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Intestinal Absorption of γ-Tocotrienol Is Mediated by Niemann-Pick C1-Like 1: In Situ Rat Intestinal Perfusion Studies

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ABSTRACT:

γ-Tocotrienol (γ-T3) is a member of the vitamin E family that displays potent anticancer activity and other therapeutic benefits. The objective of this study was to evaluate γ-T3 intestinal uptake and metabolism using the in situ rat intestinal perfusion model. Isolated segments of rat jejunum and ileum were perfused with γ-T3 solution, and measurements were made as a function of concentration (5–150 μM). Intestinal permeability (P_{eff}) and metabolism were studied by measuring total compound disappearance and major metabolite, 2,7,8-trimethyl-2-(β-carboxy-ethyl)-6-hydroxychroman, appearance in the intestinal lumen. γ-T3 and metabolite levels were also determined in mesenteric blood. The P_{eff} of γ-T3 was similar in both intestinal segments and significantly decreased at concentrations ≥25 μM in jejunum and ileum ($p < 0.05$), whereas metabolite formation was minimal and mesenteric blood concentrations of γ-T3 and metabolite remained very low. These results indicate that γ-T3 intestinal uptake is a saturable carrier-mediated process and metabolism is minimal. Results from subsequent in situ inhibition studies with ezetimibe, a potent and selective inhibitor of Niemann-Pick C1-like 1 (NPC1L1) transporter, suggested γ-T3 intestinal uptake is mediated by NPC1L1. Comparable findings were obtained when Madin-Darby canine kidney II cells that express endogenous NPC1L1 were incubated with increasing concentrations of γ-T3 or γ-T3 with increasing concentrations of ezetimibe. The present data show for the first time that γ-T3 intestinal absorption is partly mediated by NPC1L1.

γ-Tocotrienol (γ-T3) is one form of naturally occurring vitamin E present in palm, wheat germ, and rice bran (Sundram et al., 2002; Sookwong et al., 2007). Although the vitamin E family of compounds is divided into two subgroups, tocopherols and tocotrienols, all the members in both subgroups possess the same general structural features of an aromatic chromanol head and a 16-carbon hydrocarbon tail. However, tocotrienol isoforms have an unsaturated phytyl tail, whereas tocopherols have a saturated phytyl tail (Fig. 1). Recent studies have shown that γ-T3 may provide significant health benefits, including anticancer (Shah and Sylvester, 2005) and anticholesterolemic (Song and DeBose-Boyd, 2006) activity, as well as acting as a potent antioxidant (Tomeo et al., 1995).

However, studies have also established that it is very difficult to obtain therapeutic levels of γ-T3 in the blood and target tissues by simple oral administration (Sylvester and Shah, 2005). After its oral administration, γ-T3 is absorbed from the intestine and transported to the systemic circulation through the lymphatic pathway (Ikeda et al., 1996). The pharmacokinetics of γ-T3 in rats (Yap et al., 2003) and humans (Yap et al., 2001) has been reported previously. In humans, although the absolute bioavailability was not determined, γ-T3 relative bioavailability increased 3.5-fold when administered with food (Yap et al., 2001), whereas in rats, γ-T3 oral bioavailability has been found to be as low as 9% (Yap et al., 2003). In addition, in fasting humans, plasma tocotrienol concentration was not significantly increased after tocotrienol supplementation (Hayes et al., 1993). γ-T3 is a lipophilic compound with poor solubility (<0.01 μg/ml), and its intestinal absorption increases when taken with food. Food enhances γ-T3 solubility owing to the formation of mixed micelles as a result of the stimulation of bile salts and pancreatic enzyme secretions. Furthermore, food increases the lymph lipid precursor pool inside the enterocytes that eventually will enhance lymphatic transport (Yap et al., 2001; Kamran et al., 2007). Although such increase in γ-T3 solubility when administered with food is significant to enhance its bioavailability, γ-T3 absorption is not complete (Yap et al., 2001, 2003). These findings suggest the existence of other barriers and/or additional mechanisms that are involved in γ-T3 oral absorption and/or transport by primary enterocytes. Tsuzuki et al. (2007) investigated the importance of the intestinal uptake properties of γ-T3 on its plasma disposition in vitro and in vivo. Compared with α-tocopherol, Caco-2 cellular uptake and transport of γ-T3 were rapid and consistent with results obtained from in vivo studies in mice. However, maximal plasma levels of γ-T3 were much lower than those observed for α-tocopherol (Yap et al., 2003; Tsuzuki et al., 2007).

