Regulation of intestinal NPC1L1 expression by dietary fish oil and docosahexaenoic acid

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

Department of Veterans Affairs and Department of Internal Medicine, University of Iowa, Iowa City, IA 52242

Abstract **To address the effect of the n-3 fatty acid, docosahexaenoic acid (22:6), on proteins that play a role in cholesterol absorption, CaCo-2 cells were incubated with taurocholate micelles alone or micelles containing 22:6 or oleic acid (18:1). Compared with controls or 18:1, 22:6 did not interfere with the cellular uptake of micellar cholesterol. Apical cholesterol efflux was enhanced in cells incubated with 22:6. Cholesterol trafficking from the plasma membrane to the endoplasmic reticulum was decreased by 22:6. 22:6 decreased Niemann-Pick C1-Like 1 (NPC1L1) protein and mRNA levels without altering gene or protein expression of ACAT2, annexin-2, caveolin-1, or ABCG8. Peroxisome proliferator-activated receptor δ (PPARδ) activation decreased NPC1L1 mRNA levels and cholesterol trafficking to the endoplasmic reticulum, suggesting that 22:6 may act through PPARδ. Compared with hamsters fed a control diet or olive oil (enriched 18:1), NPC1L1 mRNA levels were decreased in duodenum and jejunum of hamsters ingesting fish oil (enriched 22:6). In an intestinal cell, independent of changes in ABCG8 expression, 22:6 increases the apical efflux of cholesterol. 22:6 interferes with cholesterol trafficking to the endoplasmic reticulum by the suppression of NPC1L1, perhaps through the activation of PPARδ.** **Moreover, a diet enriched in n-3 fatty acids decreases the gene expression of NPC1L1 in duodenum and jejunum of hamster.**—Mathur, S. N., K. R. Watt, and F. J. Field. **Regulation of intestinal NPC1L1 expression by dietary fish oil and docosahexaenoic acid.** ***J. Lipid Res.*** **2007.** 48: **395–404.**

Supplementary key words intestine • Niemann-Pick C1-Like 1 • cholesterol • n-3 fatty acids • cholesterol trafficking

Hypercholesterolemia is a significant risk factor for atherosclerosis. In addition to de novo cholesterol synthesis, cholesterol derived from the diet also contributes to the amount of cholesterol circulating in plasma (1). Moreover, reducing the intestinal absorption of dietary and biliary cholesterol will decrease plasma cholesterol levels (2). It is clear, therefore, that the intestine plays an important role in maintaining total body cholesterol homeostasis. During the past several years, numerous proteins, including ACAT2, caveolin-1, annexin-2, scavenger receptor class B type I, ABCG5, and ABCG8, have been implicated in facilitating/regulating cholesterol absorption by the intestinal absorptive cell (3–8). None of these proteins, however, has turned out to be the "putative" cholesterol transporter in the intestine, as individually these proteins are not essential for the uptake of luminal cholesterol by the absorptive cell (5, 7, 9–11). In the past few years, Altmann and his group (12–15) have described a plasma membrane transporter, Niemann-Pick C1-Like 1 (NPC1L1), which has been shown to play a critical role in the absorption of cholesterol by the intestine. Mice lacking NPC1L1 have a marked reduction in cholesterol absorption and do not get hypercholesterolemic in response to a high-cholesterol diet. The level of gene expression of NPC1L1 along the length of the small intestine in mice parallels the efficiency of cholesterol absorption, with the highest levels found in proximal intestine (duodenum and jejunum) and less expression found in distal intestine (ileum). NPC1L1 also contains an extracellular signal peptide, 13 putative transmembrane sequences, N-linked glycosylation sites, a conserved N-terminal "NPC1" domain, and a putative sterol-sensing domain (12, 16), all characteristics to suggest that this protein can function as a cholesterol transporter. Although NPC1L1 was felt to reside only in the brush-border membrane (BB) of intestinal cells, there is some newer evidence to suggest that it can also exist intracellularly and will translocate to the plasma membrane when cellular cholesterol is depleted (12, 15, 17). These studies and those of others have strongly suggested that NPC1L1 is the putative cholesterol transporter in the intestine (12, 14, 15, 17–19).

Polyenoic fatty acids derived from fish oil (n-3 fatty acids) have been shown to decrease cholesterol absorption (20–22) and reduce plasma cholesterol in both animal models and humans (23–29). The mechanism for how fish oil-derived fatty acids interfere with cholesterol

Abbreviations: BB, brush-border membrane; NBBM, non-brush-border membrane; NPC1L1, Niemann-Pick C1-Like 1; PPARδ, peroxisome proliferator-activated receptor δ; SREBP-1c, sterol-regulatory element binding protein 1c; 18:1, oleic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid.

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

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absorption is not clear. The primary aim of this study, therefore, was to investigate whether the fish oil-derived fatty acid, docosahexaenoic acid (22:6), would decrease intestinal NPC1L1 expression as a possible mechanism for why these fatty acids alter cholesterol absorption.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]cholesterol (48.3 Ci/mmol) and [^{14}C]acetate (56.5 mCi/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Delipidated fetal calf serum was from Intracel (Issaquah, WA). Protease inhibitor cocktail, sodium taurocholate, Tri Reagent, and oleic acid (18:1) were from Sigma Chemical (St. Louis, MO). 22:6 and anti-human ACAT2 polyclonal antibody were from Cayman Chemical (Ann Arbor, MI). Anti-human annexin-2 (sc-9061) and anti-human caveolin-1 (sc-894) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody anti-human ABCG8 was purchased from Novus Biochemicals (Littleton, CO). Rabbit polyclonal antibody anti-human ABCG5 was purchased from Alpha Diagnostics International, Inc. (San Antonio, TX). Rabbit polyclonal antibody anti-human NPC1L1 and anti-human β-actin were purchased from ABCAM (Cambridge, MA). The bicinchoninic acid protein assay kit, goat anti-rabbit HRP-conjugated antibody, and the SuperSignal West Femto maximum sensitivity substrate chemiluminescent detection kit were from Pierce Biotechnology, Inc. (Rockford, IL). Fish oil (50:05 triglyceride) was a generous gift from Ocean Nutrition Canada Limited (Bedford, Nova Scotia, Canada). Olive oil was purchased from a local grocery store. The peroxisome proliferator-activated receptor δ (PPARδ) agonist GW501516 was purchased from Alexis Biochemicals through AXXORA, LLC (San Diego, CA).

