl CoA (23000 dpm/nmole), and 1 mg BSA in a final volume 200 μl ACAT buffer. The reaction mixture was incubated at 37°C for 10 min, during which time the activity was found to be linear with protein concentration. The reaction was stopped by the addition of 2 ml of 1:1 chloroform methanol. To each reaction 15,000 dpm of [$^3$H] cholesteryl oleate was added as internal standard and the lipids were extracted in chloroform layer by addition of 0.7 ml of water. The cholesteryl esters were separated from other lipids on a silica gel G plate (Uniplate, 250 microns, Analtech, Inc. Newark, DE), using solvent system containing hexanes/diethyl ether/acetic acid (90/10/1, v/v/v). The recovery of [$^3$H] cholesteryl oleate radioactivity in the band was used to calculate the net amount of [1-$^{14}$C]oleoyl CoA incorporated into cholesteryl esters.

### Estimation of accumulation of sterol intermediates

The accumulation of cholesterol intermediates was determined by a method previously described (14). Briefly, CaCo-2 cells were preincubated for 1 h with 5 mM taurocholate micelles containing 0, 0.01, or 0.025 mM of ezetimibe. To label newly synthesized cholesterol, 0.15 ml of DMEM containing labeled [$^{14}$C] acetate was added to the apical medium containing the treatments and the incubation was continued for another 6 h. The final concentration of the acetate in the apical medium was 60 μM with specific activity of 130 dpm/pmol. At the end of this incubation, the cells were washed two times with ice cold DMEM to remove unincorporated radiolabel. The cells were harvested in 2 ml of 80% methanol containing 0.5 M NaOH. The lipids were saponified at 100°C for 1 h, followed by addition of 2 ml water. The nonsaponifiable lipids were extracted twice with 2 ml hexane. The combined hexane extract was washed once with water to remove alkali and residual fatty acids. An aliquot was taken and counted to determine total sterol synthesis. The remaining lipid extract was used to separate cholesterol and its intermediates on a silica gel thin-layer chromatography plate using hexane:diethylether:acetic acid (80/20/1, v/v). The radioactivity in the sterol fractions was determined using TLC scanner from Bioscan, Inc., Washington, DC. The cholesterol intermediates were identified, as described earlier (14, 16, 17).

### Oxidation of plasma membrane cholesterol in live cells

Cells were labeled for 18 h with 2.5 μCi/well of [$^3$H] cholesterol in the presence of 1% fetal calf serum and DMEM. One hour before the end of this incubation ± 0.025 mM ezetimibe was added to the apical medium in ethanol (final concentration: 0.1% ethanol). The cells were washed two times to remove unincorporated labeled cholesterol. The cells were then incubated for 4 h at 37°C with micellar solutions containing 5 mM taurocholate, 0.05 mM oleic acid, ± 0.025 mM ezetimibe or various amounts of cholesterol (0, 0.025, 0.050, 0.1 or 0.2 mM). The lower chambers received 2.5 ml DMEM alone. At the end of this incubation, cells were washed three times with DMEM and the cells were incubated for 1 h at 37°C in absence of the treatments with 1 ml of phosphate buffered saline, pH 7.4, containing 7 units/ml of cholesterol oxidase. The cells were then washed, lipids extracted and saponified, and radioactivity in cholesterol and cholestenone was determined as described above for trafficking of newly synthesized cholesterol to the plasma membrane.

### Effect of ezetimibe on synthesis and secretion of triglyceride-rich lipoproteins

CaCo-2 cells were incubated with 5 mM taurocholate micelles containing 0.25 mM [$^3$H] oleic acid (160 dpm/pmole/dish), ± 0.01 or 0.025 mM ezetimibe, ± 0.2 mM cholesterol for 12 h at 37°C. At the end of the incubation, cells were washed three times with DMEM to remove unincorporated radiolabel. Lipids were extracted with 1.5 ml of hexanes/isopropyl alcohol/water (3:2:0.1, v/v/v). The 2.5 ml basal medium was mixed with 5 ml of chloroform/methanol, 1:1, v/v and the chloroform layer containing lipids was collected. The lipid extracts from cells and medium were dried under nitrogen, taken up in 1 ml of chloroform and aliquots were taken for counting to estimate total [$^3$H] oleate incorporated into lipids. The remaining lipid extract was used to separate phospholipids, fatty acids, triacylglycerols, and cholesteryl esters on a silica gel thin-layer plate using hexane: diethylether:acetic acid (80/20/1, v/v). The radioactivity in the lipid fractions was determined using TLC scanner from Bioscan, Inc., Washington, DC.

### Apolipoprotein B (apoB) mass

CaCo-2 cells were incubated for 22 h with 5 mM taurocholate micelles alone, 5 mM taurocholate micelles containing 0.2 mM cholesterol, 0.25 mM of oleic acid with or without 0.01 mM ezetimibe. At the end of the incubation, basal medium and cells were harvested and apoB mass was estimated by a sandwich ELISA, as previously described (18).

### RNA estimation by real time quantitative RT-PCR

RNA was extracted from cells and subjected to DNase treatment followed by reverse transcription for 4 h at 50°C with SuperScript III (Invitrogen, Carlsbad, CA). The transcriptase was inactivated for 15 min at 70°C. The cDNA was mixed with the appropriate primers for the gene and the 2× Sybr Green PCR master mix (Applied Biosystems, Foster City, CA) and the real-time RT-PCR was performed using a BIO-RAD iCycler system. The thermal cycler parameters were: hold for 2 min at 50°C and 10 min at 95°C for one cycle, followed by amplification of cDNA for 45 cycles with melting for 15 s at 95°C and annealing and extension for 1 min at 60°C. In this real-time PCR procedure, using Sybr Green dye, the mass of PCR product generated is estimated after each PCR cycle and threshold cycle number is determined in the exponential phase of the curve. The values were normalized using 18S rRNA as endogenous internal standard. The relative expression of the gene was calculated using comparative threshold cycle ($C_T$) method (19) (**Table 1**).

### Isolation of nonbrush-border and brush-border membrane fractions of CaCo-2 cells

Isolation of the brush-border membrane fraction (BB) was based on a procedure described by Hauser et al. (20). The cell preparations were kept at 4°C throughout the isolation procedure. The CaCo-2 cell monolayer was washed once with 1.5 ml DMEM. To each filter, 0.5 ml of 2 mM Tris, 50 mM mannitol buffer, pH 7.8, containing protease inhibitors was added and the cells were scraped with a plastic spatula and transferred to a tube. Cells from six filters were combined for each sample. The cells were kept in the buffer for 15 min and then homogenized by passing 10 times through a 22-gauge needle. The homogenate was centrifuged at 500 *g* for 15 min to sediment the nuclei. The supernatant was transferred to another tube and the nuclear pellet was suspended in fresh buffer, passed five times through a 22-gauge needle to homogenize any remaining intact cells. This suspension was centrifuged at 500 *g*. The second supernatant was combined with the first supernatant. To the combined supernatant 100× $MgCl_2$ was added to obtain a final concentration of 10 mM $MgCl_2$. The mixture was kept on ice for 15 min and then centrifuged at 1500 *g* for 15 min to sediment the nonbrush-border membrane fraction (NBBM). The resulting supernatant was collected and centrifuged at 30000 *g* to sediment BB. The NBBM and BB fractions were suspended in the homogenization buffer and protein was estimated by bicinchoninic acid assay.