Oral absorption of drugs can be influenced by specific transporters and metabolizing enzymes present in the endothelial cells lining the

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ABBREVIATIONS: γ-T3, γ-tocotrienol; γ-CEHC, 2,7,8-trimethyl-2-(β-carboxy-ethyl)-6-hydroxychroman; NPC1L1, Niemann-Pick C1-like 1; PEG4000, polyethylene glycol 4000; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MRM, multiple-reaction monitoring; P_{eff}, effective permeability; F_{met}, fraction metabolized; MDCK, Madin-Darby canine kidney; SR-B1, scavenger receptor class B type 1.

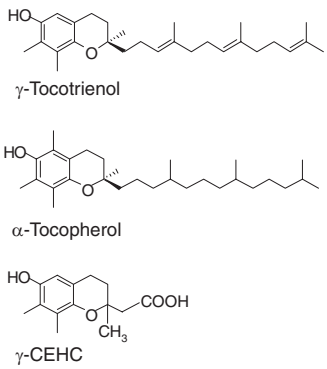

FIG. 1. Structures of γ-T3, α-tocopherol, and γ-CEHC.

gastrointestinal tract. Although no previous studies have examined the role of transporters in the intestinal absorption of γ-T3, its metabolism has been investigated. γ-T3 metabolism is catalyzed by the human-metabolizing enzyme CYP4F2 (Sontag and Parker, 2007) into 2,7,8-trimethyl-2-(β-carboxy-ethyl)-6-hydroxychroman (γ-CEHC) (Swanson et al., 1999). γ-CEHC has been shown to inhibit the production of prostaglandin E2 by inhibiting cyclooxygenase-2 enzyme, which plays a key role in inflammation and its associated diseases (Jiang et al., 2000). CYP4F2 is expressed in human liver, kidney, and intestinal cells (Kikuta et al., 1999). When γ-T3 was coadministered to rats with ketoconazole, a CYP4F2 inhibitor (You et al., 2005), urinary excretion of γ-CEHC decreased and γ-T3 concentration in the jejunum increased 3 h after coadministration (Abe et al., 2007). These findings suggest that CYP4F-dependent metabolism of γ-T3 could be a critical determinant of its intestinal absorption. However, more studies are required to investigate the intestinal contribution to γ-T3 metabolism.

At present, very little is known about γ-T3 absorption by intestinal epithelial cells. The purpose of the current study was to characterize the intestinal uptake and metabolism of γ-T3 using the in situ single-pass intestinal perfusion model in rats. We investigated the jejunal and ileal permeability and jejunal metabolism of γ-T3. The permeability of γ-T3 was measured at different concentrations ranging from 5 to 150 μM, and the intestinal metabolism was estimated by measuring the fraction of γ-T3 metabolized in the intestine. The mesenteric blood levels of γ-T3 and its metabolite γ-CEHC were also evaluated. In addition, because γ-T3 intestinal uptake exhibited nonlinear kinetics, we investigated the possible contribution of the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) in its transport across the rat intestinal membrane using the in situ model, in addition to in vitro cell uptake and inhibition studies.

