Drug Metabolism and Pharmacokinetics

Transport via Niemann-Pick C1 Like 1 contributes to the intestinal absorption of ubiquinone

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Abstract

Ubiquinone, which is a component in the electron-transport systems of mitochondria, is essential for various activities related to energy metabolism, but the detailed absorption mechanism of ubiquinone is not clear. On the other hand, Niemann-Pick C1 Like 1 (NPC1L1) is involved in the intestinal absorption of fat-soluble components such as cholesterol. In this study, we investigated whether the intestinal absorption of ubiquinone was transported by NPC1L1 as is cholesterol. In this study, coenzyme q10 (CoQ10) and coenzyme q9 (CoQ9) were used as models of ubiquinone. The transport activity of ubiquinone was increased significantly in NPC1L1-overexpressed cells compared with that in pMAM2-BSD vector-transfected MDCK cells (mock cells) and the uptake of ubiquinone was decreased in the presence of ezetimibe, an inhibitor of NPC1L1. These results indicate that NPC1L1 mediates the transport of ubiquinone. Furthermore, to clarify the effect of NPC1L1 on the intestinal absorption of CoQ10, emulsified CoQ10 was orally administered to Wistar rats, and the plasma concentration was measured. The plasma concentration of CoQ10 was significantly decreased by coadministration of ezetimibe and CoQ10 compared to that with administration of only CoQ10. This result indicates that the intestinal absorption of CoQ10 is mediated by NPC1L1.

Keywords: Coenzyme Q10 (PubChem CID: 5281915); Coenzyme Q9 (PubChem CID: 5280473); Niemann-Pick C1 Like 1; ezetimibe; intestinal absorption; mixed micelle

1. Introduction

Cholesterol homeostasis is maintained by *de novo* syntheses in the liver and intestinal absorption from the diet. Cholesterol biosynthesis is mediated by the mevalonate pathway, and this pathway is also involved in the production of ubiquinone in mammalian cells [1]. Ubiquinone has a benzoquinone ring linked to a polyisoprenyl chain of 9 or 10 units in mammalian species. It is known that the number of isoprene side chains of ubiquinone is mainly 10 in humans and 9 in rats [2,3]. Ubiquinone, which is a component in the electron-transport systems of mitochondria, is essential for various activities related to energy metabolism [4]. Ubiquinone also functions in its reduced form as an antioxidant, protecting biological membranes and serum low-density lipoprotein from lipid peroxidation [5,6]. Many studies have also indicated a beneficial antioxidant effect of ubiquinone as supplementation, and ubiquinone has received much attention due to its antioxidant activities [7,8].

The tissue concentration of ubiquinone decreases with aging [9], and it is therefore important to take ubiquinone orally as supplements for health. Ubiquinone is present in some foods including sardines, mackerels, green leafy vegetables such as spinach, soybeans, peanuts and beef liver. We usually take 3-5 mg of ubiquinone per a day

from these foods [10]. However, absorption of ubiquinone from the gastrointestinal tract is poor due to its low solubility. The bioavailability of ubiquinone is very low, less than 10% in rats [11], and its low absorption is improved by using an emulsion formulation containing the surfactant Tween 20 or Tween 80 [12]. It is thought that ubiquinone absorbed from the gastrointestinal tract is reduced to ubiquinol to show antioxidant effects, but the detailed transport route of emulsified ubiquinone is not clear. On the other hand, Niemann-Pick C1 Like 1 (NPC1L1), a transport carrier expressed in small intestinal epithelial cells, is involved in the intestinal absorption of fat-soluble components such as cholesterol [13]. Ezetimibe, an inhibitor of NPC1L1, is a widely used medicine to inhibit the absorption of cholesterol from the diet for patients with hyperlipidemia [14]. If the intestinal absorption of ubiquinone is regulated by a mechanism similar to that for cholesterol, ubiquinone may be transported by NPC1L1.

In this study, we investigated whether the intestinal absorption of ubiquinone was transported by NPC1L1 as is cholesterol by using NPC1L1-overexpressed Madin-Darby canine kidney (MDCK) cells. A study on the absorption of ubiquinone using male Wistar rats was also performed to clarify whether transport via NPC1L1 contributes to the intestinal absorption of ubiquinone.

2. Materials and Methods

2.1. Chemicals and reagents

In this study, coenzyme q10 (CoQ10) and coenzyme q9 (CoQ9) were used as models of ubiquinone. CoQ10 powder (MW 863.34, PubChem CID: 5281915), cholesterol, sodium taurocholate and (+/-)-α-tocopherol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). CoQ9 powder (MW 795.23, PubChem CID: 5280473) was purchased from Cayman Chemical Company (Ann Arbor, MI, U. S. A.). L-α-Phosphatidylcholine was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ezetimibe ((4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-4S-(4-hydroxyphenyl)-2-azetidinone) and retinol were purchased from LKT Laboratories, Inc. (St. Paul, MN, U. S. A.). All-trans retinol [15-3H (N)] was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U. S. A.). (+)-δ-Tocopherol was purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, U. S. A.). Other reagents without further description were purchased from FUJIFILM Wako Pure Chemical Corporation. All of the reagents were of the highest grade available and used without further purification.