Cell culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in DMEM (GIBCO, Grand Island, NY) with 4.5 g/l glucose and supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Once the flasks reached 80% confluence, the cells were split and seeded at a density of 0.2×10^5 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.

Micelle preparation

To prepare 5 mM taurocholate ± 0.25 mM fatty acid ± 0.5 mM cholesterol micellar solutions, appropriate volumes of the stock solutions prepared in ethanol 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 for 24 h with 5 mM taurocholate micelles alone or 5 mM taurocholate micelles containing 0.25 mM 18:1 or 22:6 in DMEM. At 1, 3, and 6 h before harvesting of the cells, 0.15 ml of micellar solution containing 5 mM taurocholate, 2 μCi of [^{3}H]cholesterol, and 0.2 mM unlabeled cholesterol was added to 1.5 ml of apical medium containing the treatments. 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. The cell lipids were extracted with 1.5 ml of hexane-isopropyl alcohol-water (3:2:0.1, v/v/v). The radioactivity in the cellular lipid extract was estimated by counting in a Packard liquid scintillation counter.

Cholesterol efflux

Cells were labeled for 4 or 18 h as indicated in the figure legends with 2.5 μCi/well [^{3}H]cholesterol in the presence of 1% fetal calf serum and DMEM. After extensive washing to remove unincorporated labeled cholesterol, treatments were added to the upper chambers while the lower chambers received DMEM alone. Samples of the media from both chambers were collected as indicated in the figure legends. Aliquots from the media from both chambers were taken for counting to estimate [^{3}H]cholesterol efflux as indicated in the figure legends. After rinsing the cells with DMEM, cell lipids were extracted with hexane-isopropyl alcohol-water (3:2:0.1, v/v/v). The lipid extracts from cells were dried under nitrogen and taken up in 1 ml of chloroform, and aliquots were taken for counting to estimate cell-associated [^{3}H]cholesterol. In some experiments, cell lipids were separated by thin-layer chromatography to estimate label in unesterified and esterified cholesterol (30).

Esterification of plasma membrane cholesterol

Cells were incubated for 24 h with 5 mM taurocholate micelles with or without 0.25 mM 18:1 or 22:6 at 37°C. The basal medium was replaced with 2.5 ml of cold DMEM. The apical medium was replaced with 1 ml of cold DMEM containing 1% delipidated fetal calf serum and 2.5 μCi of [^{3}H]cholesterol, and cells were incubated for 2 h at 4°C to label plasma membrane cholesterol. Radiolabeled cholesterol was added to this medium in ethanol (1.2% final concentration). 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 the treatments to be tested (see figure legends). After incubation, cells were washed with cold phosphate-buffered saline, and the lipids were extracted twice directly from the cells on the filter by adding 1 ml of hexane-isopropanol-water (3:2:0.1, v/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 (31).

Estimation of cholesterol synthesis and accumulation of intermediates

Estimation of cholesterol synthesis and the accumulation of cholesterol intermediates was determined by a method described previously (32). Briefly, CaCo-2 cells were incubated for 24 h with 5 mM taurocholate micelles alone or micelles containing 0.25 mM 18:1 or 22:6. 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 three times with DMEM to remove unincorporated radiolabel, and 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 the addition of 2 ml of water. The nonsaponifiable lipids were extracted twice with 2 ml of 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 plate using hexane-diethyl ether-acetic

acid (80:20:1, v/v/v). The radioactivity in the lipid fractions was determined using a TLC scanner from Bioscan, Inc. (Washington, DC).

Measurement of cellular cholesterol mass by gas-liquid chromatography

Cholesterol and cholesteryl ester mass were determined by gas-liquid chromatography using cholestane as an internal standard (33). Briefly, cellular lipids were extracted with 1.5 ml of hexane-isopropyl alcohol-water (3:2:0.1, v/v/v) containing 5 μg of cholestane as an internal standard. An aliquot of the lipid extract was analyzed by gas-liquid chromatography to estimate unesterified cholesterol. In the remaining lipid extract, the cholesteryl esters were separated from unesterified cholesterol by thin-layer chromatography on silica gel plates using a solvent system of hexane-diethyl ether-acetic acid (80:20:1, v/v/v). Because cholestane and cholesteryl esters have the same mobility in this system, the band containing these lipids was scraped and mixed with 0.8 ml of water followed by 0.2 ml of 10 M NaOH and 3 ml of methanol. The esters were hydrolyzed at 100°C for 30 min. The mixture was cooled to room temperature, and cholesterol and cholestane were extracted twice with 4 ml of hexane. The cholesterol and cholestane in the hexane extract were then analyzed by gas-liquid chromatography.

Isolation of non-brush-border membrane and BB fractions of CaCo-2 cells

Isolation of the BB fraction was based on the procedure described by Hauser et al. (34). The cell preparations were kept at 4°C throughout the isolation procedure. The CaCo-2 cell monolayer was washed once with 1.5 ml of DMEM, and 0.5 ml of 2 mM Tris and 50 mM mannitol buffer, pH 7.8, containing protease inhibitors were added to each filter. 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 and 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 1,500 *g* for 15 min to sediment the non-brush-border membrane (NBBM) fraction. The resulting supernatant was collected and centrifuged at 30,000 *g* to sediment BB fractions. The NBBM and BB fractions were suspended in the homogenization buffer, and protein was estimated by the bicinchoninic acid method.