Materials and Methods

Materials and Reagents. γ-T3 was provided by First Tech International Co., Ltd. (Hong Kong). γ-CEHC was supplied by Eisai Co., Ltd. (Tokyo, Japan). Ezetimibe was donated by Schering Plough (Kenilworth, NJ). Cholesterol and α-tocopherol were obtained from Sigma-Aldrich (St. Louis, MO). Sodium taurocholate and phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). [1,2-^{14}C]Polyethylene glycol 4000 (PEG4000; specific activity = 0.75 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled PEG4000 was purchased from Spectrum Chemical (Gardena, CA). Supplies for cell culture were obtained from American Type Cell Culture Collection (Manassas, VA). Other chemicals and reagents were obtained from VWR (West Chester, PA).

Animals. Male Sprague-Dawley rats weighing 260 to 400 g were acquired from Harlan Laboratories (Houston, TX). All the animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Monroe, and all the surgical and treatment procedures were consistent with the Institutional Animal Care and Use Committee policies and procedures. Rats were maintained on a 12-h light/dark cycle before the study and were fasted 12 to 18 h with water ad libitum before each experiment.

In Situ Rat Intestinal Perfusion Model. After overnight fasting, rats were anesthetized with intramuscular injection of 50 mg/kg ketamine and 10 mg/kg xylazine mixture, followed by intraperitoneal injection of 40 mg/kg pentobarbital. The small intestine was exposed by midline incision; approximately 15 cm of upper jejunum (proximal to the duodenum) and ileum (caudal to the cecum) was externalized. The segments were then flushed with warm normal saline to remove intestinal contents and cannulated with glass cannulas inserted at the inlet and the outlet of each segment and were secured by ligation with silk suture. The inlet tubing of each segment was connected to a 30-ml syringe that was placed in an infusion pump (Harvard Apparatus Inc., Holliston, MA). Animals, perfusion solutions, and pump were enclosed in a Plexiglas (Widgett Scientific Inc., Baton Rouge, LA) thermostatically controlled chamber set at 30°C. The perfusate was pumped through the lumen at 0.14 ml/min flow rate. The perfusate solution consisted of γ-T3 prepared as mixed micelles in 1.6 g/l sodium taurocholate and 0.575 g/l phosphatidylcholine in phosphate buffer composed of 3.9 g/l potassium dihydrogen phosphate and 7.7 g/l potassium chloride. The pH was adjusted to 6.5 with sodium hydroxide.

γ-T3 perfusate solutions were investigated in the concentration range of 5 to 150 μM. [^{14}C]PEG4000 (0.02 μCi/ml) with 0.1% radioactive PEG4000 was added to the perfusate solution as a marker for water secretion or absorption.

The exposed segments were covered by saline-soaked gauze and a plastic film. The first 40-min presteady-state outlet perfusate was discarded, which represents the stabilization period to reach steady state. Subsequently, the perfusate was collected in vials at 10-min intervals for 100 min. Blood samples, obtained at the end of the perfusion studies from the mesenteric vein, were withdrawn as reported previously (Kaddoumi et al., 2006). Blood samples were centrifuged to separate plasma. Blood collection was followed by rapid removal of the intestinal perfused segments and soaked in ice-cold saline. Plasma and tissues samples were kept frozen at −20°C until analysis. Similar in situ jejunal perfusion experiments were conducted with α-tocopherol using 10 and 50 μM concentrations prepared in mixed micelles as described above for γ-T3. In experiments other than control, perfusate contained, in addition to γ-T3, the NPC1L1 inhibitor ezetimibe at different concentrations.

Sample Preparation. *γ-T3 sample preparation.* Perfusate samples were diluted with acetonitrile (1:10 or 1:30, depending on the perfusate concentration). γ-T3 was then analyzed by direct injection of 20 μl onto the high-performance liquid chromatography (HPLC) system.