### Immunoblot analysis of NPC1L1 protein

Equal amounts of protein (40 μg) from BB fractions in 1× Laemmli sample buffer were separated by SDS/PAGE on an 8% porous gel (21) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). After rinsing in Tris-buffered saline (TBS) (25 mM Tris-HCl, pH 7.5, 150 mM sodium chloride), the membrane was air-dried for 15 min, washed with water:methanol (1:1, v/v) followed by methanol alone. After drying for 10 min at room temperature, the membrane was incubated for 1 h with the specific primary antibody for the given protein. The blot was incubated at room temperature for 1 h with the primary antibody that was diluted 1500-fold in TBS containing 0.05% Tween-20, 5% nonfat dry milk (Blotto). After washing in TBS containing Tween-20, the membrane was then incubated at room temperature for 1 h with goat anti-rabbit antibody cross-linked to horseradish peroxidase and diluted 100,000-fold in Blotto. The membrane was washed thoroughly in TBS containing Tween-20 and horseradish peroxidase was detected with superSignal west femto maximum sensitivity substrate chemiluminescent detection kit (Pierce, Rockford, IL). Relative values for protein mass were normalized to the density of actin on the immunoblot to account for differences in loading and transfer of the proteins.

TABLE 1. Primers used in this study

| Primer Name | Sequence | Accession # |
|---|---|---|
| Human NPC1L1-F | 5′-TAT-GGT-CGC-CCG- AAG-CA-3′ | AF192522.1 |
| Human NPC1L1-R | 5′-TGC-GGT-TGT-TCT- GGA-AAT-ACT-G-3′ | |
| 18S- F | 5′-TAA-GTC-CCT-GCC-CTT-TGT-ACA-CA-3′ | M33069 |
| 18S- R | 5′-GAT-CCG-AGG-GCC-TCA-CTA-AAC-3′ | |

F, forward; NPC1L1, Niemanni-Pick C1-Like 1; R, reverse.

### Other analyses

Protein content was estimated using the bicinchoninic acid assay kit (Pierce, Rockford, IL). SPSS software (SPSS Inc., Chicago, IL) was used to analyze data by general linear model univariate ANOVA or general linear model repeated measures followed by Tukey's *t*-test to compare the treatments. The data in Fig. 3 were analyzed by Student's *t*-test.

## RESULTS

### Effect of ezetimibe on micellar cholesterol uptake

To investigate whether NPC1L1 facilitates the uptake of cholesterol from bile salt micelles, cells were incubated for 10 h with or without increasing concentrations of ezetimibe. Cells were then incubated for 1, 3, and 6 h with taurocholate micelles containing 0.25 mM oleic acid, and 0.05 mM labeled cholesterol ± ezetimibe. The amount of labeled cholesterol taken up by the cells and the percent of the sterol esterified were determined. The results are shown in **Fig. 1**. In a dose-dependent manner, cells incubated with the NPC1L1 inhibitor, ezetimibe, contained less micellar unesterified cholesterol at each of the time points. Moreover, ezetimibe prevented the esterification of the micellar cholesterol that was taken up. Although the effect of ezetimibe on the uptake of cholesterol was modest, ~23% at the highest concentration, the results do suggest that NPC1L1 is playing a role in the uptake of cholesterol into the intestinal cell. It was our suspicion, however, that this modest reduction in cholesterol uptake by ezetimibe could not explain the significant inhibition of cholesterol absorption that is observed in vivo. In addition, ezetimibe appeared to have a profound effect on the esterification of micellar cholesterol. Thus, the role of intestinal NPC1L1 in intracellular cholesterol trafficking was addressed.

### Effect of ezetimibe on trafficking of newly synthesized cholesterol to the plasma membrane

To address the trafficking of cholesterol from the endoplasmic reticulum to the plasma membrane, cells were incubated for 1 h with or without ezetimibe. [$^{14}$C] -labeled acetate was then added and the incorporation of labeled acetate into total nonsaponifiable sterols and sterols found

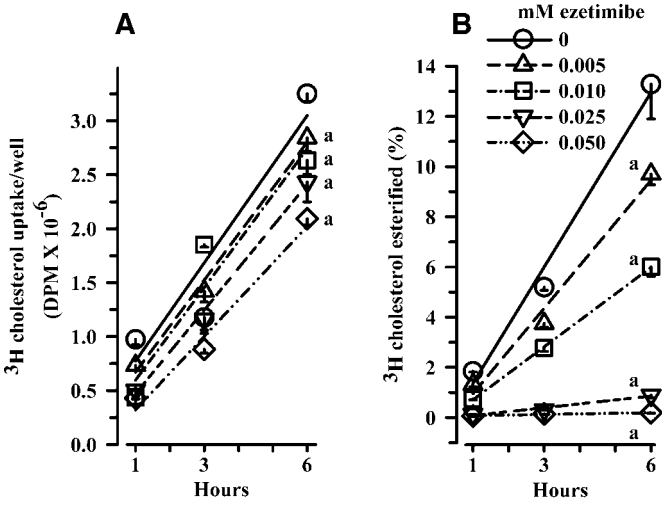


**Fig. 1.** Ezetimibe decreases uptake of micellar cholesterol. Cells were incubated for 10 h with various concentrations of ezetimibe. A: At 1, 3, or 6 h before the end of the incubation, cells were incubated with 5 mM taurocholate micelles containing 0.25 mM oleic acid, 0.05 mM of labeled cholesterol, in the presence or absence of the ezetimibe. B: Micellar cholesterol taken up and esterified by the cells was estimated as described in Experimental Procedures. Each value represents the mean ± SEM of three dishes at each time point. [a] $P < 0.05$ versus corresponding control without ezetimibe at all three time points.