Immunoblot analysis of ACAT2, annexin-2, caveolin-1, ABCG8, actin, and NPC1L1 proteins

Equal amounts of protein from NBBM or BB fractions in 1× Laemmli sample buffer were separated by SDS-PAGE on an 8% porous gel (35) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). After rinsing in TBS (25 mM Tris-HCl, pH 7.5, and 150 mM sodium chloride), the membrane was air-dried for 15 min and 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 primary antibody was diluted 1,500-fold in TBS containing 0.05% Tween-20 and 5% nonfat dry milk (Blotto). After washing in TBS containing Tween-20, the membrane was then incubated 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 the SuperSignal West Femto maximum sensitivity substrate chemiluminescent detection kit (Pierce). 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.

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 (**Table 1**) and 2× SYBR Green PCR master mix (Applied Biosystems), and real-time RT-PCR was performed using the Bio-Rad iCycler system. The thermal cycler parameters were as follows: hold for 2 min at 50°C, 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 an endogenous internal standard. The relative expression of the gene was calculated using the comparative threshold cycle method (36).

Measurement of NPC1L1 mRNA in hamster intestine

The cDNA used to estimate NPC1L1 mRNA abundance was collected from a previous study (29). Samples were stored at −80°C.

TABLE 1. Primers used in this study

Primer Name	Sequence	Accession Number
Human annexin-2 F	5′-CCT-GTG-AGG-GTG- ACG-TTA-GCA-3′	BC001388.2
Human annexin-2 R	5′-GGA-AGG-CCA-GGC- AAT-GC-3′	
Human caveolin-1 F	5′-TCC-TTC-CTC-AGT- TCC-CTT-AAA-GC-3′	NM_001753.3
Human caveolin-1 R	5′-GCC-CGT-GGC-TGG- ATG-A-3′	
Human ABCG8 F	5′-AGC-CGC-CCT-CTT-GTT-CAT-G-3′	XM_055525.1
Human ABCG8 R	5′-GAG-TAA-CAT-TTG-GAG-ATG-ACA-TCC-A-3′	
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′	
Hamster NPC1L1 F	5′-CCT-GAC-CTT-TAT-AGA-ACT-CAC-CAC-AGA-3′	AY437866
Hamster NPC1L1 R	5′-GGG-CCA-AAA-TGC-TCG-TCA-T-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, Niemann-Pick C1-Like 1; R, reverse.

To ensure the quality of the cDNA and validate the results, 18S and sterol-regulatory element binding protein 1c (SREBP-1c) mRNA levels were estimated simultaneously. The relative threshold cycle values for RT-PCR for 18S and SREBP-1c were similar to our previous results, and the pattern of SREBP-1c gene expression in the intestine was exactly as reported previously (29).

Other analyses

Protein content was estimated using the bicinchoninic acid kit (Pierce). Statistical analysis was performed by one-way ANOVA for comparison of the treatments.

RESULTS

Effects of 18:1 or 22:6 on the uptake of micellar cholesterol

To address whether 22:6 interferes with cholesterol uptake from bile salt micelles, CaCo-2 cells were incubated for 24 h with 5 mM taurocholate micelles or micelles containing 0.25 mM 22:6. To compare 22:6 with another fatty acid, cells were also incubated with micelles containing 0.25 mM 18:1, a fatty acid found in abundance in a Western diet. At 1, 3, and 6 h before harvesting the cells, 0.15 ml of a micellar solution containing 5 mM taurocholate, 2 μCi of [^{3}H]cholesterol, and 0.2 mM unlabeled cholesterol was added to the apical medium containing the treatments. The amount of cholesterol taken up by cells was then estimated. The results are shown in **Fig. 1**. Compared with cells incubated with 18:1, at 1 and 3 h, cell-associated labeled cholesterol was actually somewhat greater in cells incubated with 22:6. Compared with cells incubated with micelles alone, cells incubated with 22:6 took up similar amounts of micellar cholesterol. By 6 h, however, the uptake of cholesterol was similar for controls and both fatty acids. Thus, compared with 18:1, 22:6 did not decrease the amount of micellar cholesterol taken up by the cell.

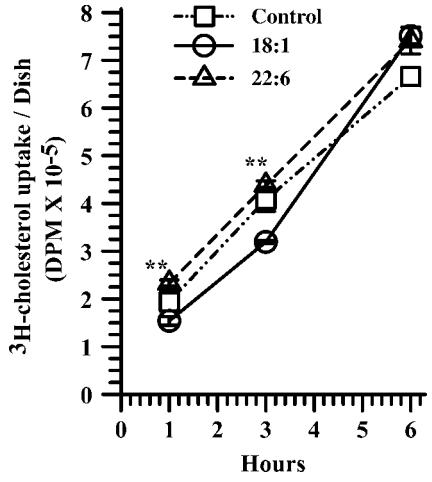

Fig. 1. Effects of oleic acid (18:1) or docosahexaenoic acid (22:6) on the uptake of micellar cholesterol. CaCo-2 cells were incubated for 24 h, with the 5 mM taurocholate micelles containing 0.25 mM 18:1 or 22:6. At 1, 3, and 6 h before harvesting the cells, the apical medium was supplemented with 0.15 ml of a micellar solution of 5 mM taurocholate, 2 μCi of [^{3}H]cholesterol, and 0.2 mM unlabeled cholesterol. At the end of the incubation, the cells were washed thoroughly and cellular lipids were extracted. The radioactivity in the cellular lipid extract was then estimated. The values shown are means ± SEM of three dishes at each time point. ** $P <$ 0.01 (significantly different from 18:1).

Effects of 18:1 or 22:6 on apical efflux of plasma membrane cholesterol

To examine the effects of 22:6 on apical cholesterol efflux, CaCo-2 cells were prelabeled with cholesterol for 4 h at 37°C. After extensive washing to remove unincorporated label, cells were incubated for 20 h with taurocholate micelles containing increasing concentrations of 18:1 or 22:6. The results are shown in **Fig. 2**. Compared with cells incubated with micelles containing 18:1, significantly more cholesterol effluxed from the apical membrane to micelles containing 22:6 (left panel). Similarly, in a time course of 24 h, significantly more apical membrane cholesterol effluxed to micelles containing 22:6 than to micelles alone or micelles containing 18:1 (right panel). Thus, micellar 22:6 enhances the efflux of cholesterol from the plasma membrane into the lumen.