Extraction of γ-T3 from the intestinal tissues was conducted as follows. Collected tissues were allowed to thaw at room temperature and then immediately homogenized (T10 basic Ultra-Turax homogenizer; IKA Works, Inc., Wilmington, NC) in normal saline containing 1% ascorbic acid (1:1, tissue weight to saline volume). One hundred microliters of the homogenate was then vortex-mixed with acetonitrile (1:6, v/v) for 30 s. Samples were then centrifuged (Eppendorf centrifuge 5804R; Eppendorf AG, Hamburg, Germany) at 10,000*g* for 10 min. From the collected supernatant, 20 μl was injected onto the HPLC system. γ-T3 extraction from plasma was performed using a modification of the method described by McIntyre et al. (2000). In brief, 100 μl of 3% sodium lauryl sulfate solution containing 1% ascorbic acid and 125 μl of ethanol were added to 50 μl of plasma and mixed. This step was followed by the addition of 500 μl of hexane, vortex mixing for 30 s, and centrifugation at 10,000*g* for 10 min. From the organic layer, 400 μl was transferred into a vial and evaporated to dryness (CentriVap concentrator; Labconco Corporation, Kansas City, MO). The residues were then reconstituted with the mobile phase, from which 10 μl was injected onto the liquid chromatography/tandem mass spectrometry (LC/MS/MS) system.

γ-CEHC sample preparation. γ-CEHC was extracted from the perfusate and plasma samples by liquid-liquid extraction method using ethyl acetate. In brief, 1% ascorbic acid (5 μl) and HCL (12 N, 4 μl) were added to 50 μl of plasma sample, vortex-mixed, and centrifuged for 10 min at 10,000*g*. The organic layer was then transferred and evaporated to dryness followed by reconstitution with mobile phase before injection onto the LC/MS/MS system.

Quantification of γ-T3 and γ-CEHC. Quantification of γ-T3 in the perfusate and tissue homogenate samples was achieved by an isocratic Prominence HPLC system (Shimadzu, Columbia, MD). The system consisted of SIL 20-AHT autosampler, SPD-20A UV/VIS detector, and LC-20AB pump connected to a DGU-20A3 degasser. Data acquisition was achieved by LC Solution software version 1.22 SP1 (Shimadzu). The chromatographic condi-

tions were Luna 5-μm C18 column (250 × 4.6 mm i.d.; Phenomenex, Torrance, CA), and mobile phase consisted of methanol, ethanol, and acetonitrile (40:30:30, v/v/v) delivered at 1.0 ml/min flow rate. The wavelength was set at 210 nm. The total run time was 12 min with retention times of 6.0 and 10.3 min for γ-T3 and α-tocopherol (10 μM, used as internal standard for γ-T3 experiments), respectively. Standard curves for γ-T3 in the perfusate and tissue homogenate were prepared in the ranges of 0.5 to 150 μM and 0.4 to 195 μM, respectively. Validation experiments were performed for both perfusate and intestinal tissue homogenates. The method was found to be accurate and precise with interday precision of 1.7% for the perfusate and 13% for the tissue homogenate. The limit of detection of γ-T3 in both matrices was 0.1 μM.

γ-T3 in plasma and γ-CEHC in perfusate and plasma samples were analyzed by LC/MS/MS. In brief, the chromatographic separation was performed on a 250 × 4.6-mm Luna 5-μm PFP column (Phenomenex) using Agilent 1100 series LC system (Agilent Technologies, Santa Clara, CA) and 3200 Qtrap LC/MS/MS system (Applied Biosystems, Foster City, CA). The mobile phase used for γ-T3 separation was similar to that used with the HPLC system with the addition of 0.05% acetic acid. The mobile phase used for the metabolite separation consisted of water and methanol (20:80, v/v) containing 0.05% acetic acid. The analytes were detected by mass spectrometry using electrospray ionization interface operated in positive and negative modes for γ-T3 and γ-CEHC, respectively. Instrument control and data acquisition were carried out by the Analyst 1.4.1 software (Applied Biosystems/MDS Sciex, Foster City, CA). The analytes were detected and quantified by MS/MS in multiple-reaction monitoring (MRM) method. The following transitions (precursor > product) were used for quantification: γ-T3, 411 > 151; γ-CEHC, 263 > 219.

