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ORIGINAL ARTICLE

Inhibition of Niemann-Pick C1-Like 1 by Ezetimibe Reduces Dietary 5β,6β-Epoxycholesterol Absorption in Rats

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Abstract

Purpose Oxycholesterols (OCs) are produced from cholesterol by oxidation of the steroidal backbone and side-chain. OCs are present in blood and evidence suggests their involvement in disease development and progression. However, limited information is available regarding the absorption mechanisms and relative absorption rates of dietary OCs. Although ezetimibe is known to inhibit intestinal cholesterol absorption via Niemann-Pick C1-Like 1 (NPC1L1), whether it also inhibits dietary OC absorption is unclear.

Methods We investigated the effects of ezetimibe on OC absorption in rats fed an OC-rich diet containing 10 different OCs. We collected lymphatic fluid using permanent cannulation of the thoracic duct and quantified OC levels.

Results Ezetimibe treatment significantly reduced the apparent absorption of 5β,6β-epoxycholesterol (5,6β-epoxy) and its levels in the proximal intestinal mucosa in OC-fed rats. Using in silico analyses, the binding energy of NPC1L1 N-terminal domain (NPC1L1-NTD) and 5,6β-epoxy was found to be similar to that of NPC1L1-NTD and cholesterol, suggesting that polar uncharged amino acids located in the steroidal part of 5,6β-epoxy were involved.

Conclusion Our results indicate that ezetimibe-mediated inhibition of dietary OC absorption varies depending on the specific OC, and only the absorption of 5,6β-epoxy is significantly reduced.

Keywords Ezetimibe · Dietary oxycholesterols · Intestinal absorption · Lymphatic lipid transport · Permanent thoracic lymph duct cannulation

Introduction

Cholesterol oxidation products (oxycholesterols; OCs) are present in blood, suggesting that OCs are related to the development and progression of various diseases such as osteoporosis [1–3], atherosclerosis [1–4], Alzheimer's disease [1–4], cataracts [1–4], diabetes [2–4], and fatty liver [4]. OCs are produced from cholesterol by spontaneous and/or enzymatic oxidation of the steroidal backbone and side-chain. OCs have been found in several foods, especially foods from animal sources [5, 6]. It has been estimated that approximately 1% of the cholesterol consumed in a mixed western diet is comprised of OCs [5, 6]. In agreement, a previous study from our laboratory showed that the daily intake among Japanese people of cholesterol and 7 molecular species of OCs, namely 5-cholesten-3β,7α-diol (7α-hydroxycholesterol, 7α-OH), 5-cholesten-3β,7β-diol (7β-hydroxycholesterol, 7β-OH), cholestan-5α,6α-epoxy-3β-ol (5α,6α-epoxycholesterol, 5,6α-epoxy), cholestan-5β,6β-epoxy-3β-ol (5β,6β-epoxycholesterol, 5,6β-epoxy, cholestan-3β,5α,6β-triol (β-cholestanetriol, β-triol), 5-cholesten-3β-ol-7-one (7-ketocholesterol, 7-keto), and 5-cholesten-3β,25-diol (25-hydroxycholesterol, 25-OH), was 258 ± 14 and 2.15 ± 0.32 mg, respectively [7]. Because plasma OC levels are increased with an increase in intake of OCs [8], the regulation of dietary OC absorption is important in disease prevention.

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Ezetimibe (SCH 58235; 1-(4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxyphenyl)-2-azetidinone) was developed during an investigation to find dietary cholesterol absorption inhibitors in the intestine [9], and was subsequently approved for treating hypercholesterolemia. It is well known that ezetimibe targets Niemann-Pick C1-Like 1 (NPC1L1) protein, which is a critical mediator of intestinal dietary cholesterol absorption [10]. Cholesterol- and ezetimibe-binding sites on NPC1L1 have been mapped to the cysteine-rich N-terminal domain and the second extracellular loop of the protein, respectively [11]. A recent study suggested that cholesterol is internalized by cells with NPC1L1 through clathrin/AP2-mediated endocytosis, and ezetimibe inhibits dietary cholesterol absorption by blocking this NPC1L1 internalization [12]. Previous studies showed that NPC1L1 also mediates alpha-tocopherol (vitamin E) and phytonadione (vitamin K) transports [13, 14]. Therefore, it is interesting whether NPC1L1 has an ability to transport dietary OCs. Although the cellular transporter of dietary OCs in the intestines remains unclear, OCs are transported to the bloodstream via the lymphatic system after being absorbed in the intestines [15, 16]. OCs that are structurally similar to cholesterol may be absorbed via NPC1L1. A previous study in humans showed that ezetimibe, 10 mg per day for 30 days, decreases the postprandial serum levels of dietary 5,6α-epoxy and 7-keto, suggesting that ezetimibe inhibits their absorption [17]. This absorption study was conducted in 7 subjects before and after the administration of ezetimibe for 30 days. Therefore, it is difficult to discern the decrease in 5,6α-epoxy and 7-keto absorption by ezetimibe from their reduced production in vivo via ezetimibe-mediated inhibition of cholesterol absorption. In addition, it remains unclear whether ezetimibe inhibits the absorption of other molecular species of dietary OCs in addition to 5,6α-epoxy and 7-keto. Therefore, more detailed investigations of lymphatic OC transport using animal models are essential for understanding OC absorption.

The lymphatic transport of dietary lipids in rats has often been measured by infusing a lipid emulsion into the stomach or duodenum for quantitative measurement [18]. However, this method fails to consider the interaction of the lipid emulsion with other dietary components [19]. Because dietary lipids are packaged in chylomicrons that are transported in the mesenteric lymph, the lymph duct-cannulated animal model is advantageous in that it allows direct lipid absorption measurements. In this study, we permanently cannulated the thoracic duct to measure the lymphatic transport of dietary OCs in rats under near-physiological conditions. This method is superior because it evaluates the lymphatic transport of dietary lipids during actual dietary fat absorption from a normal diet without the stress of animal restraint [20–22].

