

Niemann-Pick C1-Like 1 Mediates α -Tocopherol Transport

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ABSTRACT

Dietary lipids and fat-soluble micronutrients are solubilized in mixed micelles and absorbed in the small intestine. Based on an assumption that cholesterol and other fat-soluble molecules share a number of transport mechanisms and the fact that Niemann-Pick C1-like 1 (NPC1L1) is critical for intestinal cholesterol absorption, we hypothesized that some fat-soluble molecules may be transported by NPC1L1. To investigate this hypothesis, we compared the cellular uptake and inhibitory effects of ezetimibe, the molecular target of which is NPC1L1, between cholesterol and some fat-soluble molecules using rat NPC1L1-overexpressing Caco-2 cells. The *in vitro* analysis suggested that NPC1L1 mediates the uptake of α -tocopherol (vitamin E) in an ezetimibe-sensitive manner as well as the uptake of cholesterol but does not mediate the uptake of retinol (vitamin A) or cyclosporin A. To confirm the ezetimibe-sensitive

uptake of α -tocopherol *in vivo*, we performed an *in vivo* absorption study using rats and the results suggested a physiologically significant role of NPC1L1-mediated α -tocopherol absorption. Furthermore, using human NPC1L1 overexpression system, we demonstrated that both cholesterol and α -tocopherol uptake was also significantly increased by the overexpression of human NPC1L1 and ezetimibe inhibited their uptake. Mutual inhibition studies of cholesterol and α -tocopherol in human NPC1L1-mediated uptake revealed the inhibitory effect of cholesterol and the stimulatory effect of α -tocopherol on the NPC1L1-mediated transport of both substrates. The present data suggest, for the first time, that NPC1L1 has the ability to transport α -tocopherol and that ezetimibe is able to inhibit the intestinal absorption of α -tocopherol.

Intestinal absorption is an important process in the maintenance of cholesterol homeostasis in the body. Although the mechanism of cholesterol uptake from the intestinal lumen is poorly understood, the identification of ezetimibe as a potent selective inhibitor of intestinal cholesterol absorption suggests that this process is mediated by a specific transport system (van Heek et al., 2001; Patel et al., 2003). Through studies designed to understand the mechanism by which ezetimibe inhibits cholesterol absorption, Niemann-Pick C1-like 1 (NPC1L1) was identified as a critical factor for intestinal cholesterol absorption (Altmann et al., 2004), because NPC1L1 knockout mice exhibited reduction in intestinal cholesterol absorption, and the low level of remaining cholesterol absorption was insensitive to ezetimibe treatment (Altmann et al., 2004; Davis et al., 2004). These results, together with the finding that NPC1L1 is expressed in the apical membrane of the small intestine, particularly in the jejunum, where most sterol absorption takes place (Altmann et al.,

2004; Davis et al., 2004; Sané et al., 2006), suggest that NPC1L1 is involved in the intestinal absorption of cholesterol and is a target of ezetimibe.

In our previous study, we established a Caco-2 cell line that stably overexpresses rat NPC1L1 (Yamanashi et al., 2007). Using these cells, we demonstrated that the uptake of cholesterol and plant sterols from micelles was increased by rat NPC1L1 overexpression and that the uptake was inhibited by ezetimibe. Under physiological conditions, cholesterol is present as mixed micelles formed by bile salts and phospholipids in the intestinal lumen. Other fat-soluble micronutrients, such as fat-soluble vitamins and parts of fat-soluble drugs, are also known to be solubilized in mixed micelles and then absorbed in the intestine. Cholesterol and other fat-soluble molecules, which also exhibit absorption from biliary micelles, may be taken up in the intestinal lumen via the shared pathway.

In the current study, we focused on the possibility that NPC1L1 mediates the uptake of fat-soluble micronutrients, which are incorporated into micelles in addition to the uptake of cholesterol. To test this possibility, we examined the inhibitory effect of ezetimibe on the uptake of α -tocopherol (vitamin E), retinol (vitamin A) and cyclosporin A as examples of fat-soluble micronutrients and drugs by using rat

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ABBREVIATIONS: NPC1L1, Niemann-Pick C1-like 1; BSA, bovine serum albumin; TBS-T, Tris-buffered saline/Tween 20; HA, hemagglutinin; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

NPC1L1-overexpressing Caco-2 cells. In addition, to confirm ezetimibe-sensitive uptake of α -tocopherol *in vivo*, we performed an *in vivo* absorption study using rats. Furthermore, we constructed human NPC1L1 overexpressing Caco-2 cells and examined the transport activity of human NPC1L1 for cholesterol and α -tocopherol.

The present study suggests that the uptake of α -tocopherol, the most relevant form of vitamin E, is partly mediated by NPC1L1 in enterocytes, and its transport is inhibited by ezetimibe. Vitamin E is used clinically for the prevention of arteriosclerosis, which involves cholesterol accumulation. NPC1L1, which is known to be a cholesterol transporter, might protect the body from the harmful effects of excess cholesterol by dual transport of cholesterol and α -tocopherol.

Materials and Methods

Materials. [$1\alpha,2\alpha$ - ^3H]Cholesterol (44.0 Ci/mmol), D- α -[5-methyl- ^{14}C]tocopherol (57.0 mCi/mmol), and [N -methyl-butenyl-methyl-threonine- β - ^3H]cyclosporin A (7.00 Ci/mmol) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). [4 - ^{14}C]Cholesterol (53 mCi/mmol) and [$11,12$ - ^3H]retinol (45.5 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Sodium taurocholate was obtained from Sigma Aldrich (St. Louis, MO), whereas ezetimibe was obtained from Sequoia Research Products Ltd (Pangbourne, UK). Caco-2 cells were obtained from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). pcDNA3.1(+) vector was obtained from Invitrogen (Carlsbad, CA). BamHI and EcoRI restriction enzymes were obtained from Takara (Shiga, Japan). Anti HA-tag antibody [HA-probe (Y-11)] was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). All other chemicals used were commercially available and of reagent grade.