Under these chromatographic conditions, the detector signal was linear with respect to γ-T3 concentration over the range 15 to 366 nM γ-T3 in plasma. For the metabolite, the calibration curves were linear over the ranges 2 to 75 nM and 10 to 75 nM in the perfusate and plasma, respectively. Intraday and interday precision for γ-T3 and γ-CEHC detection in different concentrations for each matrix was evaluated and found to be <16%. MRM chromatograms of γ-T3 (A) and γ-CEHC (B) extracted from rat mesenteric plasma sample obtained from jejunal segment perfused with 50 μM γ-T3 are shown in Fig. 2.

Effective Permeability and Fraction Metabolized Determinations. [^{14}C]PEG4000 was used as a marker to calculate water flux by comparing the radioactivity counts of [^{14}C]PEG4000 in the perfusate before and after the perfusion. No significant difference was found, indicating water loss or excretion was minimal; thus, no corrections were made for the estimation of analyte concentrations. The effective permeability (P_{eff}) of γ-T3 across the rat intestine was calculated based on its loss from the perfusate according to the equation:

$$P_{eff} = \frac{-Q}{2\pi rL \times \ln(C_t/C_0)}$$

where Q is the perfusate flow rate through the segment (0.14 ml/min), r is the radius of the intestinal lumen (0.2 cm), L is the length of the perfused segment (15 cm), C_o is γ-T3 concentration at the start of the perfusion (from the entry tubing), and C_t is the steady state of γ-T3 concentration exiting the perfused intestinal segment.

The fraction of γ-T3 metabolized (F_{met}) measured in the jejunum was calculated as the concentration ratio of metabolite (γ-CEHC) over total loss of parent γ-T3 from the lumen according to the equation:

$$F_{met} = \frac{(\gamma - \text{CEHC})_{out}}{[(\gamma - \text{T3})_{in} - (\gamma - \text{T3})_{out}]}$$

In the tissue and plasma, γ-T3 and its metabolite concentrations were calculated from their corresponding working calibration curves.