In the present study, we investigated whether NPC1L1 inhibition by ezetimibe reduces lymphatic OC transport in rats. Rats were fed diets containing OCs with and without ezetimibe and their lymphatic OC transports were measured using permanent cannulation of the thoracic lymph duct. We also performed in silico analyses to compare the binding energies of the rat NPC1L1 N-terminal domain (NPC1L1-NTD) and cholesterol or an oxycholesterol found in vivo, and determined the amino acids involved in the bindings.

Materials and Methods

Materials

5-Cholesten-3β,19-diol (19-hydroxycholesterol, 19-OH), 7α-OH, 7β-OH, 25-OH, cholestan-3β,5α-diol-6-one (5α-hydroxy-6-ketocholestanol, 5α-OH-6-keto), 5α-cholestan-3β-ol-6-one (6-ketocholestanol, 6-keto), 7-keto, 5,6α-epoxy, 5,6β-epoxy, cholestan-3β,5α,6α-triol (α-cholestanetriol, α-triol), and β-triol were purchased from Steraloids Inc. (Newport, RI, USA). Zetia™ (containing 100 mg/g ezetimibe) was supplied by Bayer Yakuhin, Ltd. (Osaka, Japan). Zetia™ was powdered with a food processor (TML-160, Tescom Denki, Co. Ltd., Tokyo, Japan) and used to prepare the experimental diets. Lard (containing 642 μg/g cholesterol) was purchased from Sigma-Aldrich (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan) and used as a starting material in the preparation of OCs. OCs were prepared by heating cholesterol at 150 °C for 12 h. The heated products were applied to a silicic acid column (Silica Gel 60, 70–230 mesh, Nacalai Tesque, 3 cm^2 × 30 cm) and the resulting OCs were eluted with acetone after being washed with diethyl ether, as described previously [23]. The composition of the heat-prepared OCs is shown in Table 1.

Table 1 Composition of cholesterol-oxidation products (oxycholesterols) in the diets

	Weight %[a]
Cholesterol	0.84
7-Ketocholesterol	41.65
5α,6α-Epoxycholesterol	11.38
6-Ketocholestanol	8.36
5β,6β-Epoxycholesterol	6.60
7α-Hydroxycholesterol	3.59
β-Cholestanetriol	3.26
5α-Hydroxy-6-ketocholestanol	2.68
25-Hydroxycholesterol	1.75
α-Cholestanetriol	1.34
7α-Hydroxycholesterol	0.76
Unidentified oxidized cholesterols	17.78

[a] Each value is the means of triplicate measurements

Animals, Diets, and Permanent Thoracic Lymph Duct Cannulation

Experiment 1 Seven-week-old male Sprague–Dawley (SD) rats (Kud:SD) weighing 230–240 g were obtained from Kyudo (Kumamoto, Japan) and maintained in a temperature-controlled room (21–23 °C). The basal diet was prepared according to recommendations of the AIN-76 formulation as described previously [24] and supplemented with lard (100 g/kg diet). The OC diet was adjusted by adding OCs (0.75 g/kg diet) to the basal diet. The rats were trained to consume the basal diet twice per day between 10:00–11:00 and 16:00–17:00 for 5 days. On day 6, the rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan) prior to permanent cannulation of the thoracic lymph duct as described previously [20–22]. After the surgery, the rats were returned to their cages and given free access to isotonic glucose solution (139 mM glucose and 85 mM NaCl in distilled water). On day 7, the rats were divided into 3 groups ($n = 5$/group) after the surgery (Fig. 1). In the recuperative period, the basal diet was given to rats of the control and OC groups, whereas the basal + ezetimibe diet was given to rats of the OC + ezetimibe group to ensure the inhibitory action of NPC1L1 by ezetimibe (Fig. 1). Consistent with our previous study [21], ezetimibe was administered orally at a dose of 5 mg/kg per day to the rats from the recuperative period (day 7) to the lymph collection period (day 9). In consideration of food intakes during the recuperative period and the lymph collection period, the basal diet for the recuperative period was adjusted by adding Zetia™ (1.1 g/kg diet), and the OC diet for the lymph collection was adjusted by adding Zetia™ (3 g/kg diet). The feeding schedule was as described above during the recuperative period (days 7–8). On day 9, after a 20-min collection period, the rats were given free access to their respective diets (the basal diet as a control group, OC diet, or OC + ezetimibe diet) for 30 min. Then, the lymph was collected every hour for 7 h. The rats had free access to deionized water throughout this experiment. The collected lymph was maintained at 4 °C overnight before fibrin was removed. Butylated hydroxytoluene (BHT) (Nacalai Tesque, Kyoto, Japan), an antioxidant used as a food additive, was added to each sample, and the samples were stored at − 30 °C until lipid analysis. Following lymph collection, the rats were anesthetized with Somnopentyl and euthanized by exsanguination.

Experiment 2 Seven-week-old male SD rats (Kud:SD) were trained to consume the basal diet twice per day between 10:00–11:00 and 16:00–17:00 for 2 days. Groups of 6 rats were assigned either the basal diet or the basal + ezetimibe diet and then fed their respective diets for 2 days under the same feeding schedule. On day 5, the rats, weighting approximately 270 g (271 ± 2 and 273 ± 1 g for the OC and OC + ezetimibe groups, respectively), were administered an Intralipid™ bolus (3 mL/rat) containing 3.2 mg of OC with or without 1.5 mg of ezetimibe. We previously confirmed that lymphatic lipid transports in rats peaked at 4 h after feeding of diets [20–22]. Based on the results of previous studies, 2 h after administration, which is the midpoint of lymphatic lipid transport peaks, the rats were euthanized by exsanguination under Somnopentyl anesthesia. The small intestine was washed twice with ice-cold saline and divided into two equal lengths. The proximal intestinal mucosa was scraped off and then stored at − 30 °C until lipid analysis.

