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An Approach to Improve Intestinal Absorption of Poorly Absorbed Water-Insoluble Components *via* Niemann–Pick C1-Like 1

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Dietary and biliary cholesterol absorption contributes to the maintenance of tight control of cholesterol homeostasis. Cholesterol is present as mixed micelles formed by bile salts and phospholipids in the intestinal lumen. Recently, Niemann–Pick C1-Like 1 (NPC1L1) transporter was identified as being critical for cholesterol absorption. However, the uptake mechanism of an enveloped substrate of NPC1L1 in whole lipid emulsion particles remains unclear. In this study, we investigated the uptake mechanism of a substrate of NPC1L1 in lipid emulsion particles. We also investigated whether these particles containing cholesterol can improve the intestinal absorption of other lipophilic components *via* NPC1L1. The uptake of lysophosphatidylcholine (LPC)-4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid saccinimidyl ester (BODIPY), a fluorescently labeled phospholipid, in lipid emulsion particles containing cholesterol (1 μM) was significantly increased compared to that without cholesterol in Caco-2 cells. On the other hand, its increased uptake was significantly inhibited by ezetimibe, a selective inhibitor of NPC1L1. These results suggested that not only cholesterol but also some components in lipid emulsion particles are taken up into enterocytes *via* NPC1L1. We also examined an approach to improve intestinal absorption of a poorly absorbed water-insoluble component, coenzyme Q10 (CoQ10), by this mechanism. The uptake of CoQ10 in lipid emulsion particles containing cholesterol was significantly increased compared to that without cholesterol. Its increased uptake was significantly inhibited by ezetimibe. Though it is still not clear whether CoQ10 is a substrate of NPC1L1, there is a potential for improvement of the absorption of poorly absorbed components by lipid emulsion particles containing cholesterol.

Key words Niemann–Pick C1-Like 1; absorption; cholesterol; emulsion; coenzyme Q10

Intestinal absorption is an important process in the maintenance of homeostasis in the body. Cholesterol homeostasis is a highly regulated balance of *de novo* synthesis, dietary cholesterol absorption, and biliary clearance and excretion. Although the cholesterol uptake mechanism in the intestinal lumen is not fully understood, the identification of ezetimibe as a selective inhibitor of intestinal cholesterol absorption suggested that these processes are mediated by a specific transport system rather than by passive diffusion.[1)] In 2004, Altmann *et al.* identified Niemann–Pick C1-Like 1 (NPC1L1) as an apically localized sterol transporter in the small intestine.[2)] NPC1L1 is highly expressed in the small intestine, particularly in the upper intestine.[2,3)] NPC1L1 protein has 13 predicted transmembrane domains and extensive N-linked glycosylation sites located within the extracellular loops and it contains a sterol sensing domain (SSD).[4)] It has predicted that the substrates of NPC1L1 have sterol domains and have been mainly thought to transport the substance involving the sterol structure.

Cholesterol is present as mixed micelles formed by bile salts and phospholipids in the intestinal lumen. Intestinal cholesterol absorption begins with the micellar solubilization of both dietary and biliary cholesterol in the lumen of the small intestine. Cholesterol is then transported across the apical membrane of absorptive enterocytes and mobilized to chylomicrons for secretion into the lymph or blood *via* the basolateral membrane of enterocytes. However, not only cholesterol but also other lipophilic molecules such as vitamin E and lutein have been recently reported to be solubilized in lipid emulsion particles and then partly absorbed in the intestine *via* NPC1L1.[5,6)] NPC1L1 has been predicted to incorporate cholesterol and other parts of lipophilic molecules from mixed micelles into enterocytes *via* clathrin-mediated endocytosis.[7,8)] As just described, the intestinal uptake mechanism of substrates of NPC1L1 has been gradually demonstrated. However, the uptake mechanism of an enveloped substrate of NPC1L1 in whole lipid emulsion particles remains unclear. We also hypothesized that NPC1L1 would be a good candidate of an enhancer of intestinal absorption of poorly absorbed water-insoluble components if cholesterol is incorporated with lipid emulsion particles *via* NPC1L1 into enterocytes.

In this study, we investigated the uptake mechanism of a substrate of NPC1L1 in lipid emulsion particles. We also investigated whether lipid emulsion particles containing cholesterol, a typical substrate of NPC1L1, can improve the intestinal absorption of other lipophilic components *via* NPC1L1.