2.2. Cell culture

MDCK cells were purchased from American Type Culture Collection (Manassas, VA, U. S. A.). MDCK cells (passage number: 96-106) were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., LLC) with 10% fetal bovine serum (Bios era, Inc., Nuaillé, France), 100 IU/mL penicillin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 100 μg/mL streptomycin grown in an atmosphere of 5% CO_2 at 37˚C.

2.3. Animals

Male Wistar rats, aged 6 weeks (160-180 g in weight), were obtained from Jla, Inc. (Tokyo, Japan). All of the rats were housed in plastic cages. The housing conditions were the same as those described previously [15]. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals" (Approval number 18-0044).

2.4. Preparation of a mixed micelle and an emulsion

A mixed micelle was prepared according to the method described previously with some modifications [16,17]. In brief, cholesterol, α-tocopherol, ubiquinone and retinol

dissolved in ethanol, L-α-phosphatidylcholine dissolved in methanol, and sodium taurocholate dissolved in 95% (v/v) ethanol were mixed with or without ezetimibe, and the solvent was evaporated under N_2 gas. The dried residue was dispersed in transport buffer (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, adjusted to pH 7.5 with Tris) and sonicated in a bath sonicator (SND, Nagano, Japan) for 30 min. The micellar solution was stirred at 37˚C for 1 h and filtrated through 0.22 μm polytetrafluoroethylene membranes (Recenttec K.K., Tokyo, Japan) before the experiment.

The emulsion was prepared as described previously [12]. In brief, CoQ10 was dissolved in isopropyl myristate (Kanto Chemical Co., Inc., Tokyo, Japan), and Tween 20 and propylene glycol were added and then the mixture was thoroughly vortexed. Phosphate buffer was then added and thoroughly vortexed to prepare the emulsion. The final composition ratio of the emulsion was oil-surfactant-cosurfactant-water = 10.8 - 21.5 - 21.5 - 46.2 (wt%). The dose of CoQ10 was set to 25 mg/kg weight (1 mL/kg weight) based on our previous report [11,18].

The average particle diameter of mixed micelles and emulsions prepared by the methods described above was determined by using a quasielastic light scattering method (Zetasizer Nano ZS; Malvern Panalytical Ltd., Malvern, England).

2.5. Preparation of NPC1L1-overexpressed MDCK cells

Human NPC1L1 clone was purchased from BioCat GmbH (Heidelberg, Germany). The coding sequence of NPC1L1 cDNA (GenBank accession no. AY437865) was inserted into the pMAM2-BSD vector (Funakoshi Co., Ltd., Tokyo, Japan) and transfected into MDCK cells with lipofectamine reagent (Thermo Fisher Scientific Inc., Waltham, MA, U. S. A.). Then NPC1L1-overexpressed MDCK cells were cloned after selection by culturing for 3 days in the presence of 9 μg/mL blasticidin S.

2.6. Uptake assay in MDCK cells

For uptake study in MDCK cells, cells were seeded at a density of 5.0×10^4 cells/well on a 24-well plastic plate (Corning Incorporated, Corning, NY, U. S. A.) and cultured for 4 days. Before the experiment, cells were incubated with a medium containing 4 μg/mL dexamethasone for 48 h. After removal of the medium, cells were preincubated with transport buffer for 1 h at 37˚C. After removal of the transport buffer, cells were incubated with a micellar solution containing 2 μM retinol and 50 nM [^{3}H]-retinol, 40 μM α-tocopherol or 40 μM ubiquinone for the indicated time at 37˚C. The concentration of ezetimibe was set at 50 μM. At the end of the incubation period, cells were washed three

times with ice-cold transport buffer containing 1 mM sodium taurocholate.

For quantification of the uptake of [^{3}H]-retinol, cells were solubilized in 1% sodium dodecyl sulfate in 0.2 M NaOH. The radioactivity in the cell lysate was measured by a liquid scintillation counter (LSC-5100, Hitachi Aloka Medial, Ltd., Tokyo, Japan) to determine the uptake of retinol. For normalization, the protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc.).

For quantification of the uptake of α-tocopherol and ubiquinone, cells were incubated with 250 µL of transport buffer at -80˚C overnight to be disrupted through ice crystal formation. One hundred and fifty microliters of a sample was deprotenized with 400 µL of methanol (Kanto Chemical Co., Inc.) and extracted with 2 mL of n-hexane (Kanto Chemical Co., Inc.). After centrifugation at 2,330×g for 10 min at 25˚C, 1.8 mL of the organic layer was taken and evaporated to dryness under a N_2 gas stream. The concentration of ubiquinone was determined by using a high-performance liquid chromatography (HPLC) system equipped with an LC-10AD pump and an SPD-20AV UV-VIS detector (SHIMADZU Corporation, Kyoto, Japan) as described previously [19]. The concentration of α-tocopherol was determined by using an HPLC system equipped with an LC-20AT pump and an RF-20A XS fluorescence spectrometer (SHIMADZU Corporation) as described previously [20]. except for the injection volume of a sample into the HPLC system being 40 µL and the flow rate being set to 0.3 mL/min. For

normalization, the protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc.).