Determination of Oxycholesterols in the Lymph and Proximal Intestinal Mucosa

The OC levels in the lymph and proximal intestinal mucosa were analyzed according to a previous report [25]. Briefly, to 100 μL of the lymph was added 500 ng of 19-OH as an internal standard. The total lipids in the sample were extracted with 20 volumes of

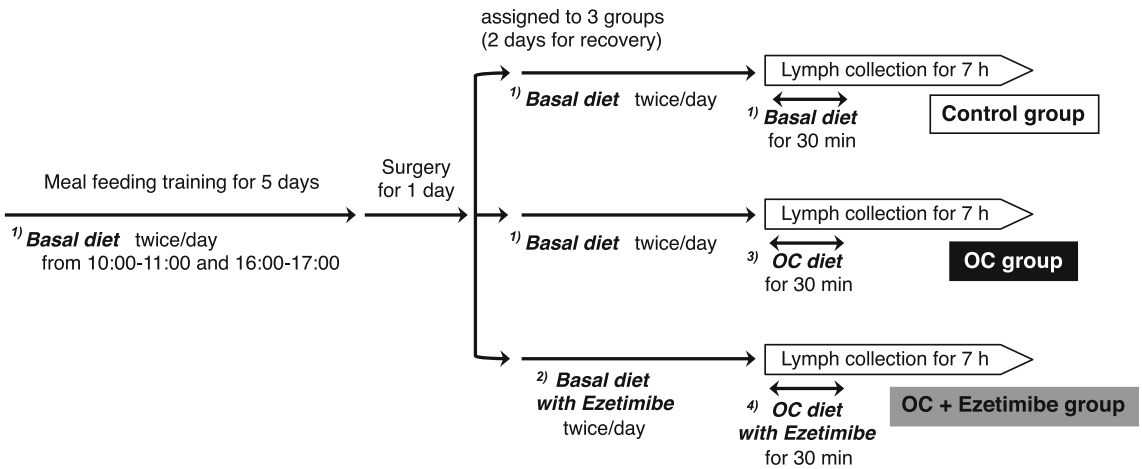

Fig. 1 Protocol for experiment 1. [1)] The basal diet was prepared according to recommendations of the AIN-76 formulation and supplemented with lard (100 g/kg diet). [2)] The basal diet plus ezetimibe was adjusted Zetia™ (1.1 g/kg diet) to the basal diet. [3)] The OC diet was adjusted by adding OCs (0.75 g/kg diet) to the basal diet. [4)] The OC diet plus ezetimibe was adjusted Zetia™ (3 g/kg diet) to the OC diet

chloroform:methanol (2:1, *v*/*v*) containing 0.01% (*w*/*v*) BHT. Total lipids were extracted from 0.5 g of proximal intestinal mucosa with 20 volumes of chloroform:methanol (2:1, *v*/*v*) containing 0.01% (*w*/*v*) BHT and then adjusted to 25 mL in a volumetric flask up. One hundred nanograms of 19-OH was added to 1 mL of this intestinal mucosal lipid extract. Freshly prepared 1 M ethanolic potassium hydroxide solution (4 mL) was added to each sample, and the lipids were allowed to saponify at room temperature overnight in the dark. To each saponified sample, 4 mL H_2O was added, and the unsaponified lipids were extracted (shaken for 5 min, upper hexane layer recovered) three times with 4 mL hexane per extraction. The extracted lipids were dried under nitrogen gas and then dissolved in acetone. The lipids were applied to a Sep-Pak Silica Vac Cartridge (Nihon Waters, Tokyo, Japan) to separate OC from cholesterol. The cartridge was washed with hexane and then eluted with hexane: 2-propanol (1:200, *v*/*v*), followed by hexane: 2-propanol (3:7, *v*/*v*), which allowed for the sequential elution of cholesterol and 19-OH plus OC, respectively. The samples were converted to trimethylsilyl ethers in a mixture of trimethylchlorosilane, hexamethyldisilazane, and dried pyridine (1:3:9, *v*/*v*/*v*) for 30 min at room temperature. Each sample was dried under nitrogen gas and then dissolved in hexane for GC-MS analysis.

The analytical conditions of GC-MS were the same as described previously [25]. A quantitative analysis was performed by the internal standard method for mass spectrometry in the selected ion monitoring mode. The relative retention time and the mass-to-charge ratio of each oxycholesterol was as follows: 0.899 and *m*/*z* 457 for 7α-OH; 1.000 and *m*/*z* 366 for 19-OH; 1.044 and *m*/*z* 457 for 7β-OH; 1.061 and *m*/*z* 475 for 5,6β-epoxy; 1.083 and *m*/*z* 135 for 5,6α-epoxy; 1.231 and *m*/*z* 404 for β-triol; 1.265 and *m*/*z* 446 for 6-keto; 1.277 and *m*/*z* 457 for α-triol; 1.295 and *m*/*z* 473 for 7-keto; 1.321 and *m*/*z* 131 for 25-OH; and 1.429 and *m*/*z* 319 for 5α-OH-6-keto. Figure 2 shows a representative chromatogram, using a programmed multiple-selection ion detector for cholesterol and each OC isolated from the lymph of rats fed the OC diet. To quantify each OC, calibration curves measuring the peak area of each OC versus that of the internal standard were used. Peak identification was confirmed by the relative retention time and a mass spectral comparison with authentic standards. The coefficient of variation (CV) values for the OCs in the lymph of rats fed the basal diet or OC diet, respectively, were as follows: 10.7 or 4.0% for 7α-OH; 8.1 or 1.3% for 7β-OH; 15.7 or 6.0% for 5,6β-epoxy; 15.1 or 5.2% for 5,6α-epoxy; 36.6 or 1.3% for β-triol; 12.6 or 1.1% for 6-keto; 22.1 or 2.3% for α-triol; 16.4 or 5.2% for 7-keto, 8.2 or 5.8% for 25-OH; and 2.5 or 2.0% for 5α-OH-6-keto.