MATERIALS AND METHODS

Chemicals and Reagents [1α,2α-^{3}H(N)] Cholesterol (53.0 Ci/mmol) was purchased from PerkinElmer, Inc. (Boston, MA, U.S.A.). LPC (lysophosphatidylcholine)-BODIPY (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid saccinimidyl ester) and 25-NBD-chol (25-[*N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol) (molecular weight (MW): 564.76) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL,

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U.S.A.). Ezetimibe, (4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-4*S*-(4-hydroxyphenyl)-2-azetidinone, was purchased from Sequoia Research Products Ltd. (Pangbroune, UK, U.S.A.). Hoechst 33342 was purchased from DOJINDO (Kumamoto, Japan). CoQ10 powder was donated by Kougen Co., Ltd. (Shizuoka, Japan: manufactured by Zhejiang Medicine Co., Ltd., Xinchang Pharmaceutical Factory). All other reagents were of the highest grade available and used without further purification.

Cell Culture Caco-2 cells obtained from RIKEN (Ibaraki, Japan) were maintained in a plastic culture dish (AS ONE, Osaka, Japan) as described previously.[6] The medium used for growth of Caco-2 cells was Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.) with 10% fetal bovine serum (ICN Biomedicals Inc., Aurora, OH, U.S.A.), 1% non-essential amino acids (Gibco-Invitrogen, Carlsbad, CA, U.S.A.), 4 mM glutamine (Invitrogen, Grand Island, NY, U.S.A.) and 100 IU/mL penicillin-100 μg/mL streptomycin (Sigma). Monolayer cultures were grown in an atmosphere of 5% CO_2/95% air at 37°C. The cells were given fresh growth medium every 2 d. When the cells had reached confluence after 4–6 d in culture, they were harvested with 0.25 mM trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2PO_4, 1.47 mM KH_2PO_4), resuspended, and seeded into a new flask.

Western Blot Analysis To assess the presence of NPC1L1, Caco-2 cells were seeded on 6-well plates and prepared for Western blotting. The cells were scraped and centrifuged at 1000×***g*** for 3 min at 4°C. The pellet was suspended in 1 mL of PBS and centrifuged at 1000×***g*** for 3 min at 4°C. The resulting pellet was suspended in 100 μL lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS). The suspension was allowed to stand for 5 min on ice and was sonicated for 15 min at 4°C. The suspension was then centrifuged at 21500×***g*** for 15 min at 4°C. The supernatants were kept at −20°C as samples. The protein concentrations in these samples were determined by the method of Lowry *et al.* with bovine serum albumin (BSA) as a standard.[9] The samples were denatured at 100°C for 3 min in a loading buffer containing 2% SDS, 0.0585 M Tris–HCl, 25% glycerin and 0.04% Bromophenol Blue and separated on 5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes at 90 mA for 90 min. The membranes were blocked for 1.5 h with block buffer (5% Skim milk powder in PBS-T (0.05% Tween 20, 20 mM Tris (pH 7.5), 137 mM NaCl)) and incubated with primary antibodies (goat anti-NPC1L1 polyclonal antibody (Cell Signaling, #49063) diluted 1 : 200 in 0.5% skim milk powder in PBS-T) overnight at room temperature and washed five times with PBS-T for 10 min each time. The membranes were then incubated with secondary antibody (horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G (IgG) antibody (SIGMA, #T5168) diluted 1 : 2000 in PBS-T) for 1 h at room temperature and washed five times with PBS-T for 10 min each time. The bands were visualized by enhanced chemiluminescence with ImageQuant LAS4000 (GE Healthcare) according to the protocol instructions of ImmunoStar® Zeta (Wako Pure Chemical Industries, Ltd.).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) RT-PCR was performed with some modification as described previously.[6] Total RNA was prepared from Caco-2 cells using an RNAiso Plus (TaKaRa Bio Inc., Shiga, Japan). Single-strand cDNA was made from 1 μg total RNA by reverse transcription using a Rever Tra Ace (TOYOBO, Osaka, Japan). PCR was performed using Quick Taq® HS DyeMix (TOYOBO) through 30 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 30 s with hNPC1L1-specific primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers. The sequences of the specific primers of NPC1L1 were the same described previously.[6] The sense sequence "5′-CCA TCA CCA TCT TCC AGG AG-3′" and the antisense sequence "5′-CCT GCT TCA CCA CCT TCT TG-3′" were for human GAPDH. The PCR products were subjected to electrophoresis on a 1% agarose gel and then visualized by ethidium bromide staining with UV light. Image processing was performed with ATTO Image Saver AE-6905.

Preparation of Emulsion Formulation for Uptake Study and Measurement of Emulsion Particle Size To prepare emulsion formulation, a substrate was dissolved in isopropyl myristate (final concentration of oil: 1%) and sodium taurocholate as surfactant was added to an incubation buffer (5 mM D-glucose, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)), pH 7.4 ajusted with 1 M Tris) to give a final concentration of 500 μM. They were mixed thoroughly with a vortex mixer (TAITEC Co., Ltd., Saitama, Japan) and an ultrasonic homogenizer (U200H, IKA Labortechnik, Germany) to produce an emulsion.