Male Wistar rats were obtained from SLC Inc. (Shizuoka, Japan). All animals used in this study were housed in temperature- and

humidity-controlled animal cages with a 12-h dark/light cycle and with free access to water and standard animal chow (MF, Oriental Yeast, Tokyo, Japan). All experiments involving rats were conducted by using protocols approved by the Animal Studies Committee of the University of Tokyo.

Construction of Human NPC1L1-Overexpressed Caco-2 Cells. Human NPC1L1 cDNA was amplified by polymerase chain reaction from Caco-2 mRNA. The complete NPC1L1 cDNA (GenBank accession number AY437865) was amplified with the BamHI site at the 5' end, and with the EcoRI site and HA tag (YPYDVPDYA) sequence attached to the 3'-end by polymerase chain reaction and then inserted into pcDNA3.1(+) vector plasmid. NPC1L1 in pcDNA3.1(+) was transfected into Caco-2 cells grown on a six-well plate with FuGene 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the user's manual. Then, Caco-2 cells were selected by culturing in the presence of 500 $\mu\text{g/ml}$ G418 sulfate (Nacalai Tesque, Osaka, Japan) and 16 $\mu\text{g/ml}$ ezetimibe.

Cell Culture. Caco-2 cells stably transfected with rat NPC1L1-HA cDNA (rat NPC1L1 cells; Yamanashi et al., 2007), human NPC1L1-HA cDNA (human NPC1L1 cells), or pcDNA3.1(+) vector (control cells) were cultured in Eagle's minimal essential medium (Nacalai Tesque) with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel), 100 units/ml penicillin and streptomycin (Nacalai Tesque), 1% nonessential amino acid (Invitrogen, Tokyo, Japan) and G418 sulfate (500 $\mu\text{g/ml}$) at 37°C in an atmosphere supplemented with 5% CO_2 .

Western Blot Analysis. For Western blotting, the cell pellet was resuspended in 1 ml of buffer A (50 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin, and 5 $\mu\text{g/ml}$ aprotinin) with mild sonication. After centrifugation (1500g for 15 min), the supernatant was recentrifuged (20,000g for 60 min). The crude membrane fraction was resuspended in buffer A and stored at -80°C before being used for Western blot analysis. The protein concentrations were determined by the method of Lowry (Lowry et al., 1951), with bovine

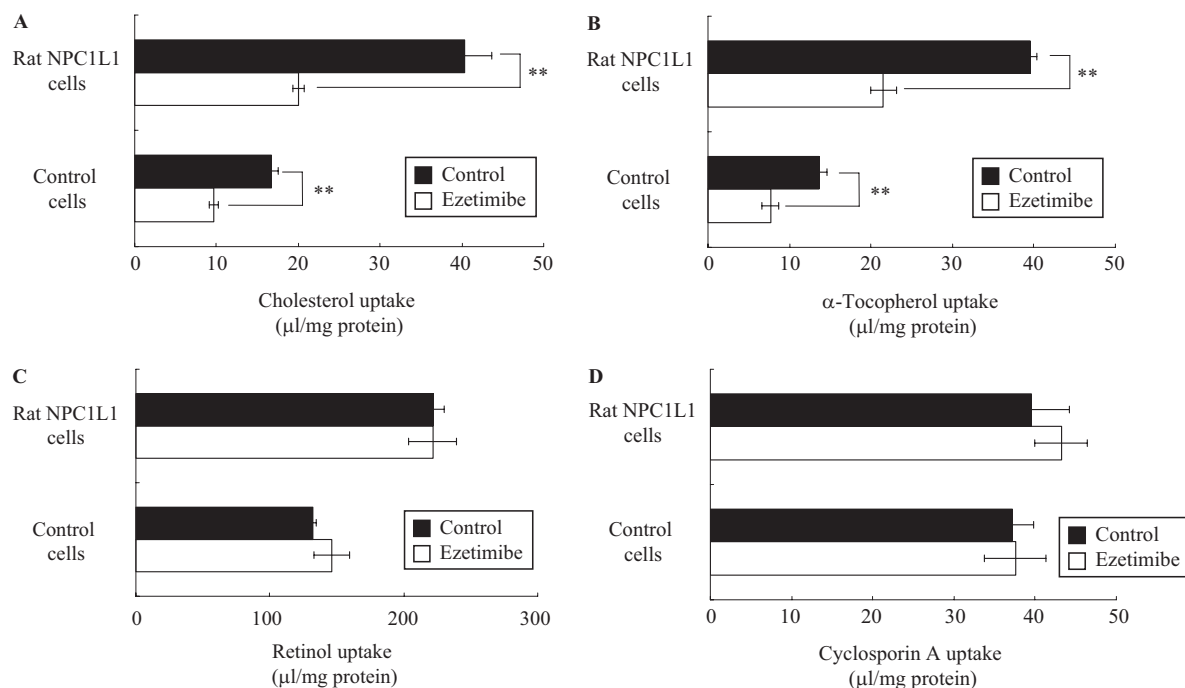


Fig. 1. NPC1L1-mediated transport and ezetimibe sensitivity of the uptake of cholesterol, α -tocopherol, retinol, and cyclosporin A. The uptake of cholesterol (A), α -tocopherol (B), retinol (C), and cyclosporin A (D) by control and rat NPC1L1 cells was examined at 37°C for 30 min in a medium containing 2 mM taurocholate, 50 μM phosphatidylcholine, 1 μM [^3H]cholesterol (0.04 $\mu\text{Ci/ml}$), and 0.06 $\mu\text{Ci/ml}$ [^{14}C] α -tocopherol (A and B) or in a medium containing 2 mM taurocholate, 50 μM phosphatidylcholine, 1 μM [^{14}C]cholesterol (0.053 $\mu\text{Ci/ml}$), and 0.08 $\mu\text{Ci/ml}$ [^3H]retinol (C) or 0.08 $\mu\text{Ci/ml}$ [^3H]cyclosporin A (D) in the presence and absence of 20 μM ezetimibe. Each column and horizontal bar represents the mean \pm S.D. ($n = 3$). **, significantly different by Student's t test ($p < 0.01$).