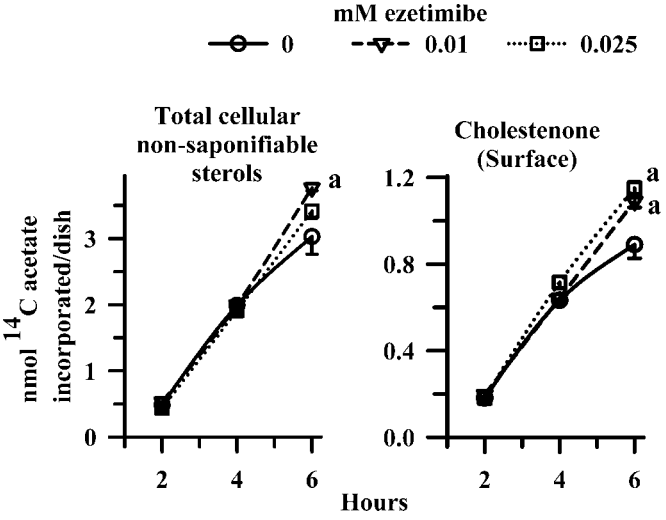


**Fig. 2.** Ezetimibe does not alter trafficking of newly synthesized cholesterol to the plasma membrane. Following a 1 h preincubation of cells with or without ezetimibe, newly synthesized sterols were labeled with [$^{14}$C] acetate in the presence of 5 mM taurocholate micelles and 0, 0.01, or 0.025 mM of ezetimibe (see Experimental Procedures). At 2, 4, and 6 h, cells were fixed with 1% glutaraldehyde. Synthesis of total cellular sterols (left panel) and cholesterol on the cell surface (cholestenone, right panel) were estimated as described in Experimental Procedures. Each value represents the mean ± SEM of three individual dishes at each time point. [a] $P < 0.05$ versus corresponding control without ezetimibe.

on the cell surface was estimated as described in Experimental Procedures (**Fig. 2**). In cells incubated with ezetimibe, the rate of incorporation of acetate into total sterols was similar to controls at 2 and 4 h. By 6 h, there was a modest increase in the amount of label found in total sterols in cells incubated with ezetimibe (left panel). Similarly, at 2 and 4 h, comparable amounts of labeled sterols were found on the cell surface. By 6 h, however, more labeled sterols were found on the cell surface in cells incubated with ezetimibe (right panel). Thus, by 6 h, ezetimibe modestly increases total sterol synthesis. Importantly, however, ezetimibe is not interfering with the trafficking of cholesterol from the endoplasmic reticulum to the plasma membrane suggesting that NPC1L1 is not playing a role in this pathway. Reasons for the observed increase in cell-surface sterols in ezetimibe treated cells at 6 h will become evident in subsequent experiments.

### Effect of ezetimibe on trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

To estimate cholesterol movement from the plasma membrane to the endoplasmic reticulum, plasma membrane cholesterol was first labeled by incubating cells for 2 h at 4 degrees with [$^3$H] cholesterol. After washing, cells were then warmed to 37°C with or without ezetimibe, together with micelles alone, or micelles containing oleic acid, to drive triacylglycerol-rich lipoprotein assembly and secretion. The amount of plasma membrane cholesterol that was esterified was determined to estimate the trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum, the site of ACAT. The results are shown in **Fig. 3**. When intestinal cells are driven to produce lipoproteins by fatty acid influx, more plasma membrane cholesterol moves to the ACAT pool for esterification (and eventual secretion; open bars). In the presence of ezetimibe (hatched bars), however, there is a marked reduction in the esterification of plasma membrane cholesterol, suggesting that by interfering with the function of NPC1L1, the movement of cholesterol from the plasma membrane to the endoplasmic reticulum is disrupted.

It could be argued that the above results and those shown in Fig. 1 are explained by ezetimibe acting as an ACAT inhibitor. This possibility was addressed in two ways. Following incubation of cells with either ezetimibe or PD 128,042 (a potent ACAT inhibitor), total membranes were prepared and ACAT activity was determined (**Fig. 4A**). Compared with ACAT activity in membranes prepared from control cells, ACAT activity in cells incubated with 0.01 and 0.025 mM of ezetimide, was decreased by 5 and 18%, respectively. In comparison, ACAT activity was inhibited by 92% in membranes prepared from cells incubated with PD 128,042. When either ezetimibe or PD 128,042 was added directly to the ACAT assay (Fig. 4B), ezetimibe decreased ACAT activity by 20% at 5 μM; whereas, PD 128,042 at 5 μM, essentially abolished ACAT activity. Thus, as others have stated and we would agree, ezetimibe is a very weak ACAT inhibitor and will not account for our observations above, or its inhibitory effect on cholesterol absorption in intact animals/humans (22, 23).

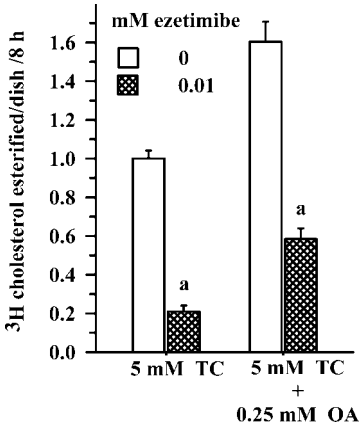


**Fig. 3.** Ezetimibe decreases the trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum. Plasma membrane cholesterol was labeled by incubating cells for two h at 4°C with [$^3$H] cholesterol. Cells were then incubated for 8 h at 37°C with taurocholate (TC) ± 0.25 mM oleate (OA), with or without 0.01 mM ezetimibe. Influx of plasma membrane cholesterol to endoplasmic reticulum was estimated by measuring the amount of labeled cholesteryl esters formed as described in Experimental Procedures. Each value represents the mean ± SEM of six dishes from two separate experiments normalized to controls (5 mM TC alone). The percent of labeled cholesterol esterified for controls was 1.3 and 2.4 for the two experiments. [a] $P < 0.001$ versus corresponding control without ezetimibe.

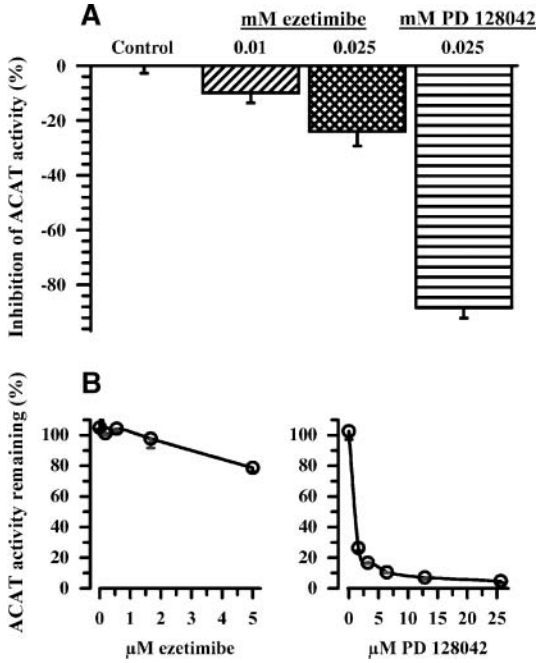


**Fig. 4.** Effect of ezetimibe or PD128042 on ACAT activity. A: ACAT activity was estimated in total membranes prepared from CaCo-2 cells incubated for 18 h with or without 0.010 or 0.025 mM ezetimibe or 0.025 mM PD128042. B: The indicated amounts of ezetimibe or PD128042 were added directly to the ACAT assay reaction. ACAT activity was measured at two enzyme concentrations in presence of exogenous cholesterol as described in Experimental Procedures. Activity was linear with protein concentration.