The average particle diameter of micelles prepared by the method described above was measured using a quasi-elastic light scattering method (Zetasizer Nano ZS; Malvern Instruments, Herrenberng, Germany).

Uptake Study in Caco-2 Cells For uptake study, Caco-2 cells were seeded at a cell density of 1.0–2.0×10^5 cells/cm^2 on a 24-well plastic plate (Corning Costar Inc., NY, U.S.A.). The cell monolayers were given a fresh medium every 2 d and were used at 4–6 d for the uptake experiments. Uptake values were corrected against the protein content. The protein content was measured by the method of Lowry *et al.* with BSA as a standard. Detailed methods of uptake study are described as follows.

Uptake Study of Radioisotope [^{3}H]-Cholesterol After removal of the growth medium, 0.5 mL of incubation buffer was added to wash each cell monolayer twice and 0.25 mL of incubation buffer containing a substrate, [^{3}H]-cholesterol (18.9 nM, 1 μCi/mL), was added. The monolayers were incubated for the indicated time at 37°C. Each cell monolayer was rapidly washed twice with 0.5 mL ice-cold incubation buffer without a substrate at the end of the incubation period. To quantify the radioactivity of the radiolabeled compound, the cells were solubilized in 1% SDS in 0.2 N NaOH. The samples were mixed with a scintillation cocktail to measure radioactivity. The radioactivity in the cell lysate was measured by a liquid scintillation counter to determine cellular cholesterol uptake.

Uptake Study of Fluorescent-Labeled Lipid After removal of the growth medium, 0.5 mL of incubation buffer was added to wash each cell monolayer twice and 0.5 mL of incubation buffer containing LPC-BODIPY or 25-NBD-Chol, fluorescent-labeled lipids, was added. The monolayers were incubated for the indicated time at 37°C. Each cell monolayer