serum albumin (BSA) as a standard. Twenty micrograms of crude membrane diluted with 2× SDS loading buffer was separated on an 8.5% SDS-polyacrylamide gel with a 4.4% stacking gel. The molecular weight was determined by a prestained protein marker (New England BioLabs, Beverly, MA). Proteins were transferred electrophoretically onto an Immobilon membrane (Millipore Corporation, Billerica, MA) using a blotter (Bio-Rad Laboratories, Hercules, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% BSA for 1 h at room temperature. After washing with TBS-T, the membrane was incubated for 1 h at room temperature in TBS-T containing 0.1% BSA and 800-fold diluted anti-HA tag antibody. For detection, the membrane was allowed to bind to 5000-fold diluted horseradish peroxidase-labeled anti-rabbit IgG antibody (GE Healthcare) in TBS-T containing 0.1% BSA for 1 h at room temperature. The enzyme activity was assessed using an ECL Plus Western Blotting Detection System (GE Healthcare) with a luminescent image analyzer (Bio-Rad Laboratories).

Immunohistochemical Staining. Fourteen-day-old confluent Caco-2 cells grown on a 35-mm dish were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with PBS containing 1% (v/v) Triton X-100 for 5 min and incubated with anti-HA tag antibody diluted 100-fold in PBS containing 0.1% BSA for 1 h at room temperature. Cells were washed three times with PBS, incubated with goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen) diluted 250-fold in PBS containing 0.1% BSA for 1 h at room temperature, and mounted in VECTASHIELD mounting medium with propidium iodide (Vector Laboratories, Burlingame, CA). The cellular localization of NPC1L1 was visualized by confocal laser microscopy (Olympus, Tokyo, Japan).

Micellar Sterol and Ezetimibe Preparation. Cholesterol diluted in ethanol, phosphatidylcholine diluted in methanol, tauro-

cholate diluted in 96% ethanol, and each labeled isotope were mixed with (or without) ezetimibe diluted in methanol, then evaporated to dryness with mild heating under N₂ gas. A transport buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.2 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl₂ adjusted to pH 7.4) was then added to prepare the medium for transport experiments containing cholesterol, taurocholate, phosphatidylcholine, ezetimibe (0, 5, 10, 20, or 40 μM), and labeled micellar components. The micellar solution was thoroughly vortexed and stirred at 37°C for several hours.

Micellar Sterol Uptake Assay. Cells were seeded on a 12-well plate at a density of 1.2×10^5 cells/well and cultured for 14 days to allow them to differentiate. During that period, the medium was replaced every 2 to 3 days. After 14 days, cells were washed twice with the transport buffer and then preincubated in transport buffer for 30 min. After preincubation, mixed micelles containing labeled micellar components were added, and cells were incubated for the indicated time. After incubation, cells were washed with ice-cold transport buffer and disrupted with 0.2 N NaOH overnight. The radioactivities of ³H and ¹⁴C in the cell lysate were measured simultaneously in a liquid scintillation counter to determine the cellular uptake. For normalization, the protein concentration of each culture was determined by the method of Lowry et al. (1951) with BSA as a standard. Cellular accumulation of the tracers was calculated as the distribution volume (V_d , microliters per milligram of protein), defined as the ratio of the cellular radioactivity per milligram of protein divided by the radioactivity per microliter of the uptake medium, to compare the relative activities of uptake among labeled micellar components. All results are expressed as means ± S.D., and significant differences were detected using a two-tailed Student's *t* test or ANOVA followed by Dunnett's test.

Preparation of Cholesterol and α-Tocopherol Emulsion. The emulsion was prepared as described previously (Tso et al., 1980)