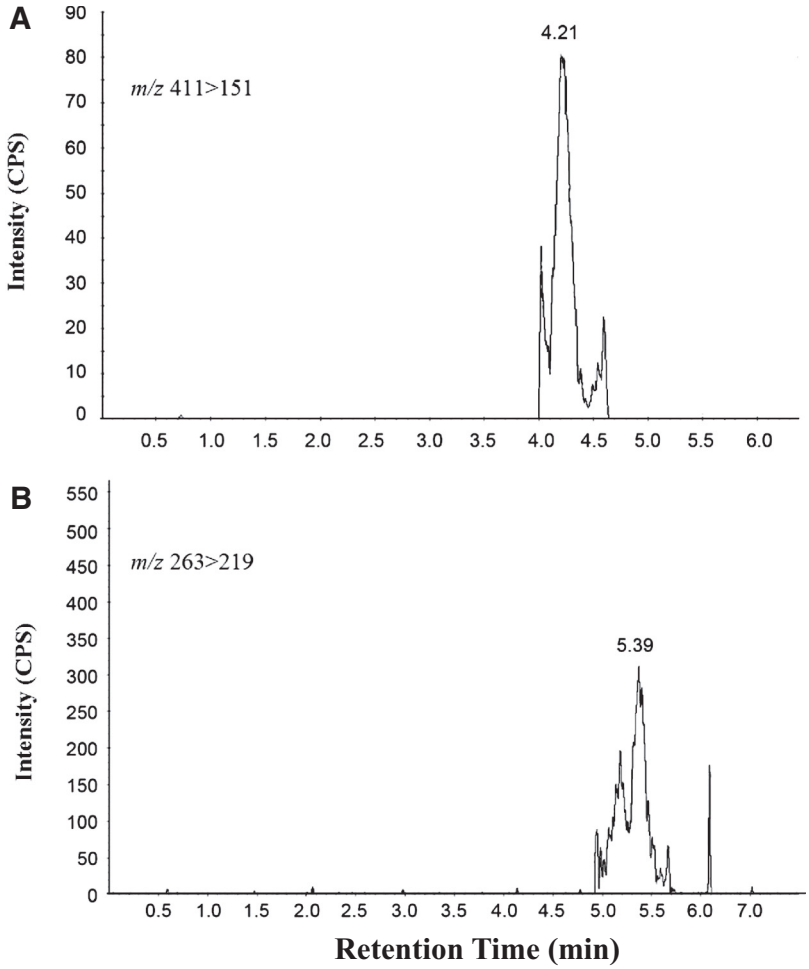

FIG. 2. MRM chromatograms of γ-T3 (A) and γ-CEHC (B) extracted from rat mesenteric plasma sample obtained from jejunal segment perfused with 50 μM γ-T3. Eluted fractions from the column containing the compound only were allowed to enter the LC/MS/MS for detection. The peaks correspond to 50.6 and 14.0 nM at 4.21 and 5.39 min for γ-T3 and γ-CEHC, respectively, of the same rat.

Cell Culture. Madin-Darby canine kidney (MDCK) II cells were a gift from Dr. R. Govindarajan (University of Georgia, Athens, GA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2.5% antibiotics (penicillin and streptomycin).

Western Blot Analysis. The analysis of NPC1L1 expression in MDCK II cells was performed as follows: 16 μg of protein extracts was resolved using 7.5% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto a nitrocellulose membrane. After blotting, the membrane was blocked using 2% bovine serum albumin in phosphate-buffered saline. The membrane was then immunoblotted with NPC1L1 rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and β-actin (C-11) primary antibodies at 1:200 and 1:3000 dilutions, respectively, and incubated overnight at 4°C. For protein detection, the membrane was subsequently incubated with secondary anti-rabbit IgG antibody for NPC1L1 and anti-goat IgG antibody for β-actin, both labeled with horseradish peroxidase, each at a 1:5000 dilution. The blots were developed using Pierce ECL Western Blotting Substrate Detection Kit (Thermo Fisher Scientific, Waltham, MA). Quantitative analysis of the immunoblots was performed using Syngene luminescent image analyzer (Scientific Resources Southwest, Inc., Stafford, TX).

Micellar γ-T3 and Ezetimibe Preparation. The micelles were prepared according to the method described by Narushima et al. (2008). In brief, cholesterol dissolved in ethanol, phosphatidylcholine dissolved in methanol, taurocholate dissolved in ethanol, and γ-tocotrienol or ezetimibe dissolved in methanol were mixed and evaporated to dryness under nitrogen. RPMI 1640 serum-free medium was then added to prepare the medium for transport experiments. Serial dilutions of the mixed micelles containing ezetimibe were done with ezetimibe-free mixed micelle solution to prepare 0, 2, 20, and 200 μM concentrations of ezetimibe mixed micelles. γ-T3 mixed micelles were prepared at 2 μM. In the inhibition studies, final concentrations of ezetimibe per well were 0, 1, 10, and 100 μM, whereas the final concentration was 1 μM/well for γ-T3.

γ-T3 Uptake and Inhibition Studies. Cells were seeded in a 48-well plate at a density of 5000 cells/well and cultured for 2 days until cells were confluent. Uptake studies were performed in triplicate. On the day of the experiment, cells were incubated with γ-T3 mixed micelles at increasing concentrations (1, 5, 10, 25, and 50 μM) for 60 min. At the end of the incubation period, cells were washed two times with 10 μM taurocholate dissolved in phosphate buffer solution to wash out nonspecific bound γ-T3. Cells were lysed and incubated with mixing for 30 min. Aliquots of 100 μl were used for γ-T3 analysis by LC/MS as described previously, and 10 μl was used for protein determination. Ezetimibe inhibition studies were conducted in the same manner except that before the addition of γ-T3 mixed micelles, cells were preincubated with different concentrations of ezetimibe mixed micelles for 30 min, followed by the addition of γ-T3 mixed micelles.

Statistical Analysis. Data are presented as mean ± S.E.M. of at least three experiments. Comparison between P_{eff}, F_{met}, and tissue-bound γ-T3 and γ-CEHC concentrations was made by one-way analysis of variance with Tukey's post hoc test (JMP 7 software; SAS Institute, Cary, NC). Differences were considered significant at p value less than 0.05.