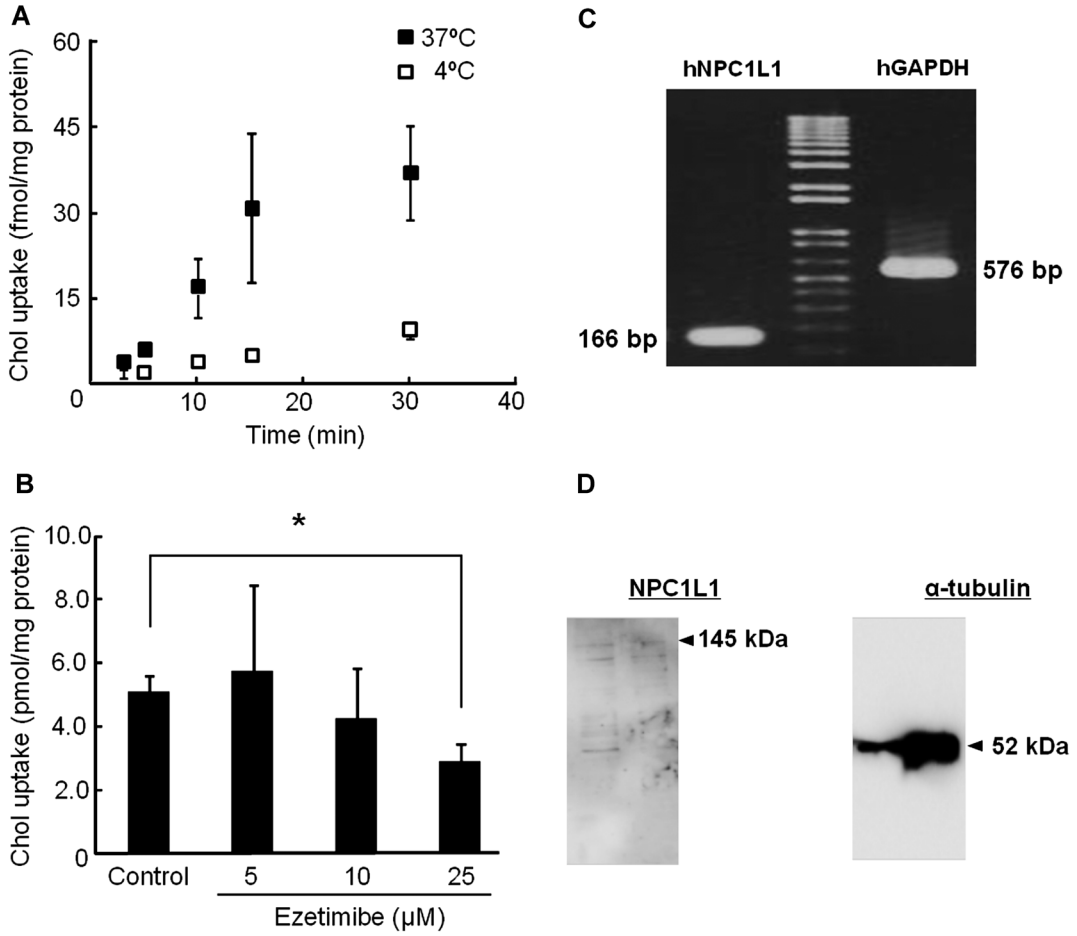

Fig. 1. Time Course (A) and Inhibitory Effect of Ezetimibe (B) on the Uptake of Cholesterol and Expression of NPC1L1 in mRNA Level (C) and in the Protein Level (D) in Caco-2 Cells

(A) The uptake of cholesterol by Caco-2 cells was examined at 37°C (closed square) or 4°C (open square) up to 30 min in a transport buffer containing 1 μM of cholesterol. Each point represents the mean with S.D. of 3 measurements. (B) The inhibitory effect of ezetimibe on the uptake of cholesterol was examined at 37°C for 5 min with ezetimibe at the indicated concentrations. Each point represents the mean with S.D. of 3–4 measurements. * Significantly different from control at $p<0.05$. (C) RT-PCR was performed with total RNA isolated from Caco-2 cells. This data is the typical result from 3 independent experiments. (D) Total cell lysates (5 μg of protein) prepared from Caco-2 cells were resolved using SDS-PAGE. Western blotting was carried out with antibody of NPC1L1 and α-tubulin. Two separate lysates were showed as similar results. This data is the typical result from 3 independent experiments.

was rapidly washed twice with 0.5 mL ice-cold incubation buffer without a substrate at the end of the incubation period. The cells were then trypsinized, suspended in PBS, and precipitated by centrifugation (2000×***g***, 3 min, 4°C). After resuspension in PBS, the cell suspension was filtered through nylon mesh (46 μm) to remove cell aggregates and dust, after which the cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The results of FACS were analyzed by the value of Geo Mean.

Uptake Study of Coenzyme Q10 After removal of the growth medium, 0.5 mL of incubation buffer was added to wash each cell monolayer twice and 0.5 mL of incubation buffer containing CoQ10 (final concentration: 300 μg/mL) was added. The monolayers were incubated for the indicated time at 37°C. Each cell monolayer was rapidly washed twice with 0.5 mL ice-cold incubation buffer without a substrate at the end of the incubation period. Three hundred microliters of phosphate buffer was added and incubated for 30 min at −80°C to break down the cell membrane.

Analytical Procedure Coenzyme Q10 concentration was determined using an HPLC system as described previously with some modifications.[10] Four hundred microliters of methanol was added to 100 μL of a sample, and the mixture was shaken for 30 s. Then 2 mL of *n*-hexane was added and the mixture was shaken for 10 min. After centrifugation at 750×***g*** for 10 min, 1.5 mL of the organic layer was taken and evaporated to dryness under a nitrogen gas stream. The residue was dissolved in 100 μL of mobile phase for HPLC injection. The column for HPLC was a GL Sciences Inertsil ODS-4 (3 μm in particle size, 3.0 mm in inside diameter ×150 mm) (Tokyo, Japan). A mobile phase containing ethanol–methanol (65 : 35, v/v) was used. Column temperature and flow rate were 40°C and 0.4 mL/min, respectively. The wavelength for detection was 275 nm. Forty microliters of a sample was injected into the HPLC system.

Data Analysis To analyze pharmacokinetics of CoQ10, the area under the curve (*AUC*) was calculated by the trapezoidal rule. Student's *t*-test was used to determine the significance of the differences between two group means. Statistical significance among means of more than two groups was determined by one-way ANOVA followed by the Turkey–Kramer test. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

NPC1L1 is enriched in the apical membrane of the small

intestine and mediates intestinal cholesterol absorption.[2,11] Cholesterol was shown to directly bind to SSD and to be transported across enterocytes. Ezetimibe binds specifically to NPC1L1 and inhibits NPC1L1-mediated accumulation of its substrate.[12] In a clinical setting, ezetimibe (Zetia®, Bayer Yakuhin Ltd.) is a widely used medicine for hyperlipidemia. NPC1L1 is a transporter that has then received much attention in recent years. NPC1L1 is thought to incorporate a substrate into enterocytes by clathrin-mediated endocytosis.[13] Lipophilic compounds in the intestinal lumen are formed into lipid emulsion particles by phospholipids such as phosphatidylcholine. The mechanism by which a substrate of NPC1L1 is incorporated into lipid emulsion particles is not fully clarified. In this study, we investigated the uptake mechanism of a substrate of NPC1L1 in lipid emulsion particles. We also investigated the potency of NPC1L1 for improvement of intestinal absorption of poorly water-soluble components.