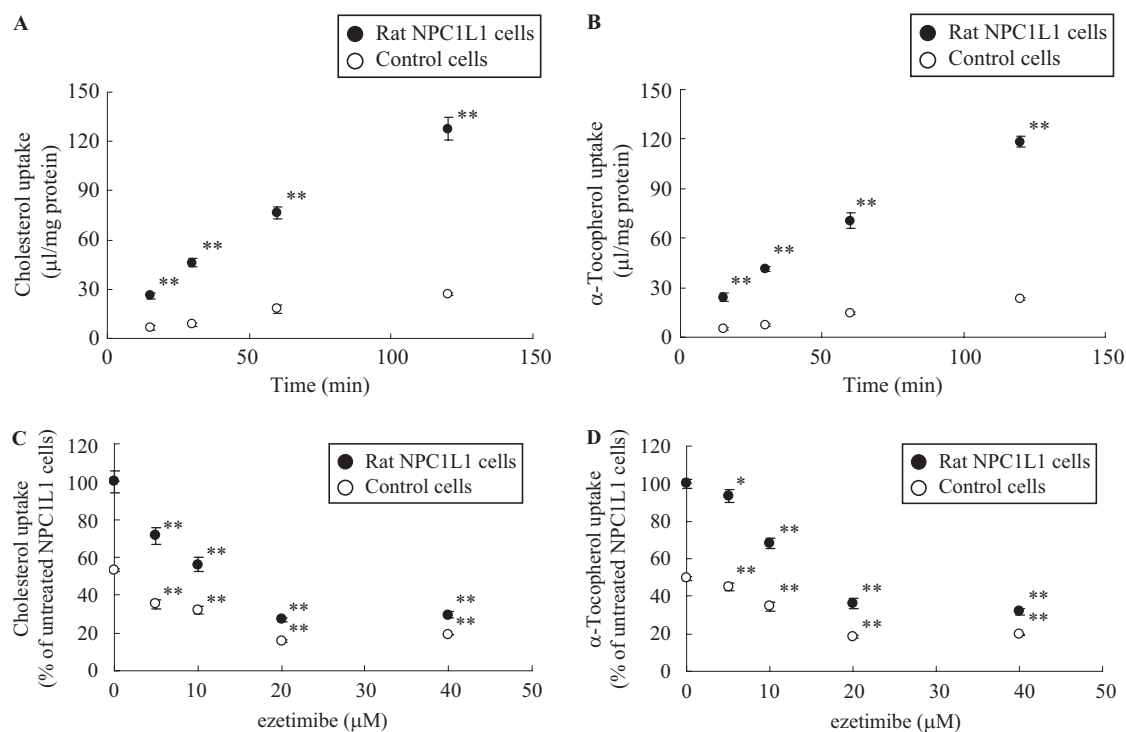


Fig. 2. Time profiles and inhibitory effect of ezetimibe on the uptake of cholesterol and α-tocopherol. The uptake of cholesterol (A) and α-tocopherol (B) by control and rat NPC1L1 cells was examined at 37°C for the indicated periods in a medium containing 2 mM taurocholate, 50 μM phosphatidylcholine, 1 μM [³H]cholesterol (0.04 μCi/ml), and 1 μM [¹⁴C]α-tocopherol (0.06 μCi/ml). The inhibitory effect of ezetimibe on the uptake of cholesterol (C) and α-tocopherol (D) was examined for 30 min with ezetimibe at the indicated concentrations. Each point and vertical bar represents the mean ± S.D. (*n* = 3). Where bars are not indicated, the S.D. is within the limits of the symbols. **, significantly different from control cells by Student's *t* test (*p* < 0.01) (A and B). *, significantly different from no treatment with ezetimibe by ANOVA followed by Dunnett's test (*p* < 0.05) (C and D). **, significantly different from no treatment with ezetimibe by ANOVA followed by Dunnett's test (*p* < 0.01) (C and D).

with minor modification. In brief, stock lipid solutions were mixed to give a final concentration of 13.3 mM triolein, 2.6 mM cholesterol, 3 mM L- α -phosphatidylcholine, 3 μ Ci/ml [3 H]cholesterol, and 0.75 μ Ci/ml [14 C] α -tocopherol. Solvent was evaporated, and 19 mM sodium taurocholate (dissolved in phosphate-buffered saline) was added to give the required lipid concentration. Then, the mixture was sonicated three times for 5 min, using an ultrasonic homogenizer (UP 200H, Hielscher ultrasonics, Teltow, Germany) to produce a stable emulsion.

Acute Absorption Study Using Wistar Rats. Male Wistar rats (181–210 g) were used for the *in vivo* study. An acute absorption study was conducted as described previously (van Heek et al., 2000) with minor modifications. Ezetimibe ethanol solution (20 mg/ml) was added directly to blank rat plasma to give a final concentration of 0.3 mg/ml. Fasted rats (18 h) were anesthetized with urethane (1.1 g/kg i.p.; Aldrich Chemical Company, Inc., Milwaukee, WI), and an intraduodenal cannula was inserted as described previously (Tso et al., 1980). After cannulation, rats received an intravenous dose of 1 ml/kg blank plasma or ezetimibe-containing plasma via the jugular vein. Immediately after drug administration, 5 ml/kg cholesterol emulsion was delivered directly into the intestine via the duodenal cannula. Three hours after cholesterol loading, rats were sacrificed, then plasma was isolated and directly analyzed to determine [3 H]cholesterol and [14 C] α -tocopherol simultaneously in duplicate. Livers were also taken, weighed, and minced. Aliquots of approximately 100 mg of tissue were solubilized using Solvable reagent (PerkinElmer Life and Analytical Sciences) and subjected to [3 H]cholesterol and [14 C] α -tocopherol measurement.

Data are expressed as a percentage of administered radioactivity per total plasma volume or total liver. The total plasma volume was

assumed to be 4% of the body weight as described elsewhere (Hawk and Leary, 1995). All results are expressed as means \pm S.E. and significant differences were calculated using a two-tailed Student's *t* test.

Results

Ezetimibe Inhibits the Uptake of Cholesterol and α -Tocopherol, but Not That of Retinol and Cyclosporin A, by Rat NPC1L1. We hypothesized that there is a possibility that NPC1L1 mediates the uptake of fat-soluble molecules in addition to cholesterol, because cholesterol and other fat-soluble molecules are known to share the micelle-dependent absorption process in the intestine. To test the hypothesis, we investigated whether ezetimibe, the molecular target of which is NPC1L1, inhibits the micellar uptake of α -tocopherol, retinol, and cyclosporin A as lipid-soluble nutrients and drugs using rat NPC1L1 overexpressing Caco-2 cells (rat NPC1L1 cells), which were constructed in an earlier study (Yamanashi et al., 2007). Figure 1 shows the inhibitory effect of ezetimibe (20 μ M) on their uptake in rat NPC1L1 cells and control cells. The uptake of cholesterol and α -tocopherol was examined using micelles containing [3 H]cholesterol and [14 C] α -tocopherol, whereas the uptake of retinol or cyclosporin A was examined using micelles containing [14 C]cholesterol and [3 H]retinol or [3 H]cyclosporin A. Cholesterol uptake was similar for [3 H]cholesterol and [14 C]cholesterol (data not shown). The uptake of cholesterol and α -tocopherol was reduced to approximately 50% by ezetimibe in rat NPC1L1 cells (Fig. 1, A and B), whereas the accumulation of retinol and cyclosporin A was not affected (Fig. 1, C and D).