Molecular Modeling of Rat NPC1L1-NTD

A previous report by Zhang et al. showed that the NPC1L1-NTD binds cholesterol and plays essential roles in cholesterol uptake [26]. We compared the amino acid residues between human and rat NPC1L1-NTD (Supplemental Fig. S1). In rats, the cholesterol-binding site of NPC1L1-NTD was highly conserved, and the crystal structure of human NPC1L1-NTD (Protein Data Bank ID, 3QNT) was available. Therefore, this PDB file was used as a template for homology modeling using MODELLER ver. 9.16 [27]. A multiple sequence alignment of NPC1L1-NTD performed on the T-COFFEE server (http://tcoffee.vital-it.ch/apps/tcoffee/do:regular) was used as the input sequence to construct the homology models of rat NPC1L1-NTD. According to a previous report [28], MODELLER was run with the following parameter options: library_schedule, autosched.slow; max_var_iterations, 300; md_level, refine.very_slow; and repeat_optimization, 10.

Docking Simulation

Docked complexes of cholesterol or 5,6β-epoxy and rat NPC1L1-NTD predicted by MODELLER were generated with the docking program AutoDock 4.2 [29]. The PDB files of cholesterol, 5,6β-epoxy, and rat NPC1L1-NTD were converted into the AutoDock format (PDBQT format), and the Gasteiger charge was added to each atom.

AutoDock 4.2 offers three types of search engine algorithms, and we used the Lamarckian genetic algorithm (LGA) in the present study. The binding area was defined using Autogrid with a grid size of 60 × 60 × 60 and grid spacing of 0.375 Å. The docking parameters were as follows: number of individuals in population, 150; maximum number of energy evaluation, 25,000,000; rate of gene mutation, 0.02; rate of crossover, 0.8. One hundred LGA runs were performed for each docking simulation.

Inter-Fragment Interaction Energy Calculation

To better analyze the interaction between NPC1L1-NTD and cholesterol or 5,6β-epoxy, the interaction energy was calculated by the fragment molecular orbital (FMO) method [30, 31] using the PAICS program developed and provided by Dr. Ishikawa, Nagasaki University Graduate School of Biomedical Sciences [32]. The NPC1L1-NTD–cholesterol and NPC1L1-NTD–5,6β-epoxy complexes generated by AutoDock were used for the preparation of the input file of the PAICS program. In this study, we did not perform energy minimizations of the complexes using the AMBER program [33] to avoid cleavage of disulfide bonds in its structure.

Statistical Analysis

All values are expressed as means ± standard error of mean (SEM). All data were analyzed with χ^2 goodness-

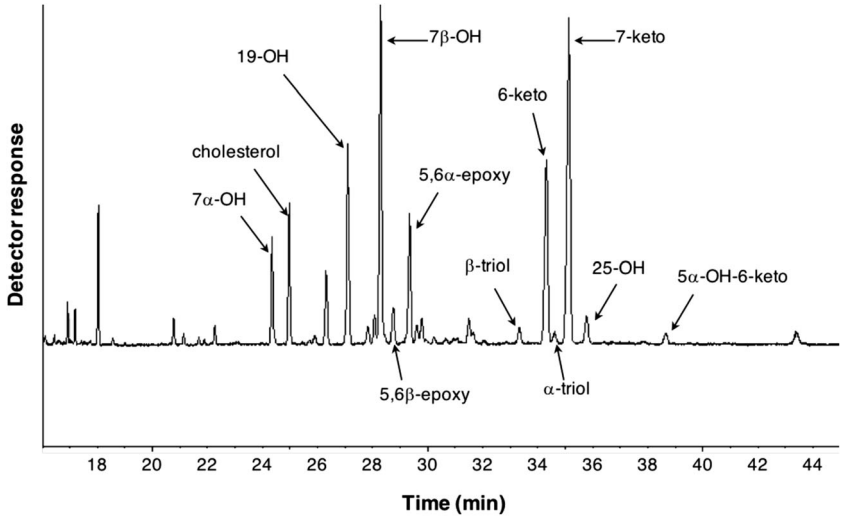

Fig. 2 Representative GC-MS chromatogram of cholesterol and OCs in rat lymph

of-fit test as a normality test. When the data was recognized as non-normal distribution, the data were normalized by logarithmic transformation and then re-analyzed with χ^2 goodness-of-fit test. Since the normality of all data was confirmed, parametric tests were used for further statistical analysis. Comparisons among 3 groups were performed by using one-way ANOVA followed by Dunnett's multiple comparison post hoc tests. Comparisons between 2 groups were performed by Student's *t* tests. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using Excel 2011 (Microsoft, USA) with the add-in software, Statcel 3 [34].

Results

Effects of Ezetimibe on Lymph Flow in Rats

The 3 groups of rats did not differ in final body weight (250 ± 6, 247 ± 2, and 254 ± 6 g for the control, OC, and OC + ezetimibe groups, respectively). After the surgery (day 7–8), the rats consumed their respective diets (basal diet or basal + ezetimibe diet) twice per day (11.5 ± 1.2, 10.8 ± 0.8, and 12.5 ± 0.7 g/day for the control, OC, and OC + ezetimibe groups, respectively). On day 9, the rats consumed the basal, OC, and OC + ezetimibe diets for 30 min (4.04 ± 0.16, 4.00 ± 0.83, and 4.30 ± 0.41 g for the control, OC, and OC + ezetimibe groups, respectively). Based on these consumption levels, the ezetimibe doses were 5.37 ± 0.38 mg/kg during the recuperative period (day 7–8) and 5.11 ± 0.55 mg/kg on day 9. The type of diet did not significantly affect food consumption. In addition, as shown in Fig. 3, there were no significant differences in the lymph flow among the groups.