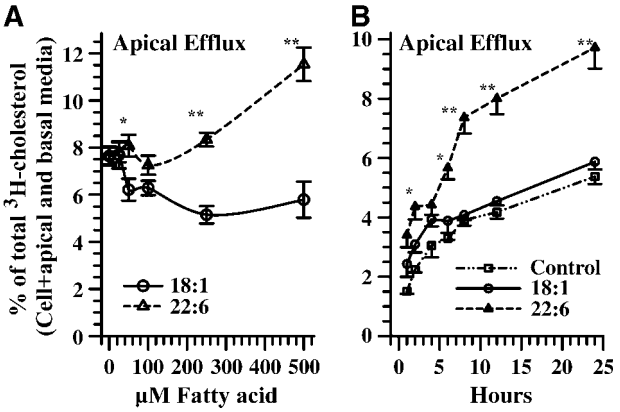

Fig. 2. Effects of 18:1 or 22:6 on the apical efflux of plasma membrane cholesterol. A: Cells were labeled for 4 h at 37°C with 2.5 μCi/dish [^{3}H]cholesterol in the presence of 1% fetal calf serum. After extensive washing to remove unincorporated label, 0, 25, 50, 100, 250, or 500 μM 18:1 or 22:6 in 5 mM taurocholate micelles was added to the upper chambers. At the end of the 20 h incubation, the total radioactivity recovered in apical medium, basal medium, and cells was determined and the percentage of [^{3}H]cholesterol effluxed into the apical medium was calculated. The values shown are means ± SEM of four dishes at each concentration of the fatty acid. * $P < 0.05$, ** $P < 0.01$ (significantly different from 18:1). B: Cells were labeled for 18 h with 2.5 μCi/well [^{3}H]cholesterol in the presence of 1% fetal calf serum. After extensive washing to remove unincorporated label, 0.25 mM 18:1 or 22:6 in 5 mM taurocholate micelles was added to the upper chambers. Aliquots from the media from both chambers were taken for counting at 1, 2, 4, 6, 8, 12, and 24 h to estimate [^{3}H]cholesterol efflux. At the end of the incubation, total radioactivity recovered in apical medium, basal medium, and cells was determined and the percentage of [^{3}H]cholesterol effluxed into the apical medium was calculated. * $P < 0.05$, ** $P < 0.01$ (significantly different from 18:1).

Trafficking of plasma membrane cholesterol to the endoplasmic reticulum

To address whether 22:6 alters the influx of plasma membrane cholesterol to the endoplasmic reticulum, cells were first incubated for 24 h with 5 mM taurocholate micelles with or without 0.25 mM 18:1 or 22:6. Plasma membrane cholesterol was then labeled at 4°C as described in the figure legends. After washing the cells, they were incubated at 37°C for 1, 3, and 6 h in the presence of the treatments. In another set of cells, 0.5 mM cholesterol was added to the micelle to "drive" more plasma membrane cholesterol to the endoplasmic reticulum. The amount of labeled cholesteryl esters was determined to estimate the amount of plasma membrane cholesterol reaching ACAT in the endoplasmic reticulum. The results are shown in **Fig. 3**. Compared with cells incubated with micelles containing 18:1, cells incubated with 22:6 contained significantly less labeled cholesteryl esters, suggesting that compared with 18:1, 22:6 caused less plasma membrane cholesterol to influx to the endoplasmic reticulum (left panel). Compared with control cells that were not stimulated to synthesize or secrete lipoproteins (micelles alone), cells incubated with 18:1 contained significantly more cholesteryl esters, whereas cells incubated with 22:6 contained significantly less. Including cholesterol in the micelle did not change these observations (right panel). Even with excessive amounts of cholesterol entering the cell, less plasma membrane cholesterol was trafficking to the endoplasmic reticulum in cells incubated with 22:6. Although both fatty acids will drive lipoprotein synthesis and secretion, compared with 18:1, 22:6 appears to interfere in some way with the trafficking of plasma membrane cholesterol to the endoplasmic reticulum.

To further address and support this observation, interference of cholesterol trafficking to the endoplasmic reticulum was estimated by measuring the accumulation of newly synthesized cholesterol intermediates in cells incubated with 18:1 or 22:6 (32, 37). **Figure 4** shows these data. Compared with control cells incubated with micelles alone, 22:6 decreased total sterol synthesis and concomi-