Overexpression of Rat NPC1L1 Results in an Increase in the Uptake of α -Tocopherol, Which Is Inhibited by Ezetimibe. The result shown in Fig. 1 suggests that NPC1L1 plays a role in the uptake of both cholesterol and α -tocopherol. To investigate the transport in more detail, we examined the time profiles for the uptake of cholesterol and α -tocopherol in control and rat NPC1L1 cells (Fig. 2, A and B). The uptake of cholesterol by rat NPC1L1 cells was approximately 4-fold higher than that by control cells up to 120 min, which is consistent with our previous observations (Fig. 2A) (Yamanashi et al., 2007). In addition, it was found that the uptake of α -tocopherol was also higher in rat NPC1L1 cells (Fig. 2B), and the transport activity of α -tocopherol was similar to that of cholesterol at each time point. For example, 120 min after the start of incubation, cholesterol uptake by rat NPC1L1 cells was 128 ± 7 μ l/mg of protein and that by control cells was 26.9 ± 0.7 μ l/mg of protein, whereas α -tocopherol uptake by rat NPC1L1 cells was 119 ± 3 μ l/mg of protein and that by control cells was 23.2 ± 0.7 μ l/mg of protein. These *in vitro* results indicate that NPC1L1 mediates α -tocopherol uptake as well as that of cholesterol.

To further characterize the NPC1L1-mediated uptake of both cholesterol and α -tocopherol, we examined the inhibitory effect of ezetimibe. As shown in Fig. 2, C and D, the uptake of both cholesterol and α -tocopherol was inhibited in a dose-dependent manner. To compare the inhibitory effect of ezetimibe on their uptake, the K_i values obtained for each uptake were determined as described previously (Yamanashi et al., 2007). The K_i values for cholesterol and α -tocopherol uptake were 10.6 ± 1.6 μ M and 17.1 ± 4.4 μ M, respectively. These results suggest that ezetimibe inhibits both cholesterol

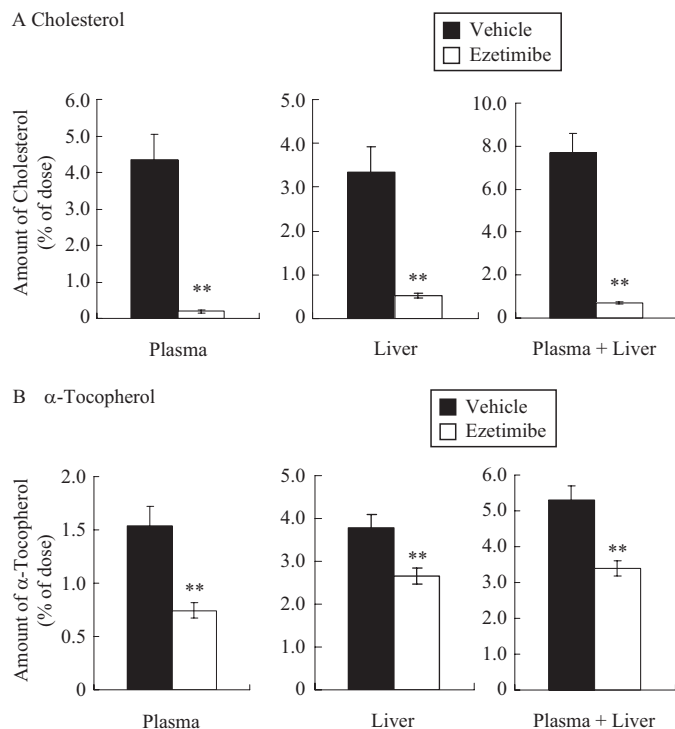


Fig. 3. The inhibitory effect of ezetimibe on the absorption of cholesterol and α -tocopherol in Wistar rats. The amount of cholesterol in plasma, liver, and plasma + liver (A) and α -tocopherol amount in plasma, liver, and plasma + liver (B) of Wistar rats were examined after intravenous administration of plasma containing 0.3 mg/kg ezetimibe or blank plasma. Open bars represent the results with 0.3 mg/kg ezetimibe ($n = 10$) and closed bars represent the results with vehicle ($n = 9$). Total plasma cholesterol absorption was calculated as described under *Materials and Methods*. Data are shown as mean \pm S.E. **, significantly different from vehicle by Student's *t* test ($p < 0.01$).

and α -tocopherol uptake mediated by NPC1L1 and that the inhibitory effect of ezetimibe on cholesterol uptake is slightly greater than that on α -tocopherol.

Ezetimibe Inhibits the Intestinal Absorption of α -Tocopherol in Vivo. To examine whether α -tocopherol absorption is inhibited by ezetimibe in vivo, we produced an emulsion containing dual radioisotopes of [3 H]cholesterol and [14 C] α -tocopherol and conducted a short-term absorption study using Wistar rats given ezetimibe intravenously (Fig. 3). The amount of labeled cholesterol in plasma and liver was significantly reduced by 0.3 mg/kg ezetimibe, which was consistent with our earlier study (Yamamoto et al., 2007). The amount of α -tocopherol absorbed by the body was significantly reduced by ezetimibe to 48% (plasma), 70% (liver), and 64% (plasma + liver), although the inhibitory effect was smaller than that of cholesterol. These results show that ezetimibe also inhibits the absorption of α -tocopherol in vivo.

Therefore, this suggests that α -tocopherol is absorbed, at least partly, via NPC1L1-dependent pathway in enterocytes.