Effects of Ezetimibe on Lymphatic Transport and Apparent Lymphatic Recovery Rates of Oxycholesterols in Rats

As shown in Fig. 4, the lymphatic transport of the 10 different OCs was significantly increased with OC intake. To investigate whether ezetimibe inhibited absorption of dietary OCs as well as cholesterol, the apparent lymphatic recovery rates of OC were calculated on the basis of their consumption (OC and OC + ezetimibe diets) (Fig. 5). The range of the absorption rate of the OCs was 5–25% (Fig. 5). There were no significant differences in the lymphatic recovery rates of β-triol; 25-OH; 5,6α-epoxy; α-triol; 7-keto; 5α-OH-6-keto; 7α-OH; 7β-OH; and 6-keto between the 2 groups (Fig. 5). In contrast, the ezetimibe-treated rats exhibited a significantly reduced lymphatic recovery rate of 5,6β-epoxy (Fig. 5).

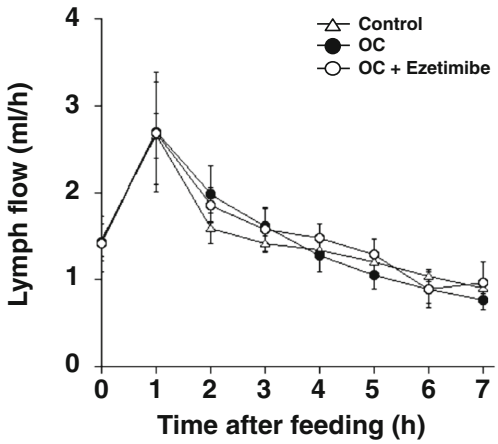

Fig. 3 Lymph flow in rats fed control (white triangles), OC (black circles), or OC + ezetimibe (white circles) diet. Values are expressed as means ± SEM ($n = 5$ per group)

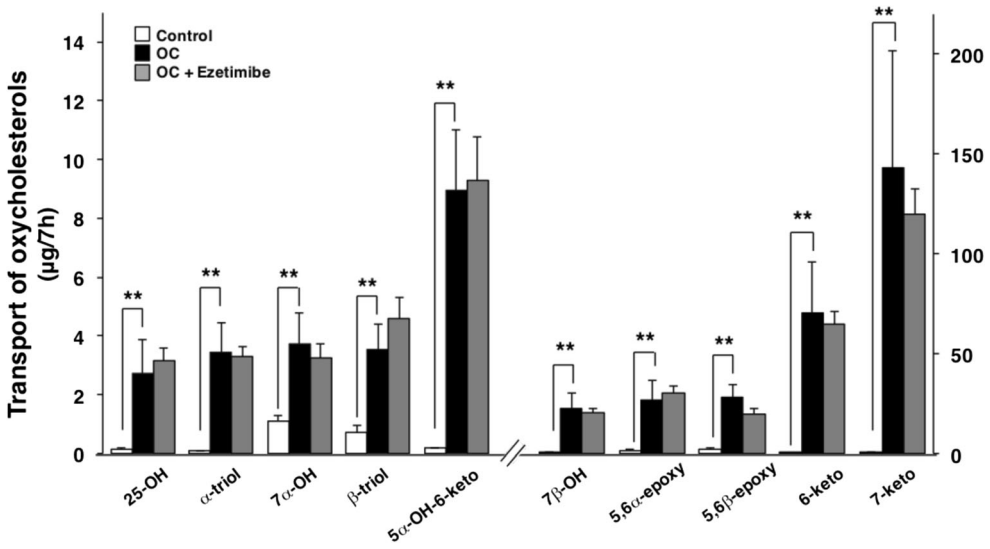

Fig. 4 Lymphatic transport of OC in rats fed control (white bars), OC (black bars), or OC + ezetimibe (gray bars) diet. Values are expressed as means ± SEM ($n = 5$ per group). Asterisks denote a significant difference (**$P < 0.01$) using ANOVA followed by Dunnett's post hoc testing

Effects of Ezetimibe on Oxycholesterol Contents in the Proximal Intestinal Mucosa of Rats

There were no significant differences in the contents of 7α-OH; 25-OH; α-triol; 5α-OH-6-keto; β-triol; 7β-OH; 5,6α-epoxy; 6-keto; and 7-keto in the proximal intestinal mucosa between the OC and OC + ezetimibe groups after 2 h of administration (Fig. 6). In contrast, the content of 5,6β-epoxy was significantly reduced in the OC + ezetimibe group compared with that of the OC group (Fig. 6).

Docking Simulation of Rat NPC1L1-NTD with Cholesterol or 5β,6β-Epoxycholesterol

To gain insight into the mechanism of inhibition of 5,6β-epoxy absorption by ezetimibe, we performed a docking simulation of rat NPC1L1-NTD with cholesterol or 5,6β-epoxy using AutoDock 4.2. We found that the top-ranked, highest binding energy between rat NPC1L1-NTD and 5,6β-epoxy was similar to that between rat NPC1L1-NTD and cholesterol (Table 2).