Cellular Uptake of Cholesterol by Caco-2 Cells In the first part of this study, [^{3}H]-cholesterol uptake in Caco-2 cells was confirmed. Figure 1A shows the time course of the uptake of [^{3}H]-cholesterol at 37°C or 4°C. The uptake of cholesterol linearly increased for 10 min after the start of incubation at 37°C. We then set the uptake time of 5 min to evaluate the initial uptake. The uptake of cholesterol at 4°C was decreased compared with that at 37°C. These results suggest that the intracellular uptake of cholesterol is involved with facilitated diffusion or some active transport system as carrier-mediated transport rather than passive diffusion. As we described, it was reported that NPC1L1 was identified as a critical factor for cholesterol absorption.[14] We then next determined the effects of various concentrations of ezetimibe on uptake of cholesterol by Caco-2 cells (Fig. 1B). The uptake of cholesterol was significantly inhibited by ezetimibe at a concentration of 25 μM. This result is roughly consistent with previous reports.[14,15] We set that ezetimibe at a concentration of 25 μM inhibited the uptake of a substrate of NPC1L1. We also assessed the presence of NPC1L1 in Caco-2 cells. NPC1L1 was detected not only in the mRNA level but also in the protein level (Figs. 1C, D). We confirmed the band of hGAPDH and α-tubulin as a positive control, respectively. Sané *et al.* also reported that Caco-2 cells were endowed with a substantial NPC1L1.[11] On the other hand, Yamanashi *et al.* constructed the NPC1L1-overexpression Caco-2 cells and performed the uptake study.[14] Although the expression of NPC1L1 was not so abundant in this study, we consider that NPC1L1 in Caco-2 cells has its functions for the following reasons: Cholesterol uptake was inhibited by ezetimibe (Fig. 1B) and the expression of NPC1L1 in mRNA level was confirmed by the methods of RT-PCR (Fig. 1C) in addition to detection of the band of NPC1L1 (Fig. 1D).

Effect of Cholesterol in Lipid Emulsion Particles on the Uptake of Fluorescently Labeled Cholesterol and Phospholipid in Caco-2 Cells We also investigated whether ezetimibe treatment inhibits the uptake of 25-NBD-Chol by Caco-2 cells. We confirmed that the uptake of 25-NBD-Chol linearly increased up to 15 min after the start of incubation at 37°C and ezetimibe inhibited the uptake with the same level in both conditions (5 and 15 min) (Fig. 2). These results suggested that 25-NBD-Chol which we use in this study was absorbed at least partly *via* NPC1L1-dependent pathway. On the other hand, Chol-NBD (22-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-23,24-bisnor-5-cholen-3β-ol) is reported to be absorbed *via* NPC1L1-independent pathway.[16,17] In terms of cholesterol uptake *via* NPC1L1, it has been reported that the N-terminal domain of NPC1L1 binds cholesterol and promotes formation of cholesterol-enriched membrane microdomains comprising lipid raft proteins flotillin-1 and flotillin-2.[8,18] Clathrin is then recruited to internalize the NPC1L1-flotillin-cholesterol membrane microdomains. Cholesterol binding the N-terminal domain of NPC1L1 released the region of a specific amino acids sequence, YVNXXF (where X stands for any amino acids) and enabled to bind Numb, a clathrin adaptor.[13] NPC1L1 and cholesterol are then internalized together through clathrin/AP2-complex-mediated endocytosis. We also consider that further studies are needed to clarify the recognition of cholesterol and its related-substances, substrates of NPC1L1, as Reboul *et al.* also suggested that the chemical modification from cholesterol to NBD-Chol has a real impact in NPC1L1 recognition.[17]