Results

Permeability of γ-T3 in Rat Jejunum as a Function of Concentration. To investigate whether γ-T3 exhibits linear or nonlinear intestinal absorption kinetics, the P_{eff} of γ-T3 was examined by perfusing the concentrations 5, 10, 25, 50, 75, 100, and 150 μM mixed micelles through the jejunum of the rats. The range of selected γ-T3 perfusate concentrations corresponds to a relevant estimated dose range of 0.5 to 15 mg in humans. Based on off-setting 2 orders of magnitude differences in humans and rats in both weight and oral fluid volume of administration, similar drug concentrations in the intestinal lumen of humans and rats would be expected over a given milligram per kilogram dose range. That is, a human dose of 10 mg taken with 8 ounces of water would be estimated to give an initial upper intestinal concentration of 100 μM.

The P_{eff} results of γ-T3 in the rat jejunum as a function of perfusion concentration are presented in Fig. 3. These results show that the permeability of γ-T3 at 10 μM perfusate concentration ($26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s; $n = 6$) is significantly higher compared with the other perfusate concentrations that ranged from 25 to 150 μM ($p < 0.05$). At the lowest perfusion concentration (5 μM) under the chromatographic conditions used, γ-T3 peak was not detected, strongly suggesting that uptake was nearly complete. Nonetheless, when the limit of detection of 0.1 μM was used to estimate the permeability at 5 μM perfusate concentration, the results obtained indicated a P_{eff} value of at least 48.5×10^{-5} cm/s ($n = 4$). This value is significantly higher than the γ-T3 P_{eff} at perfusion concentration ranging from 10 to 150 μM ($p < 0.05$).

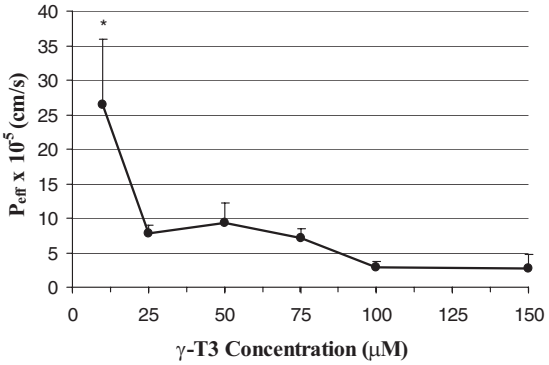

FIG. 3. P_{eff} of γ-T3 as a function of luminal concentration obtained from in situ perfused rat jejunum, $n = 3$ to 7. The error bars present the S.E.M. *, $p < 0.05$ compared with permeability from the jejunum at luminal concentrations of ≥25 μM.

Quantification of γ-T3 and γ-CEHC in Jejunal Tissue, Mesenteric Blood, and Perfusate. γ-T3 concentrations analyzed in the jejunal tissue and mesenteric blood over the same range of perfusate concentrations are shown in Table 1. In the tissue, γ-T3 concentrations increased with each corresponding increase in perfusate concentration from 10 to 150 μM. At the 5 μM perfusate concentration, γ-T3 was not detected, but blood γ-T3 was shown to be 34.1 ± 3.9 nM. However, mesenteric blood levels of γ-T3 over the entire range of perfusate concentrations showed no significant differences ($p > 0.08$) between the different treatment groups (Table 1), signifying the role of the lymphatic system in the transport of γ-T3 into the blood circulation (Ikeda et al., 1996).

γ-T3 is metabolized primarily to free and conjugated γ-CEHC (Freiser and Jiang, 2009). To measure conjugated forms of γ-CEHC in the intestine, acid hydrolysis of the perfusate and blood samples was performed according to the method by Li et al. (2008). Measurements of free and total γ-CEHC in the outlet perfusate and mesenteric blood levels were found to be less or equal to the limit of quantifi-

TABLE 1

Jejunal tissue and mesenteric plasma concentrations of γ-T3 at different perfusate concentrations

Values are expressed as mean ± S.E.M.