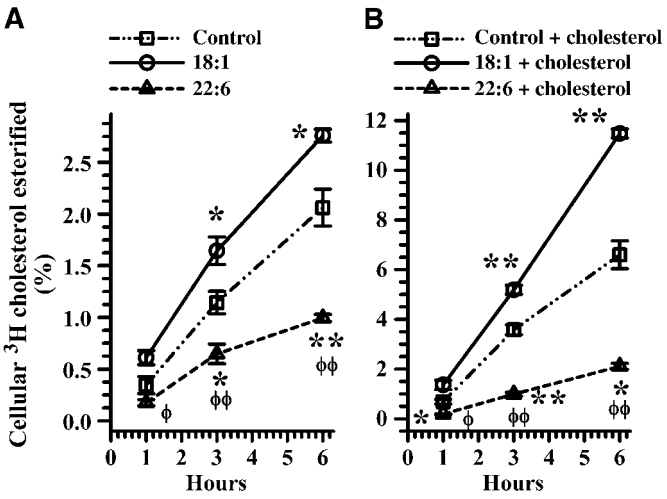

Fig. 3. Trafficking of plasma membrane cholesterol to the endoplasmic reticulum. Cells were incubated for 24 h at 37°C with 1.5 ml of medium containing 5 mM taurocholate micelles alone or micelles containing 0.25 mM 18:1 or 22:6. Plasma membrane cholesterol was labeled by incubating cells for 2 h at 4°C with 2.5 μCi of [^{3}H]cholesterol in 1 ml of DMEM containing 1% delipidated fetal calf serum. Cells were washed to remove unincorporated label. The basal medium was replaced with 2.5 ml of warm (37°C) DMEM. The cells were then incubated at 37°C with 1.5 ml of apical medium containing 5 mM taurocholate micelles alone or micelles containing 0.25 mM 18:1 or 22:6 without (A) or with (B) 0.5 mM unlabeled cholesterol for 1, 3, and 6 h. After rinsing the cells, cell lipids were extracted and aliquots were taken for counting to estimate cell-associated [^{3}H]cholesterol. The remainder of the lipid extract was analyzed by thin-layer chromatography to estimate radioactivity in unesterified and esterified cholesterol. The values shown are means ± SEM of three dishes at each time point. * $P < 0.05$, ** $P < 0.01$ [significantly different from 5 mM taurocholate micelles alone (control)]; $^{\Phi}$ $P < 0.01$, $^{\Phi\,\Phi}$ $P < 0.01$ (significantly different from 18:1).

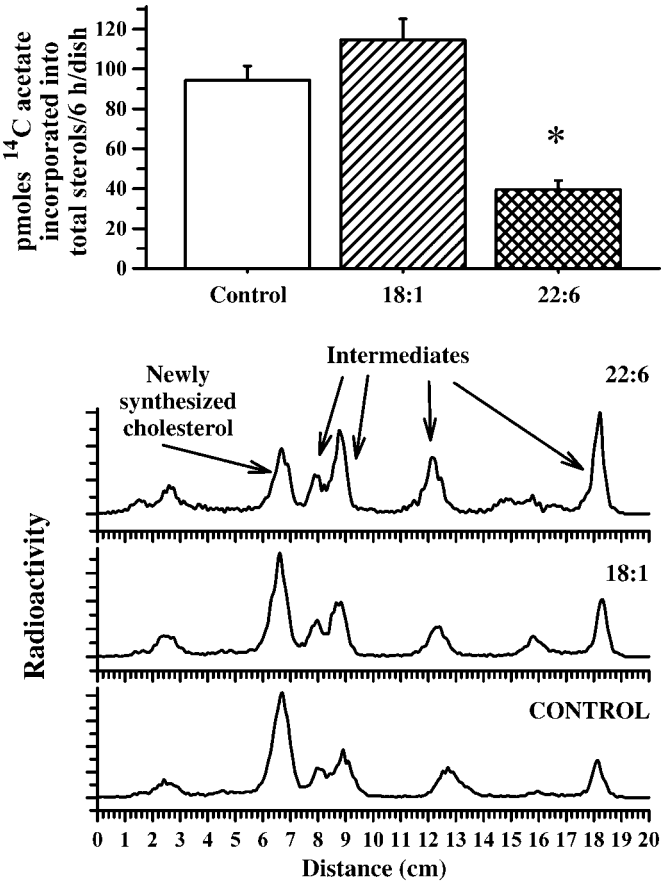

Fig. 4. Estimation of cholesterol synthesis and accumulation of intermediates. CaCo-2 cells were incubated for 24 h with 5 mM taurocholate micelles alone or micelles containing 0.25 mM 18:1 or 22:6. To label newly synthesized cholesterol, 0.15 ml of DMEM containing labeled [^{14}C]acetate was added to the apical medium containing the treatments, and the incubation was continued for another 6 h. The incorporation of label into cholesterol and its intermediates was estimated as described in Experimental Procedures. The values shown are means ± SEM of three filters. Compared with controls or cells incubated with 18:1, there was a significant accumulation of cholesterol intermediates in cells incubated with 22:6. * $P < 0.05$ versus control or 18:1.

tantly caused the accumulation of cholesterol intermediates, suggesting that 22:6 interferes with the return of cholesterol intermediates to the endoplasmic reticulum for the completion of cholesterol synthesis. In contrast to 22:6, 18:1 caused a modest increase in total sterol synthesis without the accumulation of intermediates. Thus, compared with 18:1, 22:6 will limit the amount of plasma membrane cholesterol available for esterification and transport into lymph.

This observation was substantiated further when unesterified cholesterol and cholesteryl ester mass were estimated in cells after incubation with the respective fatty acid. Compared with the amount of unesterified cholesterol in cells incubated with 18:1, unesterified cholesterol was similar in cells incubated with 22:6 (38 ± 1 vs. 41 ± 4 μg/dish; n = 4 dishes). Cholesteryl esters, however, were significantly less abundant in cells incubated with 22:6 (4 ± 0.15 vs. 2 ± 0.2 μg/dish; n = 4 dishes). In another experiment, when 0.5 mM cholesterol was supplied in micelles with the fatty acids, the amount of unesterified cholesterol in cells incubated with 18:1 was similar to that in cells incubated with 22:6 (86 ± 1 vs. 81 ± 4 μg/dish; n = 4 dishes). Cholesteryl esters, however, were significantly less abundant in cells incubated with 22:6 (121 ± 16 vs. 16 ± 0.7 μg/dish; n = 4 dishes). Thus, interfering with cholesterol influx from the plasma membrane to the endoplasmic reticulum reduces the amount of cholesteryl ester mass available for transport.