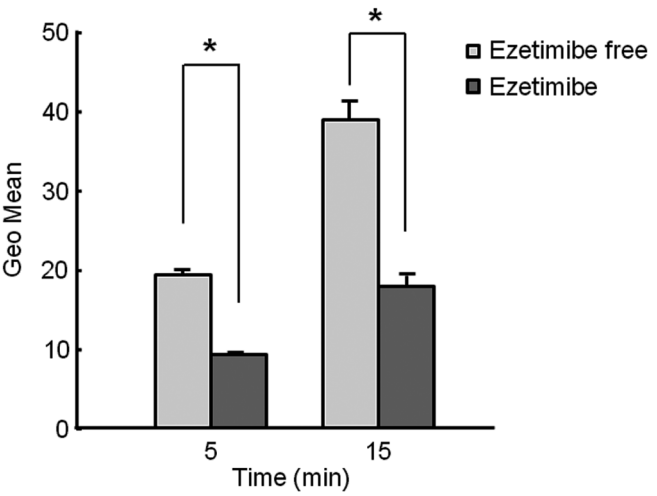

Fig. 2. Inhibitory Effect of Ezetimibe on the Uptake 25-NBD-Chol by Caco-2 Cells

The uptake of 25-NBD-Chol ((25-[*N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]-amino]-27-norcholesterol) (final concentration of 9 μM) by Caco-2 cells was examined at 37°C for 5 and 15 min in an emulsion. The inhibitory effect of ezetimibe (25 μM) on the uptake of 25-NBD-Chol was investigated. Each column represents the mean with S.D. of 3–4 measurements. * Significantly different at $p<0.05$.

We then investigated how lipid emulsion particles themselves were incorporated into Caco-2 cells as well as cholesterol (Fig. 3). LPC-BODIPY is a fluorescently labeled reagent of lysophosphatidylcholine, which is a surfactant. The uptake of this surfactant was examined with lipid emulsion particles with or without cholesterol and ezetimibe. We hypothesized that the uptake of LPC-BODIPY in lipid emulsion particles would not be different with and without cholesterol if lipid emulsion particles disintegrated near the intestinal apical membrane. On the other hand, the uptake of LPC-BODIPY in lipid emulsion particles would be increased with cholesterol compared to that without cholesterol if some components including cholesterol in lipid emulsion particles are incorporated into enterocytes *via* NPC1L1.

It was found that the uptake of LPC-BODIPY at 37°C was increased compared with that at 4°C. This result suggested that not only passive diffusion but also some active transporters are involved with the uptake of LPC-BODIPY. We also found that the uptake of LPC-BODIPY in lipid emulsion particles including cholesterol (1 μM) was significantly increased

compared with that without cholesterol. On the other hand, its increased uptake was significantly inhibited by ezetimibe. Interestingly, the uptake of LPC-BODIPY was decreased by ezetimibe even in emulsion without cholesterol compared with that with cholesterol and without ezetimibe. We thought that LPC-BODIPY would not be a substrate of NPC1L1 because there were some reports that LPC was a more hydrophilic substance than phosphatidylcholine (PC) and PC was not a substrate of NPC1L1.[8] It suggests that LPC-BODIPY, a fluorescently labeled reagent, may be a substrate of NPC1L1 in this study. Further studies are needed to determine whether LPC-BODIPY is a substrate of NPC1L1 in detail. These results in our study suggested that not only cholesterol but also some components in lipid emulsion particles are incorporated into enterocytes *via* NPC1L1 (Fig. 3).

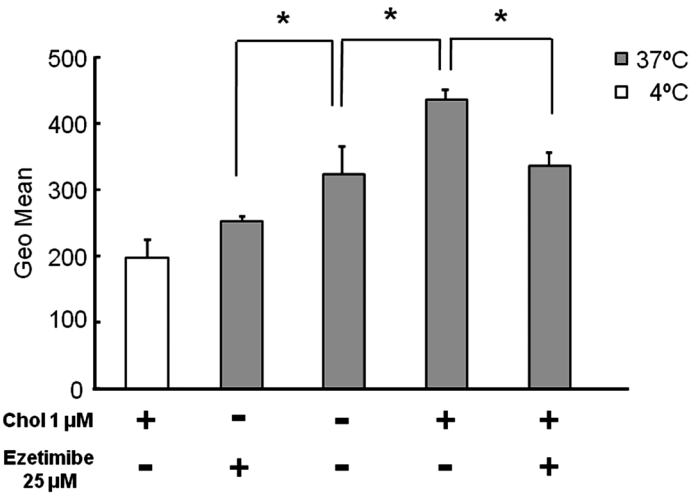

Fig. 3. Effect of Cholesterol and Ezetimibe on the Intracellular Uptake of LPC-BODIPY into Caco-2 Cells

The uptake of LPC-BODIPY (final concentration of 5 μg/mL) by Caco-2 cells was examined at 37°C or 4°C for 5 min in an emulsion with or without cholesterol. The inhibitory effect of ezetimibe (25 μM) on the uptake of LPC-BODIPY was investigated at 37°C for 5 min. Each column represents the mean with S.D. of 4–5 measurements. * Significantly different at $p<0.05$.