2.7. Oral administration, collection of samples and analytical procedures

For a single oral administration of ezetimibe, three rats were fasted for 14-16 h before the experiments to eliminate the effects of the diet. Ezetimibe was dissolved in dimethyl sulfoxide to form a 10 mg/mL solution. This solution was diluted and orally administered as a 1% (v/v) dimethyl sulfoxide solution. The dose of ezetimibe was set to 100 μg/kg weight as described previously [20]. Blood samples were collected from the jugular vein and plasma samples were obtained as described previously [21]. Bile samples were collected by inserting intramedic polyethylene tubing (Becton Dickinson and Company, Franklin Lakes, NJ, U. S. A.) into the bile duct under anesthesia. All samples were kept at -20ºC until the measurement.

For a single oral administration of CoQ10 and ezetimibe, the rats (n=13) were divided into two groups: CoQ10 group (n=6) and CoQ10+ezetimibe group (n=7). For the CoQ10 group, the emulsified CoQ10 described in section 2.4 was orally administered to rats (25 mg/kg weight). For the CoQ10+ezetimibe group, ezetimibe was spiked into the emulsion and orally coadministered with CoQ10. The dose of ezetimibe was set to 8.1

μg/kg weight. Other methods were the same as in the single oral administration of ezetimibe.

The plasma concentration of CoQ10 was determined using an HPLC system equipped with an L-7100 pump and L-7400 UV detector (Hitachi, Ltd., Tokyo Japan) as described in section 2.6. The cumulative excreted amounts of ezetimibe and its glucuronide, which is known as major metabolite, were determined using ACQUITY UPLC Quaternary solvent manager, Sample manager-FTN and Xevo TQ-S (Waters Corporation, Milford, MA, U. S. A.) as described previously [22]. Ezetimibe was extracted from the bile sample using Oasis HLB (Waters Corporation, Milford). The concentration of the ezetimibe glucuronide was calculated as an equivalent of ezetimibe by subtracting the unconjugated ezetimibe concentration from the total ezetimibe concentration measured after deconjugation reaction by β-glucuronidase treatment.

2.8. Data analysis and statistical analysis

To analyze the pharmacokinetics of CoQ10 and ezetimibe, area under the curve (AUC), maximum drug concentration (C_{max}) and time at maximum drug concentration (T_{max}) were calculated.

The means in two groups were compared by unpaired two-tailed Student's t-test or Welch test. The means in more than two groups were compared by two-way factorial analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparisons test. In two-way factorial ANOVA analysis, if the interaction between two factors was significant statistically, multiple comparisons of all groups were performed. Otherwise, comparisons between levels of factors with statistical significant differences were performed. Variability around the mean in each group was described as standard deviation (S.D.). The precision of the mean was reported as standard error (S.E.). Statistical significance was defined as $p<0.05$.

3. Results

3.1. Properties of mixed micelles

The average particle size of each micelle was about 10 nm, and there was no significant difference in the average particle size with or without ezetimibe (Table 1).

3.2. Confirmation of transport activity of NPC1L1 in MDCK cells using α-tocopherol and retinol

To analyze the transport activity of NPC1L1, uptake assays of α-tocopherol and retinol were performed using NPC1L1-overexpressed MDCK cells (NPC1L1 cells). It has been reported that NPC1L1 mediates α-tocopherol transport but does not mediate retinol transport [23]. In concordance with that report, the transport activity of α-tocopherol was increased in NPC1L1 cells compared with that in pMAM2-BSD vector-transfected MDCK cells (mock cells) at all time points (Fig. 1A). In addition, the uptake of α-tocopherol was decreased in the presence of ezetimibe in NPC1L1 cells (Fig. 1B). In mock cells, the uptake of α-tocopherol was also decreased in the presence of ezetimibe, indicating that endogenous NPC1L1 functions in MDCK cells. On the other hand, in concordance with the previous report, there was no difference in retinol uptake between NPC1L1 cells and mock cells, and no inhibition by ezetimibe was observed (Fig. 1C). These data show that transfected NPC1L1 functions normally in MDCK cells.

3.3. Contribution of NPC1L1 to the uptake of ubiquinone into MDCK cells

It is known that the number of isoprene side chains of ubiquinone varies depending on the species, being mainly 10 (CoQ10) in humans and 9 (CoQ9) in rats [2,3]. Endogenous CoQ10 was detected that was thought to be derived from fetal bovine serum

used during cell culture in MDCK cells (30.05 ± 6.72 pmol/mg protein in mock cells and 33.29 ± 6.01 pmol/mg protein in NPC1L1 cells), while endogenous CoQ9 was not detected in MDCK cells. Therefore, the uptake of CoQ10 was calculated by subtracting the value of endogenous CoQ10 detected in MDCK cells from the value of the amount of uptake obtained. Although there was large data variability, CoQ10 uptake tended to be increased in a time-dependent manner, indicating an increase in transport activity of CoQ10 in NPC1L1 cells compared with that in mock cells (Fig. 2A). Similarly, transport activity of CoQ9 increased significantly in NPC1L1 cells compared with that in mock cells (Fig. 2B).