Effects of 22:6 on ACAT2, annexin-2, caveolin-1, ABCG8, and NPC1L1 protein mass and mRNA

To address whether 22:6 altered the protein mass of any of the previously mentioned potential cholesterol transporters, cells were incubated for 24 h with micelles alone or micelles containing either 18:1 or 22:6. To determine whether the effects of 22:6 were specific for this n-3 fatty acid, cells were also incubated with micelles containing eicosapentaenoic acid (20:5), another n-3 fatty acid that is also enriched in fish oil. After incubation, the masses of caveolin-1 and ACAT2 were estimated in NBBM, as these proteins were found exclusively in this fraction. Annexin-2, ABCG8, and NPC1L1 masses were determined in BB fractions. For reasons that were not clear, ABCG5 protein was not detectable. Compared with cells incubated with taurocholate micelles alone, cells incubated with 18:1, 20:5, or 22:6 contained similar amounts of ACAT2, annexin-2, caveolin-1, and ABCG8 protein (data not shown). In contrast, there was significantly less NPC1L1 protein mass in cells incubated with 22:6 compared with cells incubated with 18:1 or micelles alone (**Fig. 5**, lower panel). There was a tendency for cells incubated with 20:5 to have less NPC1L1 mass, but this did not reach statistical significance.

mRNA abundance for these proteins was also estimated after incubation with the respective fatty acid. Annexin-2, caveolin-1, and ABCG8 mRNA levels were similar in cells incubated with taurocholate micelles alone, 18:1, 20:5, or 22:6 (data not shown). In contrast, compared with control cells or cells incubated with 18:1, NPC1L1 mRNA levels

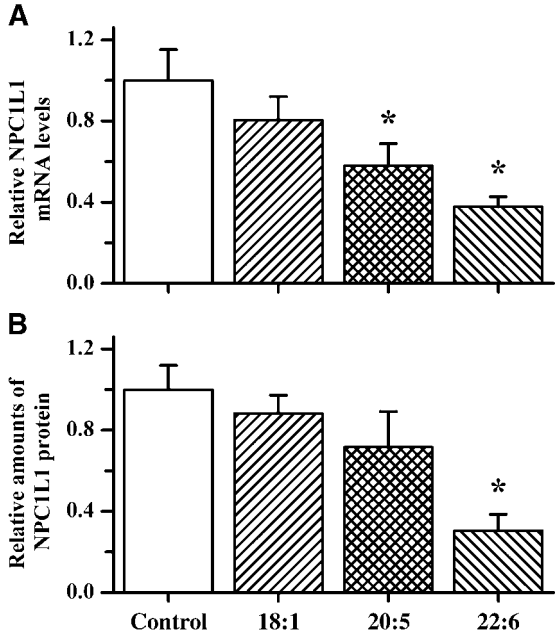

Fig. 5. Niemann-Pick C1-Like 1 (NPC1L1) protein mass and mRNA abundance. CaCo-2 cells were incubated for 24 h with 5 mM taurocholate micelles alone or micelles containing 0.25 mM 18:1, eicosapentaenoic acid (20:5), or 22:6. Brush-border and non-brush-border membrane fractions were isolated, and proteins were separated by SDS-PAGE. Annexin-2, ACAT2, caveolin-1, ABCG8, ABCG5, NPC1L1, and actin masses were estimated by immunoblotting. The relative values shown for NPC1L1 were normalized to the actin density on the blots to account for any differences in protein loading and transfer. Two or three samples were analyzed in each experiment by pooling cells from four dishes for each sample. A total of 15 samples for controls, 18:1, and 22:6 were analyzed in six separate experiments. B: For 20:5, 11 samples were analyzed in four separate experiments. Following the same experimental protocol described above, NPC1L1 mRNA abundance was determined by real-time RT-PCR as described in Experimental Procedures (A). Values shown represent means ± SEM of nine dishes. * $P < 0.05$ versus control or 18:1.

were significantly less abundant in cells incubated with 20:5 or 22:6 (Fig. 5, upper panel). Thus, 20:5 and 22:6 decrease the gene expression of NPC1L1, suggesting that fatty acids enriched in fish oil attenuate NPC1L1 expression in intestine.

Effects of diets enriched in 18:1 or 22:6 and 20:5 on hamster intestine NPC1L1 gene expression

To address whether the suppression of NPC1L1 gene expression by 22:6 and 20:5 observed in cell culture represented changes that would occur in vivo, hamsters were fed for 2 weeks a control diet or diets containing 10% olive oil (enriched in 18:1) or fish oil (enriched in 20:5 and 22:6) (29). At the end of the dietary period, the small intestine was divided into three equal segments representing duodenum, jejunum, and ileum. mRNA expression of NPC1L1 was then estimated in cells that were isolated from the three segments representing villus tip through crypt cells, as described in Experimental Procedures. The results are shown in **Fig. 6**. In all dietary

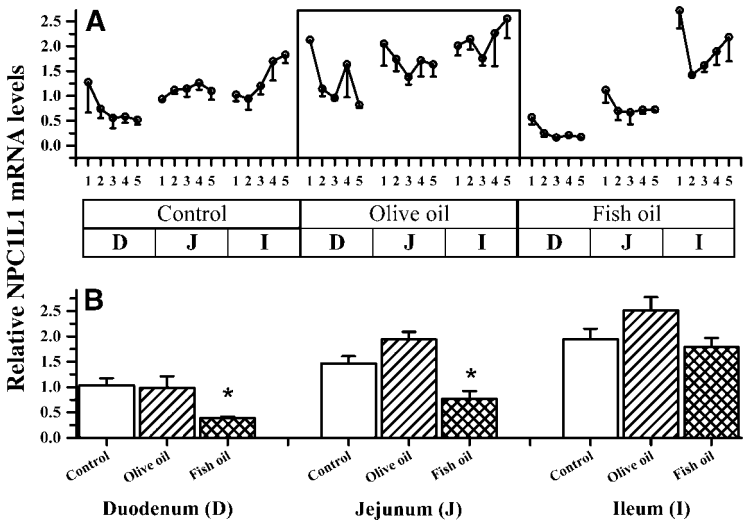

Fig. 6. Effects of diets enriched in 18:1 or 22:6 and 20:5 on hamster intestine NPC1L1 gene expression. Hamsters were fed for 2 weeks a control diet or diets enriched in olive oil or fish oil. From cell fractions isolated from duodenum (D), jejunum (J), and ileum (I), total RNA was extracted and analyzed by real-time RT-PCR as described in Experimental Procedures. A: mRNA levels in different cell populations along the villus axis, where fractions 1 through 5 represent cells from villus tips to crypts, respectively. B: Depicts the combined average mRNA levels of the three intestinal segments. The data represent means ± SEM of four animals on each diet. * $P < 0.05$ (significantly different from control or olive oil).

groups, there was a trend of NPC1L1 gene expression to be highest in the ileum compared with more proximal intestine (bottom panel). Moreover, in proximal intestine, NPC1L1 expression tended to be higher in villus tip cells compared with villus crypt cells. In the ileum, however, this trend was not apparent and, perhaps, was even reversed (top panel). In duodenal and jejunal segments, NPC1L1 mRNA levels were significantly less in animals fed the fish oil diet compared with animals fed the control diet or the olive oil diet (bottom panel). Thus, in proximal intestine, a diet enriched in n-3 polyunsaturated fatty acids resulted in the suppression of intestinal NPC1L1 gene expression.