It has been shown that when trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum is disrupted, intermediates of cholesterol accumulate because the circuit for synthesis of the sterol is incomplete (14, 16). To address whether inhibiting NPC1L1 causes the accumulation of these cholesterol intermediates, cells were again incubated for 6 h with labeled acetate in the presence or absence of ezetimibe. The amount of labeled acetate incorporated into cholesterol and its intermediates was then estimated. In **Fig. 5**, the ratio of intermediates to cholesterol in cells incubated with or without ezetimibe is shown. Compared with control cells, in a concentration-dependent manner, cells incubated with ezetimibe accumulated significantly more sterol intermediates, suggesting that NPC1L1 facilitates the trafficking of these intermediates back to the endoplasmic reticulum for completion of cholesterol synthesis. Moreover, ~0.6% of these intermediates, which accumulate in cells incubated with 0.025 mM ezetimibe, can be recovered in the apical medium with taurocholate micelles as an acceptor (data not shown). From these sets of experiments, it is clear that ezetimibe interferes with cholesterol trafficking from the plasma membrane to the endoplasmic reticulum, and this effect cannot be attributable to its weak ACAT inhibitory properties.

### Effect of ezetimibe on cholesterol oxidase-accessible cholesterol

We have postulated that cholesterol oxidase-accessible cholesterol located in "specialized microdomains" within the apical plasma membrane of intestinal cells is the cholesterol pool that is used for lipoprotein assembly/secretion (24, 25). If correct, inhibiting NPC1L1 and interfering with the influx of this apical membrane pool of cholesterol should make more apical membrane cholesterol accessible to oxidation via cholesterol oxidase.

To test this, cells were labeled overnight with [$^3$H] cholesterol. They were then incubated for 4 h in the presence or absence of ezetimibe and increasing concentrations of micellar cholesterol. After washing the cells, cholesterol oxidase was added to the live cells for 1 h and the amount of cholestenone generated was estimated. The results are shown in **Fig. 6**. As we have shown previously (25), increasing micellar cholesterol influx will modestly increase the amount of membrane cholesterol that is accessible to cholesterol oxidase (white bars). At each concentration of micellar cholesterol, the amount of cholestenone generated was significantly more in cells incubated with ezetimibe (hatched bars). Thus, inhibiting NPC1L1 with ezetimibe will cause an increase in plasma membrane cholesterol, that we postulate is localized in "specialized microdomains"; a cholesterol pool that is available for influx to the endoplasmic reticulum, esterification, and eventual secretion in a triacylglycerol-rich lipoprotein particle.

### Effect of ezetimibe on the secretion of triacylglycerol-rich lipoproteins

Because NPC1L1 facilitates the trafficking of cholesterol from the plasma membrane to the endoplasmic re-

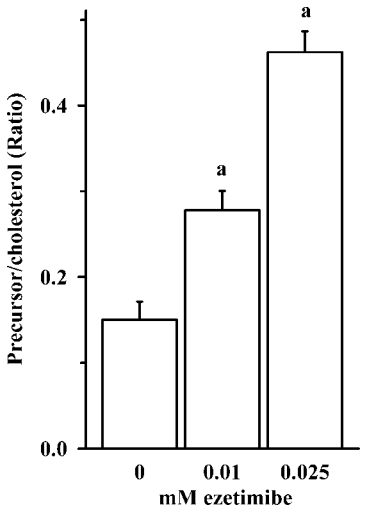


**Fig. 5.** Ezetimibe causes the accumulation of newly synthesized sterol intermediates. Newly synthesized sterols were labeled for 6 h with [$^{14}$C] acetate in presence of 5 mM taurocholate micelles with or without 0.01 or 0.025 mM of ezetimibe. After extensive washing, cells were harvested and radioactivity in newly synthesized cholesterol and intermediates was estimated as described in Experimental Procedures. The ratio of precursor/cholesterol is shown. Each value represents the mean ± SEM of six individual samples. [a] $P < 0.001$ versus cells without ezetimibe.

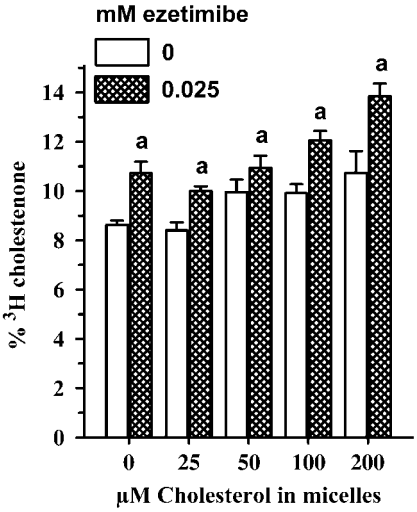


**Fig. 6.** Ezetimibe increases cholesterol oxidase-accessible cholesterol on the apical membrane. Cells were labeled for 18 h with [$^{3}$H] cholesterol. After washing, cells were incubated for 4 h with micellar solutions containing 5 mM taurocholate, 0.05 mM oleic acid, and increasing amounts of cholesterol, with or without 0.025 mM ezetimibe. Following the incubation, cells were washed and 7 units/ml of cholesterol oxidase was added for 1 h in absence of the treatments. Cholesterol oxidase-accessible cholesterol on the apical membrane of live cells was then determined as described in Experimental Procedures. Each value represents the mean ± SEM of six dishes from two experiments. [a] $P < 0.05$ versus cells without ezetimibe at the corresponding cholesterol concentration.

ticulum, it was questioned whether NPC1L1 was required for normal lipoprotein assembly and/or secretion to occur. To address this, cells were incubated with or without ezetimibe together with micelles containing cholesterol and labeled oleic acid. The amount of labeled phospholipids, triacylglycerols, and cholesteryl esters within cells and that recovered in the basolateral medium was estimated. The results are shown in **Fig. 7**. Inhibiting NPC1L1 with ezetimibe had no effect on the amount of labeled phospholipids or triacylglycerols recovered within cells or that found in the basolateral medium. As expected, however, ezetimibe did decrease the amount of labeled cellular cholesteryl esters and the amount recovered in the basolateral medium. The percent of each labeled cellular lipid recovered in the basolateral medium was similar in control cells and cells incubated with ezetimibe. In another experiment, cells were incubated for 22 h with or without ezetimibe together with micelles containing 0.25 mM oleic acid. Following the incubation, total apoB mass (apoB-48 and apoB-100) within cells and apoB secreted basolaterally was estimated. ApoB mass in control cells and cells incubated with ezetimibe was 126 ± 19 ng/mg protein and 129 ± 14, respectively. ApoB mass secreted into the basolateral medium by control cells and cells incubated with ezetimibe was 737 ± 116 ng/mg protein and 708 ± 66, respectively (not significant at $P < 0.05$, n = 6). These results suggest that ezetimibe does not alter the number of lipoprotein particles being secreted. Thus, NPC1L1 is not required for the normal assembly or secretion of triacylglycerol-rich lipoproteins. It is clear, however, that by interfering with NPC1L1 and therefore, cholesterol trafficking from the plasma membrane to the endoplasmic reticulum, less cellular cholesteryl esters will be synthesized and less will be available for secretion as part of a lipoprotein particle.