α -Tocopherol Uptake Was Also Increased and Sensitive to Ezetimibe in Human NPC1L1 Overexpressing Cells. To extrapolate the results showing that NPC1L1 should be involved in the uptake of α -tocopherol in rat NPC1L1 cells (Fig. 2) and also in vivo in rats (Fig. 3) to humans, we constructed human NPC1L1 overexpressing Caco-2 cells (human NPC1L1 cells) and examined the transport ability of human NPC1L1 as well as rat NPC1L1. To confirm the expression and cellular localization, Western blotting, and immunohistochemical staining were performed. In the Western blot analysis, human NPC1L1 expression was detected as an approximately 170-kDa band (Fig. 4A), and exogenous human NPC1L1 was observed on the apical membrane in human NPC1L1 cells (Fig. 4B) by immunohisto-

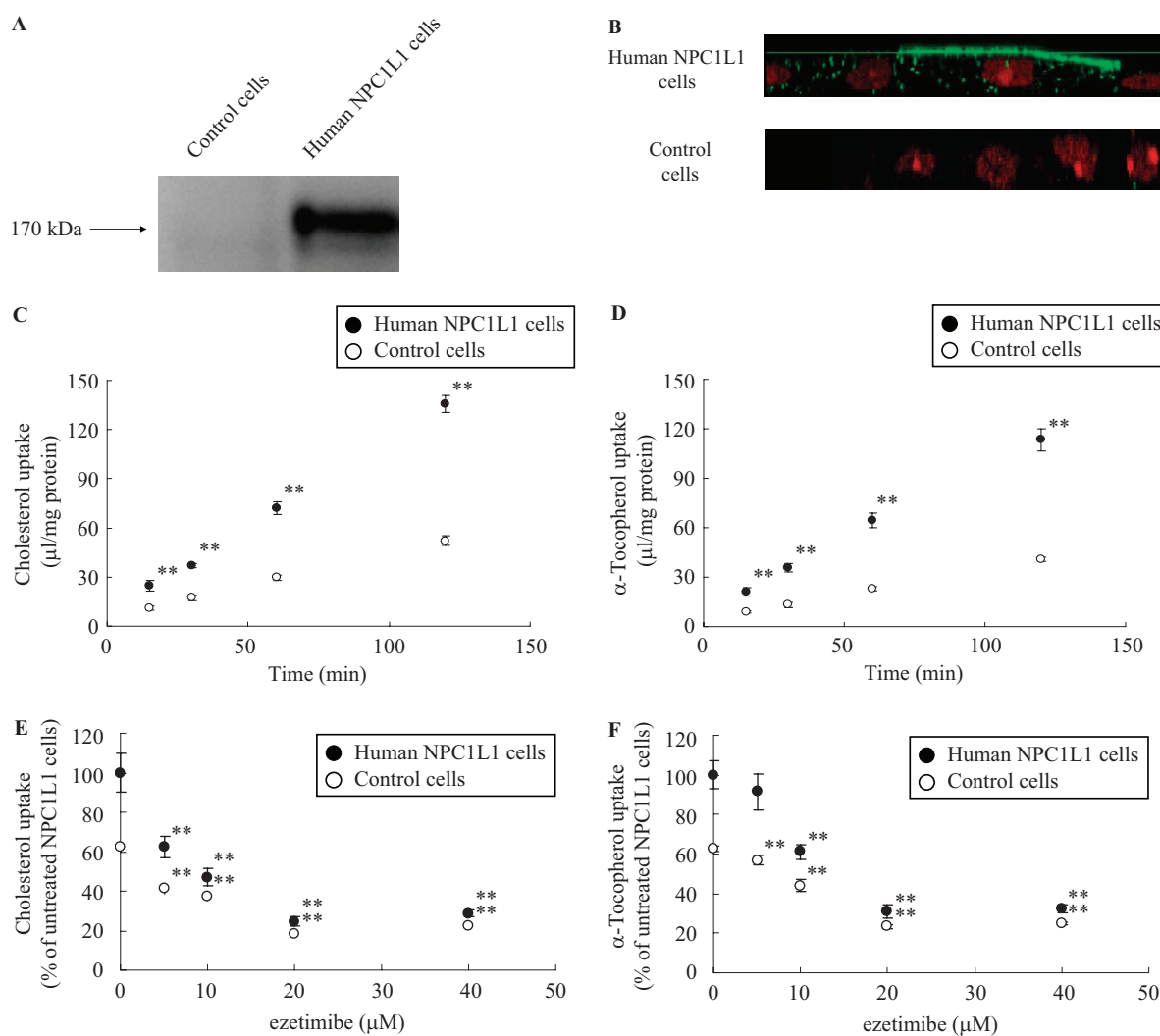


Fig. 4. Western blot analysis, cellular localization, and transport activities of human NPC1L1 on cholesterol and α -tocopherol. The expression of human NPC1L1 was determined by Western blot analysis (A). The cellular localization of human NPC1L1 was examined by immunohistochemical staining and shown in Z-section images (B). Human NPC1L1 cells and control cells were stained with anti-HA antibody (green) and nuclei were stained with propidium iodide (red). The uptake of cholesterol (C) and α -tocopherol (D) by control and human NPC1L1 cells was examined at 37°C for the indicated periods in a medium containing 2 mM taurocholate, 50 μ M phosphatidylcholine, 1 μ M [3 H]cholesterol (0.04 μ Ci/ml), and 1 μ M [14 C] α -tocopherol (0.06 μ Ci/ml). The inhibitory effect of ezetimibe on the uptake of cholesterol (E) and α -tocopherol (F) was examined for 30 min with ezetimibe at the indicated concentrations. Each point and vertical bar represents the mean \pm S.D. ($n = 3$). Where bars are not indicated, the S.D. is within the limits of symbols. **, significantly different from control cells by Student's t test ($p < 0.01$) (C and D). **, significantly different from no treatment with ezetimibe by ANOVA followed by Dunnett's test ($p < 0.01$) (E and F).

chemical staining, as is expected from the results of rat NPC1L1 (Yamanashi et al., 2007).

Furthermore, we showed that the uptake of both cholesterol and α -tocopherol by human NPC1L1 cells was linear up to 120 min and the uptake was significantly higher than that by control cells (Fig. 4, C and D), which is similar to the results of the uptake by rat NPC1L1 (Fig. 2, A and B). The clearance of α -tocopherol uptake was also similar to that of cholesterol at each time point. Moreover, we also examined the inhibitory effect of ezetimibe (Fig. 4, E and F). Ezetimibe inhibited the uptake of both cholesterol and α -tocopherol dose-dependently in human NPC1L1 cells. The K_i values obtained for cholesterol and α -tocopherol uptake were $4.9 \pm 0.7 \mu\text{M}$ and $11.0 \pm 3.6 \mu\text{M}$, respectively. The result showing that the K_i value of ezetimibe for human NPC1L1-mediated transport of α -tocopherol uptake is higher than that of cholesterol uptake is consistent with the results obtained using rat NPC1L1 cells (Fig. 2, C and D). These results suggest that human NPC1L1 is also involved in α -tocopherol uptake.