In addition, the inhibitory effect of ezetimibe on uptake of ubiquinone was investigated. Two-way ANOVA showed that the effects of the transfected gene and the presence or absence of ezetimibe were significant ($p<0.05$), that CoQ10 may be transported via NPC1L1, and that its transport may be inhibited by ezetimibe (Fig. 2C). Uptake of CoQ9 was inhibited by ezetimibe not only in NPC1L1 cells but also in mock cells (Fig. 2D). These results suggest that NPC1L1 mediates the transport of ubiquinone.

3.4. Pharmacokinetic analysis of ezetimibe and its metabolite

We next investigated the inhibitory effect of ezetimibe on the absorption of CoQ10. First, the plasma concentration and bile excretion of ezetimibe were measured to

clarify how long ezetimibe remained in the body. After a single oral administration of ezetimibe to rats, almost no ezetimibe was detected in plasma, but glucuronide, an active metabolite of ezetimibe, was detected. C_{max} was 19.77 ± 2.59 ng/mL and AUC was 62.05 ± 29.27 ng×h/mL (Table 2). On the other hand, ezetimibe glucuronide was detected in bile immediately after administration and was continuously excreted up to 12 h (Fig. 3). The cumulative amount of excretion of ezetimibe glucuronide was 5938.93 ± 1687.92 ng, that is significantly different compared with that of unconjugated ezetimibe (87.79 ± 56.23 ng) by Welch test, indicating that ezetimibe is present in the rat body as a glucuronide. In addition, a second peak was observed around 9 to 10 h after administration in the bile excretion profile of unconjugated ezetimibe.

3.5. Inhibitory effect of ezetimibe on the intestinal absorption of CoQ10

After oral administration of CoQ10, an increase in the plasma concentration was observed for 24 h (Fig. 4). On the other hand, in the coadministration of CoQ10 and ezetimibe group, the value of C_{max} was significantly decreased compared with administration of CoQ10 alone (Table 3). The AUC values up to 24 h after administration were calculated to be 2610.59 ± 825.58 ng×h/mL in the CoQ10 group, that is significantly different compared with that of CoQ10 and ezetimibe group (1495.67 ± 701.06 ng×h/mL)

by Student’s t-test (Table 3). There was no significant difference in the average particle size of emulsion with or without ezetimibe (data not shown).

4. Discussion

In this study, involvement of NPC1L1 in the transport of ubiquinone incorporated into mixed micelles was investigated. Each mixed micelle was composed of sodium taurocholate, phosphatidylcholine and cholesterol, and the average particle size of micelles prepared in this study was calculated to be 8-15 nm (Table 1). It was previously reported that mixed micelles composed of bile salts and phospholipids were spherical particles with a particle size of about 10 nm [24], and the micelles prepared in this study are considered to be similar to those prepared in the previous study.

Prior to the uptake assay of ubiquinone, to confirm the transport activity of NPC1L1, uptake assays of α-tocopherol and retinol were performed using the prepared mixed micelles. α-Tocopherol was transported by NPC1L1, while retinol was not transported by NPC1L1 even in the presence of cholesterol (Fig. 1). These results suggest that only the substrate is transferred to NPC1L1 from mixed micelles selectively. In a model of transport by NPC1L1, it was previously reported that the substrate was taken up

into cells by clathrin-mediated endocytosis [25]. This finding suggests that the whole micellar particle can be taken up into cells by NPC1L1, but the transport model by endocytosis cannot explain why cholesterol and α-tocopherol, but not retinol, were selectively transported by NPC1L1. On the other hand, it was also reported that ezetimibe-sensitive cholesterol uptake by NPC1L1 protein does not require endocytosis [26], and further investigation is needed to elucidate the detailed mechanism of transport by NPC1L1.

The results of CoQ9 and CoQ10 uptake assays using NPC1L1-overexpressed MDCK cells indicate that CoQ9 and CoQ10 may be transported via NPC1L1 (Fig. 2). There are no reports on the detailed transport route of emulsified ubiquinone. On the other hand, it was reported that other emulsified fat-soluble micronutrients such as vitamin K, α-tocopherol and siphonaxanthin are transported by NPC1L1 [22,23,27]. Therefore, it is possible that CoQ10 is also transported by NPC1L1. In this study, since only the inhibitory effect of ezetimibe on absorption of CoQ10 was evaluated, it will be necessary to examine the contribution of NPC1L1 in more detail using knockout/-knockdown techniques in the future. In addition, it remains controversial whether ubiquinone, which has a large molecular weight and long isoprene side chains, is transported by a mechanism similar to that for cholesterol and α-tocopherol. Ubiquinone may be recognized by NPC1L1 at a binding site different from that of cholesterol or α-tocopherol. It was reported that the

N-terminal domain of NPC1L1 protein was essential for cholesterol and α-tocopherol transport [28,29]. It is therefore necessary to clarify the transport mechanism of ubiquinone via NPC1L1 using N-terminal domain variants.