### Effect of intestinal cholesterol flux on NPC1L1 gene and protein expression

Because intestinal NPC1L1 is obviously involved in normal cholesterol movement from the plasma membrane to the endoplasmic reticulum and the NPC1L1 gene is purported to have a sterol sensing site, it was asked whether changes in cholesterol flux through the intestinal cell would alter NPC1L1 gene and/or protein expression. Cholesterol efflux/depletion was accomplished by incubating cells with either cyclodextrin or taurocholate micelles containing phosphatidylcholine. Micelles containing phosphatidylcholine decreased total cellular cholesterol mass by 33%. To increase cholesterol influx, cells were incubated with micelles containing either cholesterol or 25-hydroxycholesterol. After a 24 h incubation, mRNA levels for NPC1L1 were estimated (**Fig. 8**). Compared with their respective controls, NPC1L1 mRNA levels were increased 2.4- and 3-fold by taurocholate and phosphatidylcholine (TC + PC) and cyclodextrin, respectively. Cholesterol influx, either with taurocholate and cholesterol or its 25-hydroxy derivative, caused a significant decrease in NPC1L1 gene expression. 25-hydroxycholesterol significantly decreased NPC1L1 expression whether added as a micelle as shown here or in an ethanol vehicle (not shown).

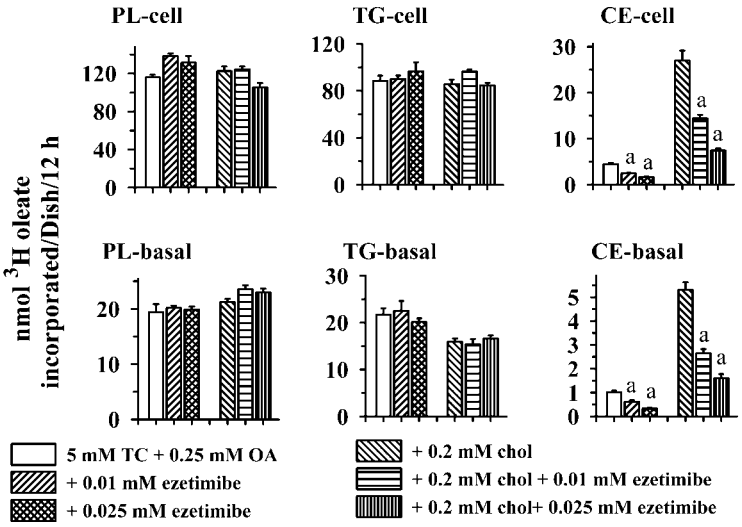


**Fig. 7.** Ezetimibe does not affect the secretion of triacylglycerol-rich lipoproteins. Cells were incubated for 12 h with 5 mM taurocholate micelles containing 0.25 mM labeled oleic acid alone or micelles containing 0.2 mM cholesterol, with or without 0.01 or 0.025 mM ezetimibe. The incorporation of labeled oleic acid into phospholipids, triacylglycerols, and cholesteryl esters within cells and in the basolateral medium was determined as described in Experimental Procedures. Each value represents the mean ± SEM of six dishes. [a] $P < 0.001$ versus cells without ezetimibe.

To address the effect of cholesterol flux on NPC1L1 mass, cells were incubated for 24 or 48 h with 25-hydroxycholesterol or taurocholate micelles containing phosphatidylcholine (TC + PC). NPC1L1 mass was then estimated by standard immunoblotting (**Fig. 9**). Two distinct bands were consistently observed, corresponding to molecular mass of ~155 and 145 kDa. Analysis was performed on the lower molecular weight band alone or both bands together. Despite observing significant changes in gene expression within 24 h of altering cholesterol flux, very little change was observed in NPC1L1 mass at 24 h (data not shown). At 48 h, however, NPC1L1 mass was significantly reduced by 25-hydroxycholesterol; whereas, cholesterol depletion (TC + PC) caused a significant increase in NPC1L1 mass. In data not shown, incubating cells with ezetimibe for 24 h did not alter mRNA levels or protein mass of NPC1L1.

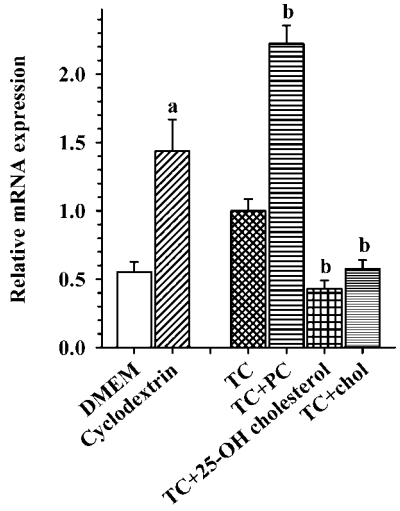


**Fig. 8.** Effect of sterol flux on NPC1L1 gene expression. Cells were incubated for 24 h with DMEM alone, DMEM+5 mg/ml cyclodextrin, 5 mM taurocholate (TC) micelles alone, TC+0.25 mM phosphatidylcholine, TC+0.01 mM 25 hydroxycholesterol or TC+0.2 mM cholesterol. RNA was isolated and the expression of NPC1L1 mRNA was estimated by real time quantitative RT-PCR as described in Experimental Procedures. Each value represents the mean ± SEM of at least nine dishes. [a] $P < 0.001$, significantly different from DMEM alone. [b] $P < 0.001$, significantly different from cells incubated with TC alone.