Cell differentiation and gene expression

As demonstrated above in proximal hamster intestine, NPC1L1 gene expression was highest in more differentiated cells of the villus tip compared with gene expression in less differentiated cells of the crypt. This suggests that there may be a gradient of expression of NPC1L1 in intestinal cells as cells differentiate up the villus. To assess whether this was true in CaCo-2 cells as they differentiate in culture, NPC1L1 mRNA levels were estimated in cells grown for 2–14 days, least to most differentiated cells, respectively. For interest, annexin-2, caveolin-1, and ABCG8 mRNA levels were also estimated. The results are shown in **Fig. 7**. It is clear that NPC1L1 expression increases with time in culture, suggesting that as intestinal cells differentiate, NPC1L1 mRNA becomes more abundant. These results support the observations obtained in hamster intestine. In contrast, the trend was opposite or nonexistent for the gene expression of annexin-2, caveolin-1, and ABCG8.

Activation of PPARδ reduces the trafficking of plasma membrane cholesterol to the endoplasmic reticulum and decreases NPC1L1 gene expression

It is recognized that polyunsaturated fatty acids, including 22:6, will activate PPARδ (38, 39). In a recent study, van der Veen et al. (40) found that activation of PPARδ resulted in the suppression of NPC1L1 expression, similar to what we observed with 22:6. Thus, we questioned the role of PPARδ activation in cholesterol trafficking. To examine this, cells were incubated for 18 h with or without

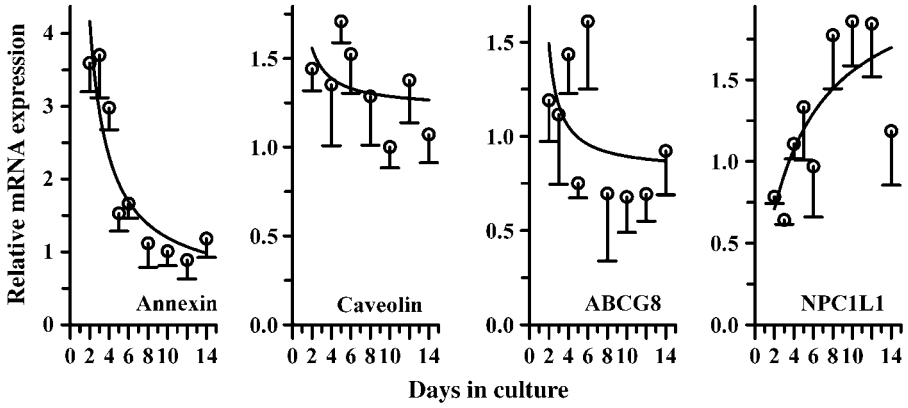

Fig. 7. Cell differentiation and gene expression. CaCo-2 cells at different stages of differentiation were obtained by harvesting the cells grown on six-well plastic dishes at 2, 3, 4, 5, 6, 8, 10, 12, or 14 days after plating. RNA was extracted and analyzed by real time RT-PCR as described in Experimental Procedures. The data represent values from two experiments with means ± SEM of eight dishes for each day.

100 nM GW501516, a specific agonist for PPARδ. Plasma membrane cholesterol was then labeled at 4°C, and the cells were warmed to 37°C in the presence of 5 mM taurocholate micelles containing 0.5 mM cholesterol. At the end of 6 h, the amount of cellular labeled cholesteryl esters was estimated. Compared with control cells incubated without the PPARδ agonist, cells incubated with GW501516 contained 25% less labeled cholesteryl esters ($P < 0.01$; n = 6 dishes), suggesting that PPARδ activation interfered with the trafficking of plasma membrane cholesterol to the endoplasmic reticulum. Similar to the observations by van der Veen et al. (40), we also found that PPARδ activation modestly decreased NPC1L1 mRNA levels by 35% ($P < 0.05$; n = 7 dishes). Thus, these data support the possibility that 22:6 activates PPARδ, which in turn leads to the suppression of NPC1L1 and a decrease in the movement of plasma membrane cholesterol to the endoplasmic reticulum.

DISCUSSION

The major new observations from this study are that compared with 18:1, dietary fatty acids derived from fish oil, 22:6 and 20:5, cause a decrease in the expression of intestinal NPC1L1 and that, in animals ingesting a diet enriched in fish oil, NPC1L1 gene expression is decreased in proximal intestine. Because NPC1L1 is clearly important in facilitating cholesterol absorption, these findings may have important clinical implications. A diet that includes the regular ingestion of fish, or is supplemented by fish oil, is likely to have beneficial effects on plasma cholesterol levels by reducing the absorption of luminal cholesterol through its action on intestinal NPC1L1 expression.