Table 1. Physicochemical Properties of Mixed Micelles with and without Cholesterol

Cholesterol (μM)	Particle size (nm)	ζ-Potential (mV)
0	236.8±3.4	−58.7±0.4
1	232.2±11.0	−55.3±0.6

The average particle sizes (diameters) and ζ-potentials in mixed micelles with and without cholesterol were measured using a quasi-elastic light scattering method. Each value represents the mean with S.D. of 3 measurements.

The Effect of Physicochemical Properties of Lipid Emulsion Particles The intracellular uptake from lipid emulsion particles in the intestine would be altered by various factors such as particle size, ζ-potential, fluidity of the membrane and kind of surfactant. Haikal *et al.* reported that the uptake of cholesterol in small-sized micelles was increased due to an easy accession to the intestinal brush-border membrane.[19] We also investigated whether there are differences between the physicochemical properties of two kinds of lipid emulsion particles. The average particle sizes (diameters) and ζ-potentials in lipid emulsion particles with and without cholesterol were measured (Table 1). The values were almost the same for the lipid emulsion particles. The polydispersity index (PDI) values of lipid emulsion particles with cholesterol and without cholesterol were 0.182±0.008 and 0.180±0.0023, respectively. PDI is a parameter calculated from a cumulants analysis of the dynamic light scattering-measured intensity autocorrelation function. This is a rough indication of homogeneity of particle size distribution. We confirmed that our prepared micelles with cholesterol and those without cholesterol had similar properties and were stable (Table 1).

Li *et al.* reported that NPC1L1 is thought to incorporate a substrate into enterocytes by clathrin-mediated endocytosis.[13] Generally, clathrin-coated pit endocytosis occurs with the intracellular uptake of 100–150 nm particles. On the other hand, our emulsion size was about over 200 nm. We might well be able to clarify the uptake mechanism in detail when we prepare <200 nm lipid emulsion particles and performed the study.

Lipid Emulsion Particles Containing Cholesterol Enhanced the Uptake of CoQ10 by Caco-2 Cells We then examined whether lipid emulsion particles including cholesterol improve the intestinal absorption of a lipophilic component *via* NPC1L1 (Fig. 4). The bioavailability of CoQ10 is very low, less than 10%.[20] CoQ10 is a ubiquitous component vital to a number of activities related to energy metabolism. CoQ10 also functions in its reduced form as an antioxidant, protecting biological membranes and serum low density lipoprotein from lipid peroxidation.[21,22] As just described, CoQ10 is a component to improve the absorption and its biological activity. We therefore selected CoQ10 as a model substance of poorly absorbed lipophilic components. The uptake of CoQ10 in lipid emulsion particles including cholesterol (1 μM) was significantly increased compared to that without cholesterol (Fig. 4). On the other hand, the increased uptake of CoQ10 in lipid emulsion particles containing cholesterol was significantly inhibited by ezetimibe at a concentration of 25 μM. We found that the uptake of CoQ10 in lipid emulsion particles was slightly decreased by ezetimibe even in lipid emulsion particles without cholesterol. These results suggest that CoQ10 may be a substrate of NPC1L1 as is the case for vitamin E. Although NPC1L1 is known to be a sterol transporter in the intestine, NPC1L1-overexpression appears to facilitate the accumulation of α-tocopherol, which does not have a sterol structure, in an ezetimibe-sensitive manner.[5,14] Abuasal *et al.* reported that the intestinal absorption of γ-tocotrienol is partly mediated by NPC1L1.[23] We also reported that NPC1L1 is partly involved in the absorption of lutein, a dihydroxycarotenoid.[6] As just described, it was suggested that some components that do not have a sterol structure are substrates of NPC1L1. So far, there has been no report showing that CoQ10 is a substrate of NPC1L1. Further studies are needed to determine whether CoQ10 is a substrate of NPC1L1.

We also performed gel-filtration chromatography with Sephadex G-50 column to confirm whether lipid emulsion particles include CoQ10. Some fractions were obtained by the gel-filtration to measure particle size, PDI and the concentration of CoQ10 in each fraction (Supplementary Fig. 1). Supplementary Fig. 1 showed the particle size distributions of CoQ10 emulsion before the gel-filtraton. It was showed that the particle size of emulsion was almost all the same in each fraction (Tables 2A, B). The tendency that PDI was small in strongly cloudy fractions was also showed. In addition, both emulsions with and without cholesterol include much CoQ10