## DISCUSSION

There is supportive evidence to suggest that NPC1L1 facilitates the uptake of cholesterol from the intestinal lumen into the absorptive cell; the first step (of potentially many) in the overall absorptive process of cholesterol. Compared with wild-type mice, in mice lacking NPC1L1, the amount of exogenous dietary cholesterol found in the wall of the proximal intestine 2 h after oral gavage of labeled cholesterol is significantly less (2). Using a similar experimental protocol, similar results were obtained in ezetimibe-treated mice and rats (5, 26). Because of the relatively short 2 h time period after giving labeled cholesterol orally, it would be difficult to argue against NPC1L1's role in the uptake of cholesterol from the lumen. Our data also support a role for NPC1L1 in the uptake of micellar cholesterol into human intestinal absorptive cells. In our studies, however, the effect of ezetimibe on cholesterol uptake was modest decreasing uptake by only ~23%, probably insufficient to account for the profound effect of this drug on cholesterol absorption. It is important to note, however, that in vivo, ezetimibe is absorbed, glucuronidated, secreted into bile, and circulates via the enterohepatic circulation back to the intestine (27). It has been shown that both ezetimibe and its glucuronide metabolite inhibit cholesterol absorption but that the glucuronide derivative is more potent. This is likely related to the enhanced binding affinity of the glucuronide metabolite to NPC1L1 (4, 8, 27–29). Thus, our observations with the parent drug may underestimate the effects of the glucuronyl metabolite. However, Sane

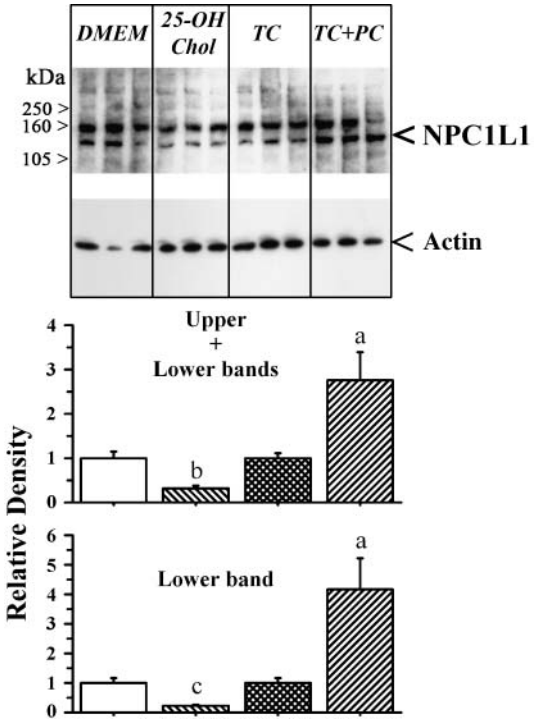


**Fig. 9.** Effect of sterol flux on NPC1L1 protein mass. Cells were incubated for 48 h with or without 0.025 mM 25-hydroxycholesterol (final concentration of ethanol, 0.1%). In another set of cells, cells were incubated for 48 h with 0.25 mM taurocholate (TC) or TC + 0.25 mM phosphatidylcholine. Brush border membrane fractions were then isolated from cells pooled from four dishes for each sample. The amount of NPC1L1 and actin were estimated by immunoblotting as described in Experimental Procedures. A representative blot from two separate experiments is shown. The density of each band was estimated using National Institutes of Health image software. Density of NPC1L1 protein was corrected using actin as a loading control. Each value represents the mean ± SEM of six individual samples. [a] $P < 0.05$, [b] $P < 0.01$ or [c] $P < 0.001$, versus respective control.

et al. (30) also observed only a modest 20% decrease in cholesterol uptake in CaCo-2 cells in which NPC1L1 expression was attenuated by siRNA. Thus, it would seem that NPC1L1 may have other transport functions in intestine that would facilitate cholesterol absorption. Moreover, it cannot be argued that low or deficient NPC1L1 abundance/expression is the explanation for the modest effect of ezetimibe on cholesterol uptake in CaCo-2 cells. In the study by Sane et al. (30), CaCo-2 cells and human small intestine contained similar amounts of NPC1L1 protein and mRNA. In addition, hamster intestine and CaCo-2 cells have similar NPC1L1 mRNA levels, as evidenced by real-time PCR Ct values of 26 versus 25, respectively (31) (unpublished observation). The elusive question is how NPC1L1 facilitates cholesterol uptake at the apical membrane. We have no data that specifically addresses this. It is not clear whether NPC1L1 acts alone or as a complex with other proteins on the apical surface. It is not even clear if NPC1L1 is the transporter itself, or is a required part of a transport complex. Due to the difficulty in reconstituting this uptake pathway in nonintestinal cells, it has been postulated that intestinal-specific proteins may be required for NPC1L1 to function as a cholesterol transporter (1); although recently, a reconstituted NPC1L1 transport system was successful in enhancing cholesterol uptake in a rat hepatoma cell line (32).

NPC1L1 is a homolog of NPC1, a protein that is critical for normal intracellular cholesterol trafficking. We postulated, therefore, that NPC1L1 might have a similar role in the absorptive pathway requiring intracellular cholesterol trafficking "downstream" from cholesterol uptake. In previous studies in CaCo-2 cells, it was always perplexing as to why so little of exogenous micellar cholesterol was being esterified and transported at a time when the cell was being driven to produce lipoproteins; lipoproteins that contained significant amounts of cholesteryl esters (24). Similar observations were also made in lymph-fistula animal models and CaCo-2 cells (33, 34). To explain this, we and others (in nonintestinal cells) have shown that the preferred substrate for ACAT is cholesterol derived from the plasma membrane (35–37). We have since demonstrated that when an intestinal cell is being driven to produce triacylglycerol-rich lipoproteins by fatty acid and cholesterol flux, it is cholesterol derived from the plasma membrane (not exogenously-derived dietary cholesterol) that is preferentially being trafficked to the ACAT pool for esterification and transport (24). This makes the trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum very important for the normal transport of cholesterol into lymph as part of a lipoprotein particle. It is also clear that the entire apical membrane cholesterol pool is not available for this transport. That would make little sense. We have postulated that there is a "clustering" of cholesterol in microdomains within the apical plasma membrane (perhaps in rafts) that is then utilized for cholesterol influx and efflux (25). Obviously, dietary cholesterol that is incorporated into the membrane will eventually be moved to these microdomains and traffic to the endoplasmic reticulum for transport; but, it is not the immediate substrate for this pathway (24). Iqbal et al. (34) have actually suggested that this newly absorbed cholesterol is secreted by a pathway that is independent of apoB, circumventing the triacylglycerol-rich lipoprotein assembly pathway. It is clear from our present results, that NPC1L1 facilitates this trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum. Interfering with NPC1L1 causes a marked decrease in the esterification of plasma membrane cholesterol and thus, causes a marked decrease in the amount of cholesterol that gets transported basolaterally. It is likely that this is the reason why ezetimibe is such a profound inhibitor of cholesterol absorption.