Mutual Inhibition Studies of Cholesterol and α -Tocopherol in Human NPC1L1-Mediated Uptake. To examine whether cholesterol and α -tocopherol share the same uptake mechanism, we performed transport assays using human NPC1L1 cells and control cells with micelles containing several concentrations of cholesterol and α -tocopherol. As shown in Fig. 5, A and B, the increase in cholesterol concentration in the micelles resulted in the lower uptake of cholesterol and α -tocopherol. In contrast, the transport activities for both cholesterol and α -tocopherol were significantly stimulated by the increase in the micellar concentration of α -tocopherol (Fig. 5, C and D).

Discussion

The data presented here suggest that NPC1L1, a cholesterol transporter, mediates the uptake of α -tocopherol and that ezetimibe also inhibits α -tocopherol uptake. Rat and human NPC1L1 overexpression increased α -tocopherol uptake as well as cholesterol uptake in vitro (Figs. 2 and 4). In the in vivo experiments on rats, ezetimibe inhibited the absorption of α -tocopherol (Fig. 3).

We showed that ezetimibe inhibits the uptake of cholesterol and α -tocopherol but does not affect the uptake of retinol and cyclosporin A (Fig. 1). It has been reported that ezetimibe does not affect the absorption of vitamins A and D in rats using a method similar to our experimental procedure (Fig. 3) (van Heek et al., 2001), which is consistent with the present study. Concerning cyclosporin A, there is a report that the plasma concentration of cyclosporin A is increased by ezetimibe treatment (Bergman et al., 2006). From our results, it appears that the interaction cannot be explained by NPC1L1-mediated uptake. The increased concentrations may be due to the involvement of other transporters, such as OATPs/SLCOs, which are hypothesized to be the cause of the interaction between cyclosporin A and some other drugs (Treiber et al., 2007). In the present study, we showed that NPC1L1 mediates the uptake of α -tocopherol. An earlier study showed that NPC1L1 is involved in the uptake of cholesterol and some phytosterols (Davis et al., 2004), which have similar structures with a steroid ring. Therefore, it is surprising that NPC1L1 has the ability to take up α -tocopherol, which has a chemical structure different from sterols.

As shown in Fig. 2, it was demonstrated in rat NPC1L1 cells that the transport activity of NPC1L1 and the inhibitory effect of ezetimibe were similar for cholesterol and α -tocopherol. To verify that NPC1L1 is involved in the ezetimibe-sensitive uptake of α -tocopherol in vivo, we examined the inhibitory effect of ezetimibe on the absorption of α -tocopherol using rats (Fig. 3). In plasma and liver, the absorption of α -tocopherol was inhibited by ezetimibe, even though the inhibitory effect of ezetimibe on the absorption of α -tocopherol was weaker than that on the absorption of cholesterol.

On the other hand, it has been reported that the serum concentrations of vitamin E were not significantly altered by a 12-week administrations of ezetimibe in a clinical study (Knopp et al., 2003). Although there was no significant difference, patients treated with ezetimibe had relatively lower serum concentrations of vitamin E. Because the report investigated the plasma α -tocopherol concentrations at steady-state after long-term administration of ezetimibe, it is possible that the serum concentrations were not dependent only on the absorption pathway. After absorption from intestine,

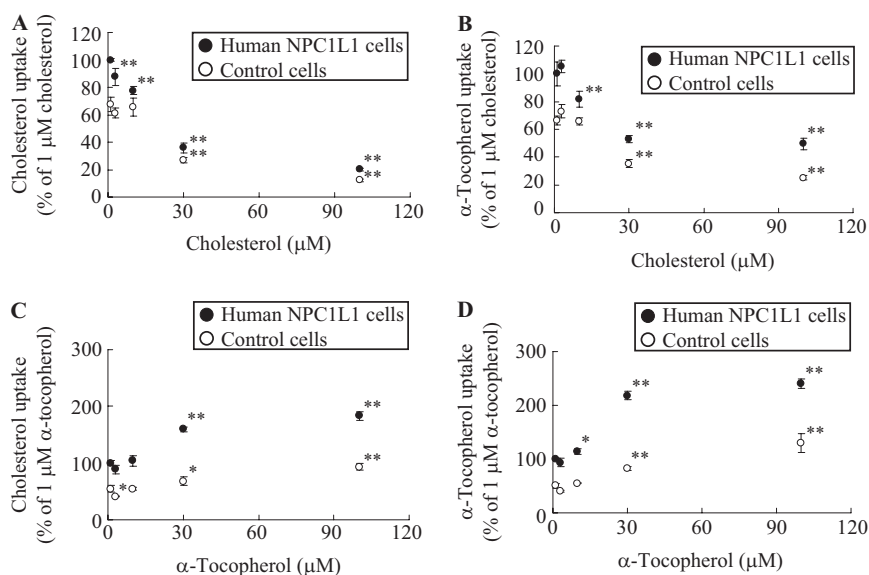


Fig. 5. Effect of increasing concentrations of cholesterol and α -tocopherol on human NPC1L1-mediated transport. The uptake of cholesterol (A) and α -tocopherol (B) by control and human NPC1L1 cells was examined at 37°C for 30 min in a medium containing 2 mM taurocholate, 50 μM phosphatidylcholine, 1 μM [^3H]cholesterol (A; 0.04 $\mu\text{Ci/ml}$), 1 μM [^{14}C] α -tocopherol (B; 0.06 $\mu\text{Ci/ml}$), and indicated concentrations of unlabeled cholesterol. In the same manner, the uptake of cholesterol (C) and α -tocopherol (D) by control and human NPC1L1 cells was examined at 37°C for 30 min in a medium containing 2 mM taurocholate, 50 μM phosphatidylcholine, 1 μM [^3H]cholesterol (C; 0.04 $\mu\text{Ci/ml}$), 1 μM [^{14}C] α -tocopherol (D; 0.06 $\mu\text{Ci/ml}$), and indicated concentrations of unlabeled α -tocopherol. Each point and vertical bar represents the mean \pm S.D. ($n = 3$). Where bars are not indicated, the S.D. is within the limits of the symbols. *, significantly different from 1 μM cholesterol (A and B) or 1 μM α -tocopherol (C and D) by ANOVA followed by Dunnett's test ($p < 0.05$). **, significantly different from 1 μM cholesterol (A and B) or 1 μM α -tocopherol (C and D) by ANOVA followed by Dunnett's test ($p < 0.01$).