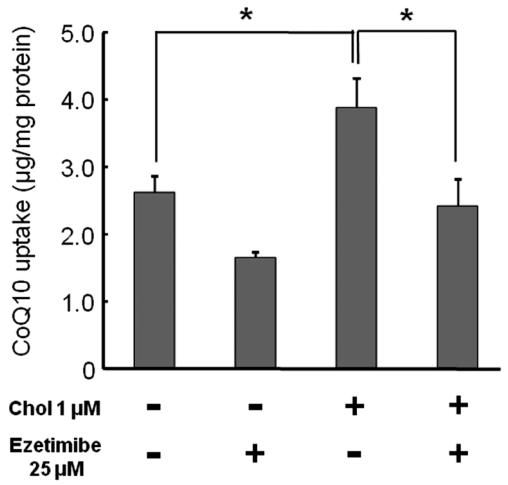

Fig. 4. Effect of Cholesterol and Ezetimibe on the Intracellular Uptake of CoQ10 into Caco-2 Cells

The uptake of CoQ10 (final concentration of 300 μg/mL) by Caco-2 cells was examined at 37°C for 15 min in an emulsion with or without cholesterol in the presence or absence of ezetimibe (25 μM). Each column represents the mean with S.D. of 4–7 measurements. * Significantly different at $p<0.05$.

in cloudy fractions. On the other hand, the concentration of CoQ10 was low in clear fractions. These results suggest that CoQ10 in lipid emulsion particles contributes largely to the uptake into enterocytes.

Oral delivery is generally the most desirable means of food and drug administration, mainly because of consumer or patient acceptance, convenience in administration and cost-effective manufacture. Absorption of components from the gastrointestinal tract is one of the important determinants of oral bioavailability, and the solubility of components is thought to be a critical issue. Generally, highly lipohilic components show very low bioavailability because of their very low hydrophilicity.[24] Some pharmaceutical devices are therefore needed to improve the absorption of such components. We have demonstrated improvement of the intestinal absorption of poorly water-insoluble functional food components.[25] It remains obscure whether these components are the substrates of NPC1L1 like cholesterol. Further investigations to obtain evidence of pharmaceutical design for theoretical improvement of intestinal absorption of these components are in progress.

CONCLUSION

In this study, we demonstrated that not only cholesterol but also some components in lipid emulsion particles are taken up to enterocytes *via* NPC1L1. The results of this study suggested that some components including cholesterol in lipid emulsion particles are incorporated into enterocytes *via* NPC1L1. We also investigated whether the intestinal absorption of poorly absorbed components can be improved by lipid emulsion particles containing cholesterol, a substrate of NPC1L1. CoQ10 was chosen as a model substance of these components. The uptake of CoQ10 was also improved with lipid emulsion particles containing cholesterol in Caco-2 cells. On the other hand, the uptake of CoQ10 in lipid emulsion particles was slightly decreased by ezetimibe, even in lipid emulsion particles without cholesterol. It is still not clear whether CoQ10 is a substrate of NPC1L1. There is a potential for improvement of the absorption of poorly absorbed components by lipid emulsion particles containing cholesterol, a substrate of NPC1L1. We consider NPC1L1 to be a beneficial transporter for improving the intestinal absorption of poorly absorbed water-insoluble components.

Table 2A. Particles Size and PDI and Content of CoQ10 in Each Fraction of CoQ10 Emulsion with Cholesterol

	Particle size (nm)	PDI	CoQ10 (% of original emulsion)
Origin	228.5	0.142	100
A	387.5	0.325	0.06
B	263.4	0.272	0.51
C	238.6	0.178	3.86
D	240.2	0.159	14.14
E	235.7	0.104	29.76
F	227.6	0.126	14.04
G	232.4	0.152	10.55
H	270.9	0.286	4.68
I	244.4	0.223	2.94
J	261.1	0.269	0.57

Table 2B. Particles Size and PDI and Content of CoQ10 in Each Fraction of CoQ10 Emulsion without Cholesterol

	Particle size (nm)	PDI	CoQ10 (% of original emulsion)
Origin	230.2	0.133	100
A	311.2	0.213	N.D.
B	291.8	0.188	2.48
C	252.8	0.151	13.10
D	238.3	0.109	21.45
E	231.5	0.088	17.97
F	223.8	0.127	15.88
G	225.5	0.125	12.52
H	229.2	0.139	9.93
I	225.4	0.148	4.42
J	228.1	0.121	2.60
K	226.0	0.155	1.99
L	224.7	0.124	1.19
M	232.5	0.144	0.89
N	232.5	0.150	0.83
O	229.9	0.134	0.55
P	229.0	0.129	N.D.

N.D., not detected.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials. Supplementary Fig. 1. Fractions obtained by the gel filtration of CoQ10 emulsion with cholesterol (A) and without cholesterol (B). Some fractions were obtained by the gel filtration of CoQ10 emulsion with cholesterol (A) and without cholesterol (B) with Sephadex G-50 column (10×40 mm).

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