Based on the *in vitro* results, the inhibitory effect of ezetimibe on the absorption of CoQ10 was examined using male Wistar rats. In Fig. 3, the dosage of ezetimibe was set to 100 μg/kg weight. In our previous study, the intestinal absorption of α-tocopherol in rats was significantly inhibited by 100 μg/kg weight ezetimibe [20]. Therefore, this dosage is considered to be appropriate for evaluating the inhibitory effect of ezetimibe. After oral administration of ezetimibe, most of the ezetimibe was present in the rat body as glucuronide (Fig. 3, Table 2). Ezetimibe is metabolized immediately *in vivo*, and it was reported that this glucuronide had higher affinity for NPC1L1 [30,31]. This result suggests that ezetimibe is present for a long time in the gastrointestinal tract near NPC1L1 by enterohepatic circulation.

In the study on absorption of CoQ10 using Wistar rats, emulsified CoQ10 was administered to rats and plasma concentration of CoQ10 was measured by HPLC. The dosage of CoQ10 is set according to the previous study [11,18]. It was also reported that the non-observed-adverse-effect level (NOAEL) of CoQ10 was considered to be 1200 mg/kg/day for male and female rats [32]. Therefore, it is considered that single administration of 25 mg/kg weight CoQ10 in this study has no toxicological effect. In Fig.

4, ezetimibe was spiked into the emulsion and orally administered (8.1 μg/kg weight). There was no significant difference in the average particle size with or without ezetimibe (data not shown). Although the morphology of emulsions was not observed in this study, but at least ezetimibe may not affect the particle size of the emulsion. In previous studies, the dosage of ezetimibe to rats was about 3 - 1000 μg/kg weight [20,22,23,31]. Compared to these values, the dosage is considered to be reasonable. Our data showed that the increase in plasma concentration of CoQ10 was decreased by co-administration with ezetimibe (Fig. 4, Table 3). This result suggests that the intestinal absorption of CoQ10 is mediated by NPC1L1. However, it is considered difficult to apply this result to humans because the lipoprotein concentration profile, gastric emptying rate, and the presence or absence of a gallbladder, which are factors affecting pharmacokinetics, are different in rats and humans. In addition, it was reported that the amino acid sequence homology of NPC1L1 between rats and humans was 77.6% and that the binding affinity of ezetimibe to NPC1L1 is higher in rats [30]. It was also reported that NPC1L1 was also expressed in the liver in humans but not in rats [13], and the effect of hepatic NPC1L1 on pharmacokinetics could therefore not be evaluated in this study. In clinical research, Berthold et al. [33] found that the combined administration of simvastatin and ezetimibe for two weeks significantly reduced human plasma CoQ10 levels compared to those with administration of simvastatin alone in healthy volunteers, suggesting that ezetimibe affects the plasma

concentration of CoQ10 in humans. On the other hand, it was reported that there was no significant difference in the plasma CoQ10 concentration between a rosuvastatin alone group and a rosuvastatin, ezetimibe and colestimide combination group in patients with heterozygous familial hypercholesterolemia [34]. More detailed studies are needed to clarify whether interactions in the absorption process observed in this study can occur in humans.

5. Conclusion

Our study showed by using MDCK cells that the transport of ubiquinone is mediated by NPC1L1 and also showed that the plasma concentration of CoQ10 after oral administration is significantly decreased by coadministration with ezetimibe. Since ezetimibe inhibits NPC1L1 for a long time by enterohepatic circulation, these results suggest that the plasma concentration of CoQ10 is decreased in patients taking ezetimibe.

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Author contributions

S.N., Y.T., M.S. and Y.S. conceived and designed the experiments. S.N. and Y.T. performed the experiments, analyzed the data. S.N., Y.T., M.S. and Y.S. wrote or contributed to the writing of the manuscript.

Conflict of interest

The authors report no conflicts of interest in this work.

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Figure legends

Fig. 1. Evaluation of the transport activity of NPC1L1 using α-tocopherol and retinol in NPC1L1-overexpressed MDCK cells.

The transport activity of NPC1L1 was confirmed by an α-tocopherol uptake assay. Cells were incubated with a transport buffer containing 2 μM cholesterol, 10 mM sodium taurocholate, 3.33 mM phosphatidylcholine and 40 μM α-tocopherol for 15, 30, 60, 120 min at 37˚C (A) (*$p<0.05$, **$p<0.01$; compared with mock by Student's t-test). The inhibitory effect of ezetimibe on NPC1L1-mediated α-tocopherol uptake was examined by incubating cells with 40 μM α-tocopherol with or without 50 μM ezetimibe for 30 min at 37˚C (B) (*$p<0.05$, **$p<0.01$ by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test). The uptake of α-tocopherol was determined by HPLC as described in Materials & Methods. As a negative control, a retinol uptake assay was performed by incubating cells with 2 μM cholesterol, 10 mM sodium taurocholate, 3.33 mM phosphatidylcholine, 2 μM retinol and 50 nM [^{3}H]-retinol with or without 50 μM ezetimibe for 30 min at 37˚C (C). The radioactivity was measured by a liquid scintillation counter to determine the uptake of cholesterol. Each point and column represents the mean ± S.D. of 3 measurements.

Fig. 2. NPC1L1-mediated transport of ubiquinone and inhibitory effect of ezetimibe on its transport in MDCK cells.