Analysis of Rat NPC1L1-NTD–5β,6β-Epoxycholesterol Interaction Using Fragment Molecular Orbital Calculation

The interaction energies between the amino acid residues of rat NPC1L1-NTD and cholesterol or 5,6β-epoxy were calculated using the FMO method (PAICS) and the binding structures from the docking simulation. In Table 3, the calculated energies are provided for residues located within 5.0 Å of the compounds; six residues that are important for 5,6β-epoxy binding were found. The predicted binding models of rat

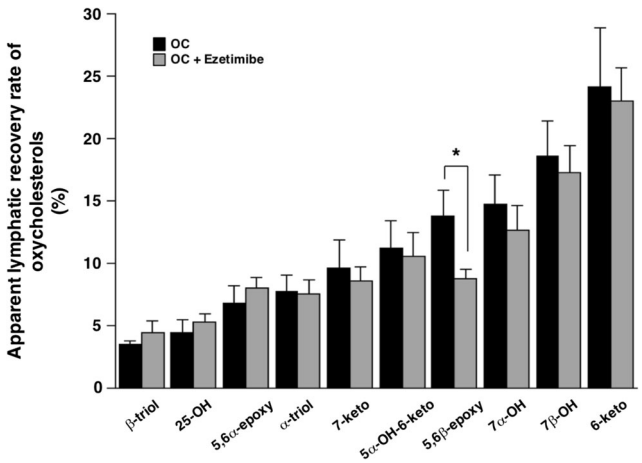

Fig. 5 Apparent lymphatic recovery rate of OC in rats fed OC (black bars) or OC + ezetimibe (gray bars) diet. Values are expressed as means ± SEM ($n = 5$ per group). Asterisks denote a significant difference (*$P < 0.05$) using Student's t tests

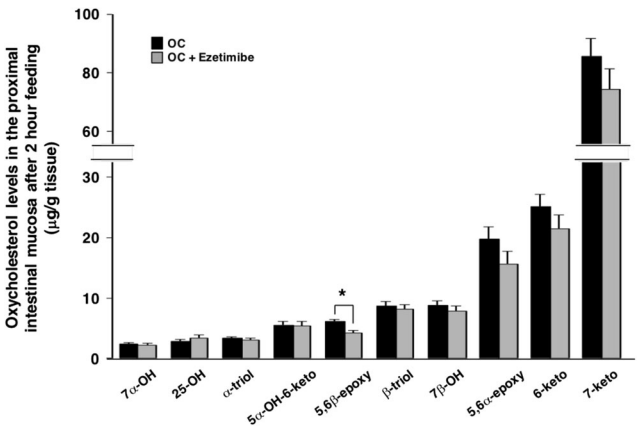

Fig. 6 OC levels in the proximal intestinal mucosa 2 h after administration of OC (black bars) or OC + ezetimibe (gray bars) diet. Values are expressed as means ± SEM ($n = 6$ per group). Asterisks denote a significant difference (*$P < 0.05$) using Student's t tests

Table 2 Binding energies of rat NPC1L1-NTD with cholesterol or 5β,6β-epoxycholesterol using the docking program, AutoDock 4.2

Compound	Binding energy (kcal/mol)	Cluster rank	Population[a]
Cholesterol	− 13.41	1	62
5β,6β-Epoxycholesterol	− 13.07	1	55

[a] This value shows the population of cluster rank. The maximum value is 100 corresponding to a docking job (100 docking runs)

NPC1L1-NTD complexed with cholesterol (Fig. 7a, c) or 5,6β-epoxy (Fig. 7b, d) are also shown.

Discussion

Using permanent thoracic lymph duct-cannulated rats, the objective of this study was to investigate whether ezetimibe inhibits the absorption of 10 different dietary OCs produced by heat. The ezetimibe-mediated inhibition of dietary OC absorption varied depending on the molecular species of OC, only significantly reducing the intestinal absorption of 5,6β-epoxy (Fig. 5). We previously observed that ezetimibe reduced the apparent lymphatic cholesterol recovery rate by 54% using permanent thoracic lymph duct-cannulated rats [21]. In the present study, ezetimibe inhibitory effect on dietary 5,6β-epoxy absorption was 36.2% (Fig. 5). The content of 5,6β-epoxy in the proximal intestinal mucosa was significantly reduced by ezetimibe (Fig. 6). Furthermore, using in silico analyses, we observed that the binding energy of rat NPC1L1-NTD and 5,6β-epoxy was similar to that of rat NPC1L1-NTD and cholesterol (Table 2), suggesting that the polar uncharged amino acids located in the steroidal region of 5,6β-epoxy were involved in the binding (Table 3 and Fig. 7d). Taken together, we conclude that dietary 5,6β-epoxy is partly absorbed via NPC1L1 in rats.

It is well known that the absorption rate of cholesterol in the intestines is approximately 50% [35]. We found that the range of absorption rates of the 10 OCs used this study was 5–25% (Fig. 5). As shown in Fig. 5, the absorption rates of the OCs varied depending on the oxidized position of the cholesterol. The absorption rate of 25-OH, a side-chain OC, was low. Carbon-5, carbon-6, and carbon-7 in the B-ring of cholesterol are easily oxidized [36], producing hydroxyl, keto, and epoxy groups. The absorption rates of α-triol and β-triol, having two hydroxyl groups at the carbon-5 and carbon-6 positions of cholesterol, were less than 10% (Fig. 5). In addition, the absorption rate of 6-keto (~ 25%), with a keto group at the carbon-6 position, was higher than that of 7-keto (~ 10%), with a keto group at the carbon-7 position (Fig. 5). Compared with that of 7-keto, increased absorption was observed with 7α-OH and 7β-OH, both with one hydroxyl group at carbon-7. However, there was no change in absorption rate due to the different configuration of the hydroxyl group between 7α-OH and 7β-OH (Fig. 5). In contrast, the absorption of 5,6α-epoxy and 5,6β-epoxy, with an epoxy group at both carbon-5 and carbon-6, varied depending on their configuration (Fig. 5). Taken together, the ability of different OC molecules to diffuse through biological membranes may vary. Baumgartner et al. [37] showed that the OC composition in erythrocyte membranes without an intracellular lipid transport system was different from the OC composition in serum. For instance, 7-keto was high in serum, whereas it was relatively low in erythrocyte membranes. In the present study, the 7-keto content in the diet was also high, whereas the absorption rate of 7-keto was low. Cell membranes may be selective for OC uptake depending on their phospholipid and fatty acid composition. To the best of our knowledge, our present study is the first report that the absorption rate of dietary 6-keto is higher than that of other OC molecules using permanent cannulation of the thoracic lymph duct. In many studies that have included OC analysis, 6-keto was not measured. The high absorptivity of 6-keto found in the present study may be due to its high capacity for simple diffusion.