NPC1L1 is not required for the normal assembly and secretion of triacylglycerol-rich lipoproteins by the intestinal cell. The present results show clearly that ezetimibe did not alter apoB secretion or the secretion of triacylglycerols, phospholipids, or cholesteryl esters. Thus, despite markedly reducing the amount of cholesteryl esters within the lipoprotein particle via its action on cholesterol trafficking to the ACAT pool, ezetimibe did not alter the

number of lipoprotein particles being secreted. In NPC1L1 deficient mice, triacylglycerol uptake and absorption were similar to that observed in wild-type mice (2). Similar results were observed in ezetimibe-treated animals and humans. In cynomolgus monkeys, ezetimibe treatment markedly decreased cholesteryl esters in postprandial chylomicrons/remnants without significantly altering unesterified cholesterol, apoB-48, or triacylglycerol content (6). In men with primary hypercholesterolemia, Tremblay et al. (38) observed no effect of ezetimibe on triacylglycerol-rich lipoprotein apoB-48 kinetics or pool size. NPC1L1, therefore, is facilitating steps of cholesterol transport prior to ACAT2 and the lipoprotein assembly and secretory pathway.

During the preparation of this manuscript, Alrefai et al. (39) published results of CaCo-2 cells showing the regulation of NPC1L1 gene expression by changes in cholesterol flux. They demonstrate that cholesterol depletion (mevinolin) increases NPC1L1 mRNA levels and sterol enrichment (25-hydroxycholesterol) decreases NPC1L1 mRNA levels. They also identified two putative sterol response elements in the promoter of NPC1L1 and implicated the SREBP-2 pathway in the regulation of NPC1L1 promoter activity by cholesterol. This finding in cell culture supports a dietary study in mice whereby a diet enriched in cholesterol causes a decrease in intestinal NPC1L1 mRNA levels (2); albeit, not all studies in mice demonstrate a significant reduction of NPC1L1 by dietary cholesterol (40). Moreover, in hamsters, NPC1L1 gene expression was not altered in intestines of animals fed a diet enriched in cholesterol or by one that depletes the intestine of cholesterol (31). In miniature pigs, ezetimibe treatment resulted in a modest increase in intestinal NPC1L1 mRNA levels (41). Just as there are species differences as to the tissue location of NPC1L1 expression (3), there are obviously differences within and among species in the regulation of NPC1L1 gene expression by changes in cholesterol flux. Our present results in CaCo-2 cells support and extend those of Alrefai et al. (39). Influx of micellar cholesterol and 25-hydroxycholesterol significantly decreased NPC1L1 mRNA levels; whereas, depleting cellular cholesterol by enhancing cholesterol efflux (cyclodextrin or micelles containing phosphatidylcholine) increased NPC1L1 mRNA levels significantly. Interestingly, within this same time frame of 24 h, NPC1L1 mass was little altered by changes in cholesterol flux. By 48 h, however, significant changes in NPC1L1 mass were observed and paralleled the changes observed in gene expression. One could perhaps argue that the half-life of NPC1L1 protein is long and 24 h is insufficient to see the effect of sterol influx on NPC1L1 protein mass. It is also possible that the cell contains post-transcriptional mechanisms for maintaining NPC1L1 protein when rates of synthesis are decreased by acute or rapid changes in mRNA levels. Obviously, this will have to await further studies on the normal turnover of NPC1L1 protein in an intestinal cell. If our results can be extrapolated to human intestine, it is tempting to speculate that the regulation of NPC1L1 expression by changes in cholesterol flux is an adaptive mechanism to prevent the organism from getting too little, or too much, cholesterol.

This study was supported by the Department of Veterans Affairs and National Institutes of Health Grant DK-067132.

## REFERENCES


1. Altmann, S. W., H. R. Davis, Jr., L. J. Zhu, X. Yao, L. M. Hoos, G. Tetzloff, S. P. Iyer, M. Maguire, A. Golovko, M. Zeng, et al. 2004. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science.* **303:** 1201–1204.
2. Davis, H. R., Jr., L. J. Zhu, L. M. Hoos, G. Tetzloff, M. Maguire, J. Liu, X. Yao, S. P. Iyer, M. H. Lam, E. G. Lund, et al. 2004. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J. Biol. Chem.* **279:** 33586–33592.
3. Davies, J. P., C. Scott, K. Oishi, A. Liapis, and Y. A. Ioannou. 2005. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. *J. Biol. Chem.* **280:** 12710–12720.
4. Van Heek, M., C. F. France, D. S. Compton, R. L. McLeod, N. P. Yumibe, K. B. Alton, E. J. Sybertz, and H. R. Davis, Jr. 1997. In vivo metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. *J. Pharmacol. Exp. Ther.* **283:** 157–163.
5. van Heek, M., C. Farley, D. S. Compton, L. Hoos, and H. R. Davis. 2001. Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. *Br. J. Pharmacol.* **134:** 409–417.
6. van Heek, M., D. S. Compton, and H. R. Davis. 2001. The cholesterol absorption inhibitor, ezetimibe, decreases diet-induced hypercholesterolemia in monkeys. *Eur. J. Pharmacol.* **415:** 79–84.
7. van Heek, M., C. Farley, D. S. Compton, L. M. Hoos, A. Smith-Torhan, and H. R. Davis. 2003. Ezetimibe potently inhibits cholesterol absorption but does not affect acute hepatic or intestinal cholesterol synthesis in rats. *Br. J. Pharmacol.* **138:** 1459–1464.
8. Garcia-Calvo, M., J. Lisnock, H. G. Bull, B. E. Hawes, D. A. Burnett, M. P. Braun, J. H. Crona, H. R. Davis, Jr., D. C. Dean, P. A. Detmers, et al. 2005. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci U S A* **102:** 8132–8137.
9. Davies, J. P., B. Levy, and Y. A. Ioannou. 2000. Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics.* **65:** 137–145.
10. Carstea, E. D., J. A. Morris, K. G. Coleman, S. K. Loftus, D. Zhang, C. Cummings, J. Gu, M. A. Rosenfeld, W. J. Pavan, D. B. Krizman, et al. 1997. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science.* **277:** 228–231.
11. Ory, D. S. 2000. Niemann-Pick type C: a disorder of cellular cholesterol trafficking. *Biochim. Biophys. Acta.* **1529:** 331–339.
12. Loftus, S. K., J. A. Morris, E. D. Carstea, J. Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. A. Rosenfeld, D. A. Tagle, et al. 1997. Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science.* **277:** 232–235.
13. Davies, J. P., and Y. A. Ioannou. 2000. Topological analysis of Niemann-Pick C1 protein reveals that the membrane orientation of the putative sterol-sensing domain is identical to those of 3-hydroxy-3-methylglutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein. *J. Biol. Chem.* **275:** 24367–24374.
14. Field, F. J., E. Born, S. Murthy, and S. N. Mathur. 1998. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum. *J. Lipid Res.* **39:** 333–343.
15. Chang, C. C., C. Y. Lee, E. T. Chang, J. C. Cruz, M. C. Levesque, and T. Y. Chang. 1998. Recombinant acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) purified to essential homogeneity utilizes cholesterol in mixed micelles or in vesicles in a highly cooperative manner. *J. Biol. Chem.* **273:** 35132–35141.
16. Metherall, J. E., K. Waugh, and H. Li. 1996. Progesterone inhibits cholesterol biosynthesis in cultured cells. Accumulation of cholesterol precursors. *J. Biol. Chem.* **271:** 2627–2633.
17. Lusa, S., S. Heino, and E. Ikonen. 2003. Differential mobilization of newly synthesized cholesterol and biosynthetic sterol precursors from cells. *J. Biol. Chem.* **278:** 19844–19851.