Cells were incubated with a transport buffer containing 2 μM cholesterol, 10 mM sodium taurocholate, 3.33 mM phosphatidylcholine and 40 μM CoQ10 for 30, 60, 120, 180 min at 37˚C (A) or 40 μM CoQ9 for 15, 30, 60, 120 min at 37˚C (B) (*$p<0.05$, **$p<0.01$ compared with mock by Student's t-test). The inhibitory effect of ezetimibe on NPC1L1-mediated CoQ10 or CoQ9 uptake was examined by incubating cells with 40 μM CoQ10 (C) or CoQ9 (D) with or without 50 μM ezetimibe for 60 min at 37˚C (*$p<0.05$, **$p<0.01$ by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test). In Fig. 2C, the result of comparison between levels of factors with significant difference in two-way factorial ANOVA was inserted as a table (LCL: Lower Confidence Limit, UCL: Upper Confidence Limit). The uptake of ubiquinone was determined by HPLC as described in Materials & Methods. Each point and column represents the mean ± S.D. of 3 measurements (A,B,D) or 6 measurements (C).

Fig. 3. Cumulative amounts of bile excretion of ezetimibe (A) and its glucuronide (B) up to 12 h after oral administration of ezetimibe.

All rats were fasted for 14-16 h before the experiments. Ezetimibe (100 μg/kg weight) was administered and bile samples were obtained for up to 12 h after

administration. The bile excretion of ezetimibe was determined by UPLC-MS/MS as described in Materials & Methods. Each column represents the mean with S.D. of 3 measurements.

Fig. 4. Contribution of inhibition of NPC1L1 to the intestinal absorption of ubiquinone.

All rats were fasted for 14-16 h before the experiments. CoQ10 (25 mg/kg weight) and ezetimibe (100 μg/kg weight) were administered and blood samples were obtained for up to 24 h after administration. The plasma concentration of CoQ10 was determined by HPLC as described in Materials & Methods. Each point represents the mean with S.E. of 5-7 measurements.

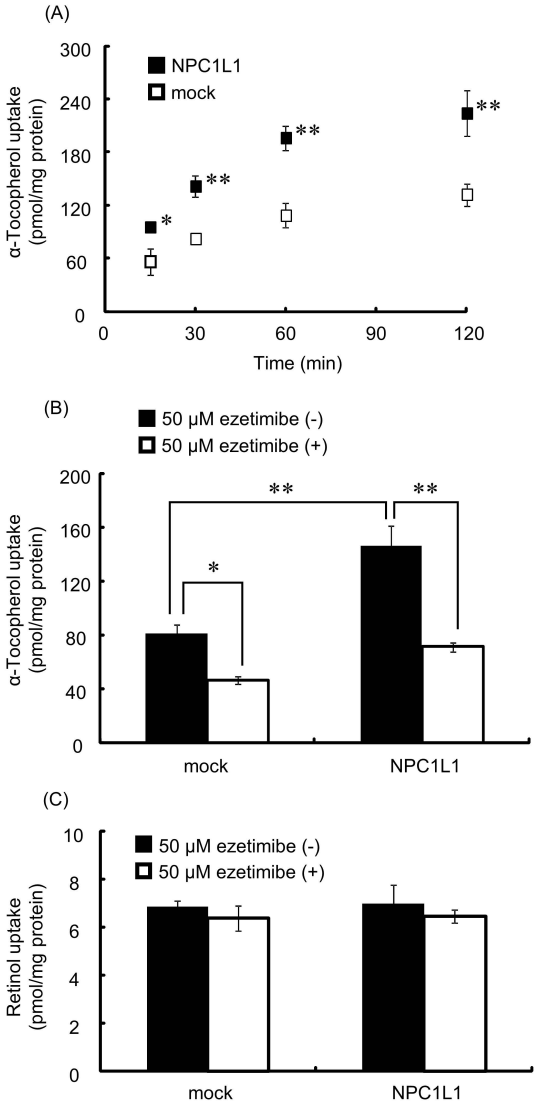
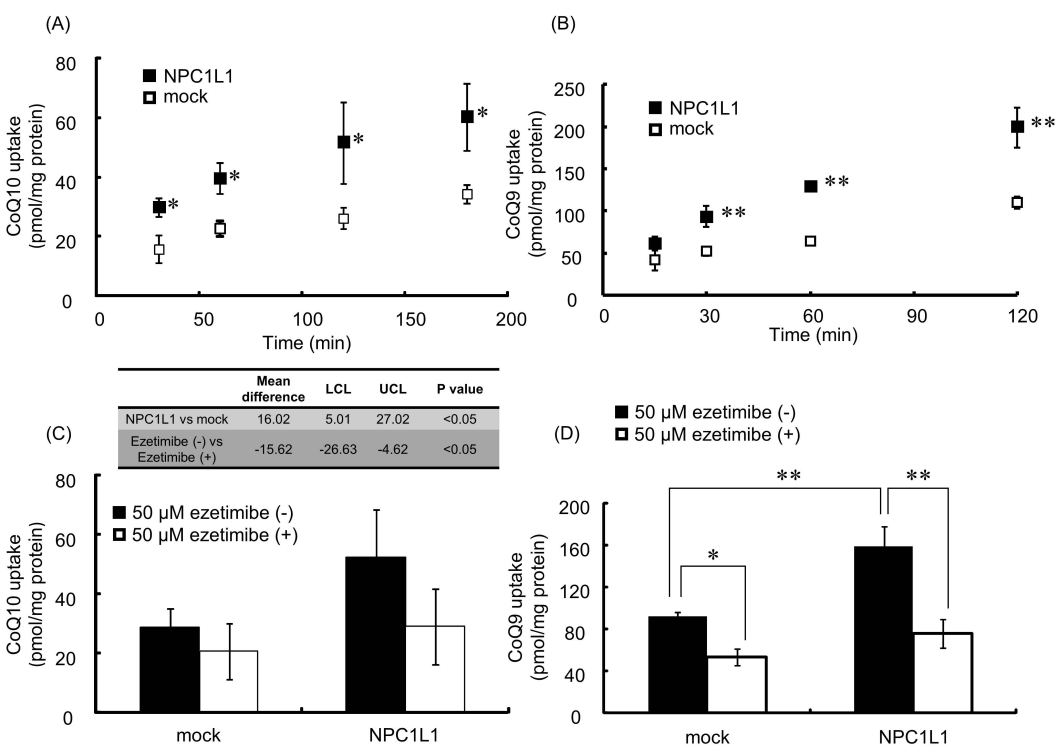
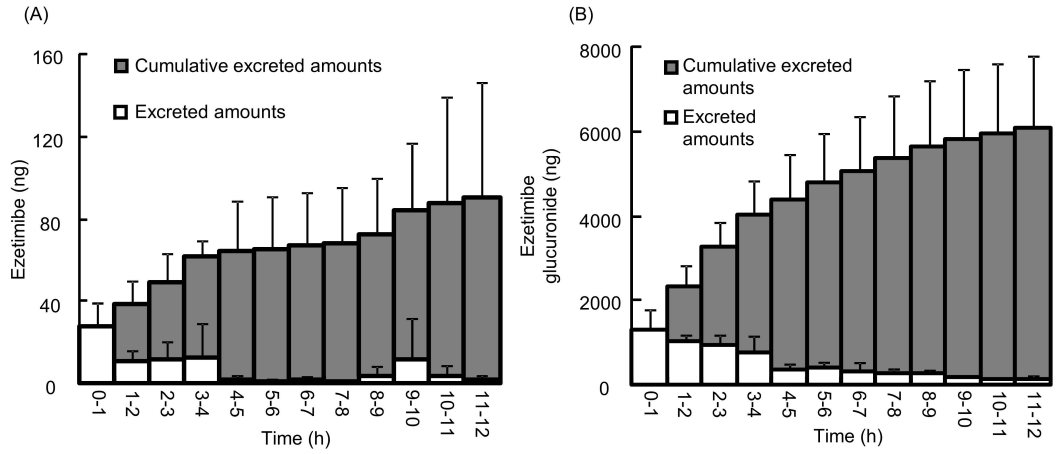