Table 3 Interaction energy of each amino acid residue of rat NPC1L1-NTD with cholesterol or 5β,6β-epoxycholesterol obtained by the FMO method (PAICS)[a]

Rat NPC1L1-NTD					
Cholesterol			5β,6β-Epoxycholesterol		
Residues	Energy (kcal/mol)	Distance (Å)	Residues	Energy (kcal/mol)	Distance (Å)
GLU 37	− 23.067	1.81	VAL 54	− 17.188	1.74
SER 102	− 6.738	1.91	HIS 124	− 13.842	1.27
VAL 54	− 5.407	1.81	SER 55	− 11.862	1.66
MET 103	− 5.258	1.46	ASN 53	− 6.704	3.53
TYR 156	− 5.033	1.77	TYR 156	− 6.003	1.89
			GLU 37	− 5.855	2.29

[a] Interaction energies (less than − 5.0 kcal/mol) of residues located within 5.0 Å are provided

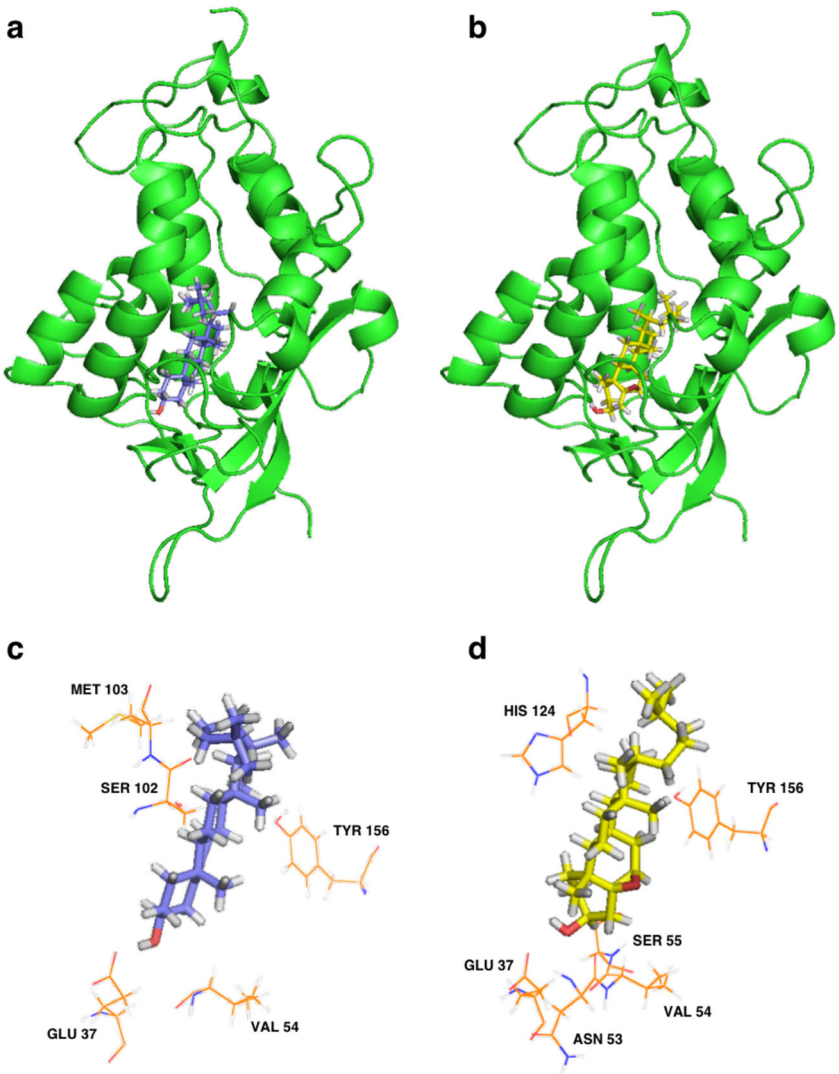

Fig. 7 Predicted binding model of rat NPC1L1-NTD complexed with cholesterol (**a**) or 5β,6β-epoxycholesterol (**b**). Coordination geometry of amino acids at the active site (**c**: rat NPC1L1-NTD and cholesterol; **d**: rat NPC1L1-NTD and 5β,6β-epoxycholesterol). The whole protein structure is shown by ribbon diagrams. Cholesterol and 5β,6β-epoxycholesterol are shown as stick models. The carbon skeletons of amino acids with attraction forces listed in Table 3 are shown in orange. The figures were made by PyMOL

In addition, a significant attenuation of OC absorption by NPC1L1 inhibition was found only with 5,6β-epoxy. The NPC1L1-NTD protrudes from the cell membranes of the small intestine, and it is thought that the cell membranes do not interact with the binding of NPC1L1-NTD and substrate. Therefore, we conclude that 5,6β-epoxy has a high binding affinity to NPC1L1-NTD.