18. Murthy, S., E. Albright, S. N. Mathur, N. O. Davidson, and F. J. Field. 1992. Apolipoprotein B mRNA abundance is decreased by eicosapentaenoic acid in CaCo-2 cells. B. Effect on the synthesis and secretion of apolipoprotein, *Arterioscler. Thromb.* **12:** 691–700.
19. Wong, M. L., and J. F. Medrano. 2005. Real-time PCR for mRNA quantitation. *Biotechniques.* **39:** 75–85.
20. Hauser, H., K. Howell, R. M. Dawson, and D. E. Bowyer. 1980. Rabbit small intestinal brush border membrane preparation and lipid composition. *Biochim. Biophys. Acta.* **602:** 567–577.
21. Doucet, J. P., B. J. Murphy, and B. S. Tuana. 1990. Modification of a discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide gel system for minigel electrophoresis. *Anal. Biochem.* **190:** 209–211.
22. Burnett, D. A., M. A. Caplen, H. R. Davis, Jr., R. E. Burrier, and J. W. Clader. 1994. 2-Azetidinones as inhibitors of cholesterol absorption. *J. Med. Chem.* **37:** 1733–1736.
23. Salisbury, B. G., H. R. Davis, R. E. Burrier, D. A. Burnett, G. Bowkow, M. A. Caplen, A. L. Clemmons, D. S. Compton, L. M. Hoos, D. G. McGregor, et al. 1995. Hypocholesterolemic activity of a novel inhibitor of cholesterol absorption, SCH 48461. *Atherosclerosis.* **115:** 45–63.
24. Field, F. J., E. Born, and S. N. Mathur. 1995. Triacylglycerol-rich lipoprotein cholesterol is derived from the plasma membrane in CaCo-2 cells. *J. Lipid Res.* **36:** 2651–2660.
25. Field, F. J., E. Born, S. Murthy, and S. N. Mathur. 1998. Caveolin is present in intestinal cells: role in cholesterol trafficking? *J. Lipid Res.* **39:** 1938–1950.
26. Altmann, S. W., H. R. Davis, Jr., X. Yao, M. Laverty, D. S. Compton, L. J. Zhu, J. H. Crona, M. A. Caplen, L. M. Hoos, G. Tetzloff, et al. 2002. The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. *Biochim. Biophys. Acta.* **1580:** 77–93.
27. van Heek, M., C. Farley, D. S. Compton, L. Hoos, K. B. Alton, E. J. Sybertz, and H. R. Davis, Jr. 2000. Comparison of the activity and disposition of the novel cholesterol absorption inhibitor, SCH58235, and its glucuronide, SCH60663. *Br. J. Pharmacol.* **129:** 1748–1754.
28. Clader, J. W. 2004. The discovery of ezetimibe: a view from outside the receptor. *J. Med. Chem.* **47:** 1–9.
29. Hawes, B. E., K. A. O'Neill, X. Yao, J. H. Crona, H. R. Davis, Jr., M. P. Graziano, and S. W. Altmann. 2007. In vivo responsiveness to ezetimibe correlates with niemann-pick C1 like-1 (NPC1L1) binding affinity: Comparison of multiple species NPC1L1 orthologs. *Mol. Pharmacol.* **71:** 19–29.
30. Sane, A. T., D. Sinnett, E. Delvin, M. Bendayan, V. Marcil, D. Menard, J. F. Beaulieu, and E. Levy. 2006. Localization and role of NPC1L1 in cholesterol absorption in human intestine. *J. Lipid Res.* **47:** 2112–2120.
31. Field, F. J., E. Born, and S. N. Mathur. 2004. Stanol esters decrease plasma cholesterol independently of intestinal ABC sterol transporters and Niemann-Pick C1-like 1 protein gene expression. *J. Lipid Res.* **45:** 2252–2259.
32. Yu, L., S. Bharadwaj, J. M. Brown, Y. Ma, W. Du, M. A. Davis, P. Michaely, P. Liu, M. C. Willingham, and L. L. Rudel. 2006. Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake. *J. Biol. Chem.* **281:** 6616–6624.
33. Klein, R. L., and L. L. Rudel. 1983. Cholesterol absorption and transport in thoracic duct lymph lipoproteins of nonhuman primates. Effect of dietary cholesterol level. *J. Lipid Res.* **24:** 343–356.
34. Iqbal, J., K. Anwar, and M. M. Hussain. 2003. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J. Biol. Chem.* **278:** 31610–31620.
35. Lange, Y., F. Strebel, and T. L. Steck. 1993. Role of the plasma membrane in cholesterol esterification in rat hepatoma cells. *J. Biol. Chem.* **268:** 13838–13843.
36. Tabas, I., W. J. Rosoff, and G. C. Boykow. 1988. Acyl coenzyme A: cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. *J. Biol. Chem.* **263:** 1266–1272.
37. Field, F. J., E. Born, H. Chen, S. Murthy, and S. N. Mathur. 1995. Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of p-glycoprotein. *J. Lipid Res.* **36:** 1533–1543.
38. Tremblay, A. J., B. Lamarche, J. S. Cohn, J. C. Hogue, and P. Couture. 2006. Effect of ezetimibe on the in vivo kinetics of apoB-48 and apoB-100 in men with primary hypercholesterolemia. *Arterioscler. Thromb. Vasc. Biol.* **26:** 1101–1106.
39. Alrefai, W., F. Annaba, Z. Sarwar, A. Dwivedi, S. Saksena, A. Singla, P. K. Dudeja, and R. Gill. 2006. Modulation of human Niemann-Pick C1 like 1 gene expression by sterol: Role of sterol regulatory element binding protein-2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292:** G369–G376.
40. Repa, J. J., K. K. Buhman, R. V. Farese, Jr., J. M. Dietschy, and S. D. Turley. 2004. ACAT2 deficiency limits cholesterol absorption in the cholesterol-fed mouse: impact on hepatic cholesterol homeostasis. *Hepatology.* **40:** 1088–1097.
41. Telford, D. E., B. G. Sutherland, J. Y. Edwards, J. D. Andrews, P. H. Barrett, and M. W. Huff. 2007. The molecular mechanisms underlying the reduction of LDL apoB100 by ezetimibe plus simvastatin. *J. Lipid Res.* **48:** 699–708.