(A)
300
240
180
120
60
0
α-Tocopherol uptake
(pmol/mg protein)
NPC1L1
mock
*
**
**
**
0
30
60
90
120
Time (min)
(B)
50 μM ezetimibe (-)
50 μM ezetimibe (+)
200
160
120
80
40
0
α-Tocopherol uptake
(pmol/mg protein)
*
**
**
mock
NPC1L1
(C)
10
8
6
4
2
0
Retinol uptake
(pmol/mg protein)
50 μM ezetimibe (-)
50 μM ezetimibe (+)
mock
NPC1L1

Fig. 1



	Mean difference	LCL	UCL	P value
NPC1L1 vs mock	16.02	5.01	27.02	<0.05
Ezetimibe (-) vs Ezetimibe (+)	-15.62	-26.63	-4.62	<0.05


(A)
160
120
80
40
0
Ezetimibe (ng)
Cumulative excreted amounts
Excreted amounts
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
Time (h)
(B)
8000
6000
4000
2000
0
Ezetimibe glucuronide (ng)
Cumulative excreted amounts
Excreted amounts
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
Time (h)

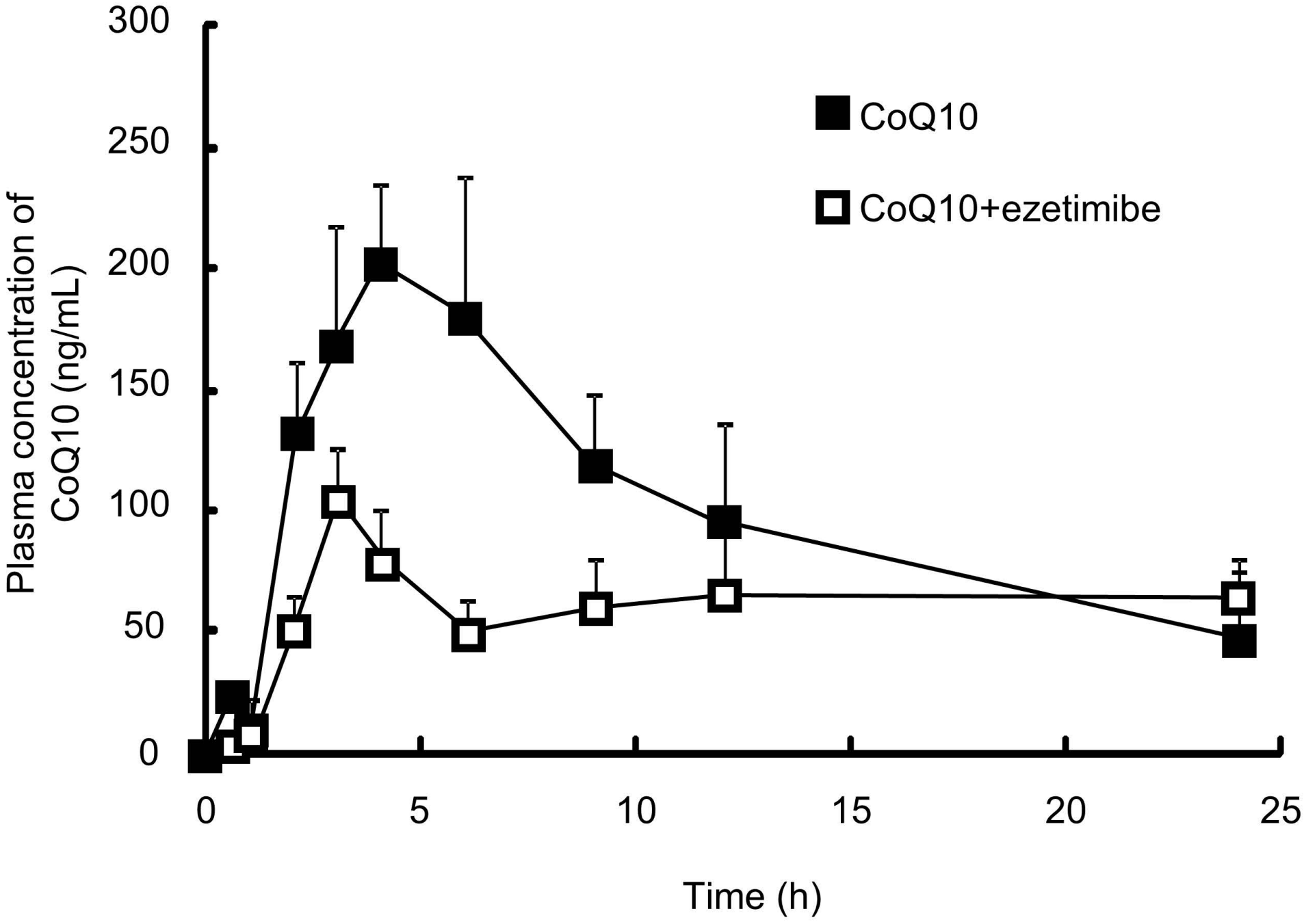

300
250
200
150
100
50
0
Plasma concentration of CoQ10 (ng/mL)
CoQ10
CoQ10+ezetimibe
0
5
10
15
20
25
Time (h)

Table 1 Average particle size (nm) and PdI value of each mixed micelle.

		Particle size (nm)	PdI
α-tocopherol	Ezetimibe (-)	13.33 ± 1.82	0.43 ± 0.034
	Ezetimibe (+)	11.60 ± 1.00	0.34 ± 0.0041
CoQ10	Ezetimibe (-)	13.77 ± 0.82	0.39 ± 0.067
	Ezetimibe (+)	14.33 ± 0.33	0.40 ± 0.021
CoQ9	Ezetimibe (-)	11.14 ± 0.54	0.29 ± 0.0045
	Ezetimibe (+)	8.19 ± 0.65	0.24 ± 0.030
Retinol	Ezetimibe (-)	10.86 ± 2.48	0.24 ± 0.064
	Ezetimibe (+)	10.63 ± 2.09	0.25 ± 0.052

Average particle size and PdI value were determined by using a quasi-elastic light scattering method. Each parameter represents the mean ± S.D. of 3 measurements.

Table 2 Pharmacokinetic parameters of ezetimibe and its glucuronide after oral administration of ezetimibe (100 μg/kg weight).

	C_{max} (ng/mL)	T_{max} (h)	AUC (ng×h/mL)
Ezetimibe	N.D.	N.D.	N.D.
Ezetimibe glucuronide	19.77 ± 2.59	3.00 ± 2.82	62.05 ± 29.27

The value of AUC was calculated by the trapezoidal method. Each parameter represents the mean ± S.D. of 3 measurements.

Table 3 Pharmacokinetic parameters of CoQ10 after oral administration of CoQ10 (25 mg/kg weight) and ezetimibe (8.1 μg/kg weight).

	C_{max} (ng/mL)	T_{max} (h)	AUC (ng×h/mL)
CoQ10	301.07 ± 83.03 **	5.00 ± 3.37	2610.59 ± 825.58 *
CoQ10+ezetimibe	127.45 ± 42.42	9.00 ± 6.99	1495.67 ± 701.06

The value of AUC was calculated by the trapezoidal method. Each parameter represents the mean ± S.D. of 5-7 measurements. *$p<0.05$, **$p<0.01$; compared with CoQ10+ezetimibe by Student's t-test.