A previous study in humans showed that ezetimibe inhibited dietary 5,6α-epoxy and 7-keto uptake into lipoproteins [17]. The 4–5 subjects were given a dose of 400 mg of 5,6α-epoxy or 7-keto in 50 mL of olive oil and 100 g of carbohydrate (mashed potatoes) with ezetimibe (10 mg/day) was added. Ezetimibe treatment significantly decreased postprandial serum levels of 5,6α-epoxy and 7-keto when each was ingested solely [17]. However, it is unknown whether this inhibitory activity of ezetimibe would also be observed when the OC molecules are ingested simultaneously. It is possible that each OC competes for the binding sites in NPC1L1. In addition, that study failed to take into account the interaction of the OCs (5,6α-epoxy and 7-keto) with other dietary components, such as protein and fiber, which can affect dietary lipid absorption [17]. In the present study, for the first time, we found that 5,6β-epoxy is an OC molecular species that preferentially binds to NPC1L1 under conditions of simultaneous intake of different OCs. Although there could be differences between humans and rats, our results indicate an important phenomenon. Future studies are needed to examine the effects of ezetimibe on dietary OC absorption in humans under actual OC intake conditions.

The formation and metabolism of the 5,6-epoxides of cholesterol (5,6α-epoxy and 5,6β-epoxy) in rat liver homogenates has been examined [38, 39]. In rat liver microsomal fractions, 5,6β-epoxy was formed at three- to fourfold the level of 5,6α-epoxy [39]. In addition, both 5,6-epoxides of cholesterol, especially 5,6β-epoxy, were converted by a microsomal hydrolase to β-triol [39]. Among the OC molecular species, 5,6α-epoxy; 5,6β-epoxy; and their principal metabolic product, β-triol, are purportedly angiotoxic [40–42].

Sevanian et al. [42] reported that the cytotoxic potencies of these OCs in rabbit aortic endothelial cells were ranked in the following order: β-triol > 5,6β-epoxy > 5,6α-epoxy. Collectively considering these findings, we believe that the inhibition of dietary 5,6β-epoxy absorption is physiologically meaningful in suppressing its cytotoxicity and that of its metabolite, β-triol, in vivo.

Several reports regarding the effects of ezetimibe after dietary OC intake demonstrated alteration of hepatic function and lipid metabolism in rats [43] and monkeys [44], and prevention of atherosclerotic plaque destabilization and rupture in apo E-deficient ($ApoE^{-/-}$) mice [45]. Terunuma et al. [43] examined the effects of ezetimibe (0.7 mg/kg body weight) on hepatic lipid and antioxidant levels in rats fed COP (containing 26.0% cholesterol, 3.0% 7α-OH, 6.0% 7β-OH, 3.0% 5,6β-epoxy, 10.0% 5,6α-epoxy, 1.8% 3,5-diencholesterol, 15.6% β-triol, 15.5% 7-keto, and 42.1% unidentified OCs) for 27 days. Although this article did not describe changes in the individual OCs, the levels of total OC in the plasma and liver were lowered by ezetimibe via increased OC excretion in the feces. Deushi et al. [44] examined the effects of ezetimibe (0.2 mg/kg body weight) on hepatic lipid content in monkeys fed high-fat diet plus 0.015% COP (containing 68.0% cholesterol, 3.7% 7α-OH, 4.3% 7β-OH, 3.9% 5,6β-epoxy, 0.5% 5,6α-epoxy, 1.6% β-triol, 4.4% 7-keto, and 13.0% unidentified OCs) for 24 weeks. Under these conditions, ezetimibe treatment significantly reduced hepatic lipid content in monkeys fed high-fat diet plus COP. Sato et al. [45] examined the effects of ezetimibe (5 mg/kg body weight) on atherosclerotic plaques in $ApoE^{-/-}$ mice fed high-fat diet plus 0.15% COP (containing 68% cholesterol, 3.7% 7α-OH, 4.3% 7β-OH, 3.9% 5,6β-epoxy, 0.5% 5,6α-epoxy, 1.6% β-triol, 4.4% 7-keto, and 13.0% unidentified OCs) for 8 weeks. Ezetimibe treatment significantly decreased plasma cholesterol levels and prevented the acceleration of plaque destabilization and rupture in the mice. Unfortunately, the COP used in the above three articles contained a large amount of cholesterol, and whether ezetimibe inhibited the absorption of cholesterol or OCs could not be distinguished. The inhibition of cholesterol absorption by ezetimibe may contribute to the suppression of OC production in vivo. Future studies are needed to investigate whether long-term inhibition of dietary OC absorption attenuates the development and progression of various diseases.

Conclusions

We found that, using permanent thoracic lymph duct-cannulated rats, the ezetimibe-mediated (NPC1L1) inhibition of dietary OC absorption varied depending on the molecular species of OC, and only the absorption of 5,6β-epoxy was significantly reduced. In addition, in silico analysis demonstrated that the binding energy of rat NPC1L1-NTD and 5,6β-epoxy was similar to that of rat NPC1L1-NTD and cholesterol, suggesting that the polar uncharged amino acids located in the steroidal region of 5,6β-epoxy were involved in the binding.

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Author Contributions B.S. wrote the manuscript. B.S., Y.F., Y. N., A.K., and H.O. participated in the experimental work and collected and analyzed data. B.S., K.I., and M.S. contributed to the study design, supervised the study, and commented on the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The handling and euthanasia of all animals were carried out in accordance with nationally prescribed guidelines, and ethical approval for the studies was granted by the Animal Care and Use Committee of Kyushu University. The authorization number was A24-034-2. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Informed consent is not applicable in this article.

Consent for Publication All authors have approved the submission for publication of this study.

Abbreviations 19-OH, 19-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 5α-OH-6-keto, 5α-hydroxy-6-ketocholestanol; 6-keto, 6-ketocholestanol; 7-keto, 7-ketocholesterol; 5,6α-epoxy, 5α6α-epoxycholesterol; 5,6β-epoxy, 5β6β-epoxycholesterol; α-triol, α-cholestanetriol; β-triol, β-cholestanetriol; Niemann-Pick C1-Like 1, NPC1L1; NTD, N-terminal domain; OC, oxycholesterol

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