α -tocopherol is delivered to the liver and then one third of the total body content of α -tocopherol is stored by α -tocopherol transfer protein in hepatocytes (Kaempf-Rotzoll et al., 2003; Hacquebard and Carpentier, 2005; Rigotti, 2007). Direct monitoring of the α -tocopherol absorption may result in a significant reduction after ezetimibe treatment.

In vitro, the transport activity of NPC1L1 and the inhibitory effect of ezetimibe on α -tocopherol uptake were almost the same as those on cholesterol, whereas the inhibitory effect of ezetimibe on α -tocopherol absorption in vivo was weaker than that on cholesterol absorption. Concerning the transport mechanism of the residual α -tocopherol absorption, which was not sensitive to ezetimibe in vivo, it is conceivable that a pathway other than NPC1L1 is involved. The most likely candidate is the scavenger receptor class B type I (SR-BI)-mediated pathway (Reboul et al., 2006), whose involvement in vitamin E uptake has been shown by both in vitro and in vivo analyses. This result indicates that SR-BI may be involved, at least in part, in vitamin E transport across enterocytes. To investigate the precise mechanism of the absorption of α -tocopherol in the intestine, the detailed experiments need to be designed using a combination of genetic knockout of NPC1L1 and SR-BI.

In the current study, to confirm the function of human NPC1L1, we constructed human NPC1L1-overexpressing Caco-2 cells as well as rat NPC1L1 cells and performed an in vitro analysis (Fig. 4). The results showed that cholesterol uptake is mediated by human NPC1L1, and the inhibition by ezetimibe could be evaluated in our model as rat NPC1L1 expression system. It was also shown that the inhibitory effect of ezetimibe in rat and human NPC1L1 cells was similar, which is not contradictory to the report that the binding of ezetimibe and ezetimibe-glucuronide, an active metabolite of ezetimibe, to NPC1L1 was not markedly different in rat and human orthologs (Garcia-Calvo et al., 2005; Hawes et al., 2007). On the other hand, there were significant differences between the tissue distribution of NPC1L1 in humans and rodents. Humans highly express NPC1L1 in the liver and intestine, whereas rodents mainly express NPC1L1 in the intestine (Altmann et al., 2004; Davies et al., 2005). In a very recent report, the hepatic overexpression of human NPC1L1 resulted in a dramatic reduction in the biliary cholesterol concentration in mice (Temel et al., 2007), which suggests that human NPC1L1 should be involved in the reabsorption of cholesterol from bile. Together with our present results showing that α -tocopherol uptake is increased in human NPC1L1 cells and that ezetimibe also inhibits this uptake, it is assumed that human NPC1L1 is also involved in the reuptake of intact α -tocopherol from bile to hepatocytes and prevents the excessive biliary loss of α -tocopherol together with α -tocopherol transfer protein, which is responsible for the storage of α -tocopherol in the liver.

Finally, NPC1L1-mediated transport of cholesterol and α -tocopherol should be discussed in relation to the transport of these two compounds mediated by other mechanisms. Indeed, it has been established that cholesterol and α -tocopherol share some transport mechanisms. Previously, it was reported that ABCA1 mediates the cellular efflux of α -tocopherol as well as cholesterol (Oram et al., 2001). In addition, it is suggested that, like cholesterol absorption, intestinal α -tocopherol absorption is mediated by the high-density lipoprotein (HDL)-mediated pathway in addition to absorp-

tion via chylomicrons (Anwar et al., 2007). The results of the current study further indicated that NPC1L1, which transports cholesterol, is also capable of transporting α -tocopherol. However, it was found that the NPC1L1-mediated transport of cholesterol and α -tocopherol was stimulated by increasing the concentration of α -tocopherol in the medium (Fig. 5, C and D). One of the possible hypotheses to account for this result is that the stimulatory effects may be caused by the allosteric effect of α -tocopherol on the NPC1L1-mediated transport. Such a stimulatory effect has also been observed for ABCC2-mediated transport; it has been reported that the ABCC2-mediated transport of substrates like estradiol 17 β -D-glucuronide was stimulated by ABCC2 substrates, such as benzylpenicillin and bile acids (Bodo et al., 2003; Zelcer et al., 2003; Ito et al., 2004).

In conclusion, the results of the present study indicate that NPC1L1, which is known as a sterol transporter, is also involved in the uptake of α -tocopherol in enterocytes and ezetimibe inhibits this uptake. Our present study showing that ezetimibe affects the acute absorption of α -tocopherol will be important from a clinical point of view; for example, administration of ezetimibe to patients who are expected to have a poor ability to absorb fat-soluble vitamins, such as cholestatic patients, may need extra attention. Furthermore, it has been reported that multiple variants of NPC1L1 are associated with reduced sterol absorption (Cohen et al., 2006). The variants may exhibit reduced absorption of α -tocopherol and, consequently, individual differences in intestinal α -tocopherol absorption may be accounted for by the NPC1L1-mediated transport mechanism, although it is also possible that some variants may be associated with the selective reduction of sterol or α -tocopherol absorption.

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