It is still not entirely clear how NPC1L1 facilitates cholesterol absorption. It could act alone by binding luminal cholesterol and facilitating its uptake into the cell, or it could be part of a transport complex that indirectly influences cholesterol transport. In our previous work in CaCo-2 cells, we demonstrated that micellar cholesterol is taken up at the apical membrane of the cell (31). This micellar cholesterol displaces membrane cholesterol, causing it to move or "cluster" into specialized microdomains consistent with membrane "rafts." When a "concentration threshold" is reached, cholesterol from these rafts is transported to the endoplasmic reticulum for esterification and transport. It is possible, therefore, that NPC1L1 could facilitate any one (or more) of these steps in cholesterol absorption either by itself or in combination with other proteins. The present results, however, favor NPC1L1 facilitating steps beyond the uptake of cholesterol into the cell. The expression of NPC1L1 in BB was decreased in cells incubated with 22:6, yet 22:6 did not interfere with cholesterol uptake from bile salt micelles. Rather, the predominant effect of 22:6 was the interference of cholesterol trafficking from the plasma membrane to the endoplasmic reticulum. Thus, NPC1L1 could facilitate the clustering of cholesterol into specialized raft domains, or it could act as a "chaperone protein" for the trafficking of cholesterol from rafts to the endoplasmic reticulum. Although our data cannot distinguish between these two possibilities, NPC1L1 expression was decreased by 22:6 in BB without altering its expression in NBBMs (personal observations). This would implicate the BB as the possible site of action for NPC1L1 and perhaps suggest that NPC1L1 facilitates the movement of plasma membrane cholesterol to rafts. Because NPC1L1 has a cholesterol-sensing domain, it is tempting to speculate that NPC1L1 acts as a detector, monitoring the concentration threshold of cholesterol within rafts and initiating the influx of cholesterol from these rafts to the endoplasmic reticulum. Neither of these processes would necessarily imply that NPC1L1 binds to the sterol itself. Although 22:6 and 20:5 decrease the expression of intestinal NPC1L1 and interfere with cholesterol trafficking, these fatty acids could also interfere with the activity or function of NPC1L1 independent of their effects on NPC1L1 gene or protein expression.

From these results, one could also argue that micelles containing 22:6 decrease cholesterol absorption by enhancing cholesterol efflux back into the lumen. This observation was not totally unexpected given the results described above. If by suppressing NPC1L1 by 22:6, cholesterol trafficking from the plasma membrane to the endoplasmic reticulum is disrupted, it would be expected that more plasma membrane cholesterol would be available for efflux. Using a similar argument, 18:1, which enhances cholesterol influx, would result in less cholesterol being available for efflux (Fig. 3, left panel). An increase in cholesterol efflux observed with micellar 22:6 was unrelated to any changes in expression of ABCG5/ABCG8, a complex that is proposed to facilitate the efflux of cholesterol from absorptive cells back into the lumen (5, 41, 42). It is also possible that changes in membrane fatty acid composition could contribute to this enhanced cholesterol efflux. It is clear that membrane fatty acid composition alters the movement of cholesterol within a membrane (43, 44). Compared with membranes enriched in monounsaturated fatty acids, membranes enriched in polyunsaturated fatty acids are more "fluid" and are more likely to transfer cholesterol to an acceptor (43–45). Compared with the influx of plasma membrane cholesterol to the endoplasmic reticulum in control cells (in the absence of fatty acid), cholesterol influx was markedly enhanced in cells incubated with 18:1. Despite an increase in cholesterol influx, neither NPC1L1 mRNA nor NPC1L1 protein mass was altered in cells incubated with 18:1. This would imply that there is sufficient NPC1L1 in intestinal cells to perform its function when cells are exposed to dietary fatty acids that drive lipoprotein synthesis and secretion. This is somewhat reminiscent of ACAT2, apoprotein B-48, and triglyceride transfer protein, proteins intricately involved in intestinal lipoprotein assembly. The expression of these essential proteins also is not enhanced in the face of acute fatty acid influx, suggesting that they are all in ample supply within the intestinal cell (46–50). This seems to make good sense in a cell that needs to efficiently and rapidly transport fat and cholesterol when challenged by a fatty meal.

PPARδ is a receptor that has been shown to enhance reverse cholesterol transport (51). Long-chain polyunsaturated

fatty acids, including 22:6, have been identified as natural activators of PPARδ (38, 39, 52). In a recent study, PPARδ activation resulted in a 2.5-fold increase in fecal cholesterol excretion in wild-type and ABCA1 null mice (40). Intestines of animals that received the PPARδ activator had a 40% decrease in NPC1L1 gene expression without altering ABCG5, ABCG8, ABCA1, or ACAT2 expression. Cholesterol absorption was decreased by some 40%, an amount felt to be insufficient to explain the total amount of fecal cholesterol lost. Although cellular cholesterol efflux was not measured, it was postulated that PPARδ activation enhanced the excretion of plasma cholesterol directly into the gut lumen. We would argue that cholesterol efflux from intestinal cells into the lumen can occur independently of changes in ABCG5/ABCG8 expression (present results and personal observations) and that there is little need to implicate a direct passage of cholesterol from plasma into gut lumen. Moreover, in that study (40) as well as ours, in CaCo-2 cells, PPARδ activation decreased NPC1L1 gene expression. As already mentioned, it is possible that in our study, 22:6 activated PPARδ, which in turn decreased NPC1L1 expression, interfering with cholesterol influx to the endoplasmic reticulum. Further studies will be necessary to more firmly establish the link between 22:6, PPARδ, and NPC1L1.

In hamster intestine, independent of the fat ingested, NPC1L1 gene expression was lowest in the duodenum compared with the jejunum or ileum. There was a trend for NPC1L1 expression to increase down the length of the small intestine, although the differences observed between segments representing jejunum and ileum were not marked. In contrast, in rat intestine, it has been shown that distal small intestine contained much less NPC1L1 mRNA than proximal intestine, and this has been used as an argument for its importance in cholesterol absorption (12). In human intestine, however, there appears to be very little difference in the expression of NPC1L1 in duodenum, jejunum, and ileum (17). What is clear from the present data, however, is that in duodenum and jejunum, NPC1L1 expression was significantly less in hamsters fed fish oil compared with animals fed olive oil or a control diet. This complements our findings in cultured intestinal cells and supports the notion that fatty acids derived from fish will have a beneficial effect on plasma cholesterol by reducing cholesterol absorption through its suppression of intestinal NPC1L1 expression.

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