	Matrix	
Perfusate Concentration	Jejunal Tissue	Mesenteric Plasma
μM (n)	*μM*	*nM*
5 (4)	BLD	34.0 ± 3.9
10 (6)	12.2 ± 2.2	22.1 ± 10.4
25 (5)	33.9 ± 6.9	27.1 ± 11.6
50 (4)	78.0 ± 3.3	30.4 ± 10.3
75 (4)	58.5 ± 8.0	10.3 ± 2.6
100 (7)	118.4 ± 12.7	39.8 ± 16.0
150 (3)	206.2 ± 53.3	24.4 ± 9.0

BLD, below limit of detection.

cation (≤2 nM in the perfusate, and ≤10 nM in the mesenteric blood), indicating that the F_{met} of γ-T3 was insignificant (<0.02%).

Regional Differences in γ-T3 Permeability. The regional differences in γ-T3 permeability across the jejunum and ileum were investigated at two concentrations (10 and 75 μM) and are shown in Fig. 4. Like that in the jejunum, permeability of γ-T3 at 10 μM was significantly higher compared with 75 μM ($p < 0.05$) in the ileum. In addition, the permeability of γ-T3 in the jejunum and ileum at both concentrations was comparable and was not found to be significantly different between the two intestinal regions ($p > 0.6$).

Jejunal Permeability of α-Tocopherol. Additional studies compared α-tocopherol and γ-T3 P_{eff} values at perfusate doses of 10 and 50 μM. At the 50 μM concentration, the α-tocopherol P_{eff} value was $11.9 \times 10^{-5} \pm 1.5 \times 10^{-5}$ cm/s ($n = 3$) and did not differ significantly from the γ-T3 P_{eff} at the same concentration ($9.3 \times 10^{-5} \pm 3.0 \times 10^{-5}$ cm/s; $n = 4$; $p > 0.4$). However, at the 10 μM concentration, the level of α-tocopherol in the outlet perfusate could not be detected, and the permeability was estimated to be $\geq 45.6 \times 10^{-5}$ cm/s ($n = 3$), corresponding to α-tocopherol permeability at concentrations that are lower than its limit of detection (0.25 μM). At 10 μM, α-tocopherol P_{eff} was significantly higher ($p < 0.05$) than that of γ-T3 P_{eff} ($26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s; $n = 6$) obtained at the same dose. Furthermore, like γ-T3, α-tocopherol displays significant decreases in permeability when the perfusate dose was increased from 10 to 50 μM ($p < 0.05$).

In Situ Inhibition Studies of γ-T3 Transport with Ezetimibe. Ezetimibe is a potent and selective inhibitor of cholesterol absorption that works by selective binding and inhibition of NPC1L1 (Weinglass et al., 2008b). To investigate the contribution of NPC1L1 to the intestinal uptake of γ-T3, ezetimibe at different concentrations (40, 100, and 200 μM) was coperfused with γ-T3 at 10 μM concentration. The results are shown in Fig. 5. At 100 and 200 μM, ezetimibe significantly reduced γ-T3 P_{eff} from $26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s (in the absence of ezetimibe) to $0.99 \times 10^{-5} \pm 0.19 \times 10^{-5}$ and $2.0 \times 10^{-5} \pm 0.26 \times 10^{-5}$ cm/s, respectively ($p < 0.05$). However, at 40 μM, although a decrease in γ-T3 permeability was observed ($22.2 \times 10^{-5} \pm 3.0 \times 10^{-5}$ cm/s), this reduction was not significantly different compared with the γ-T3 alone perfusion (Fig. 5). With regard to α-tocopherol, coperfusion with ezetimibe significantly reduced α-tocopherol (10 μM) P_{eff} only at 200 μM concentration from complete absorption ($P_{eff} \geq 45.6 \times 10^{-5}$ cm/s) in the absence of ezetimibe to $3.6 \times 10^{-5} \pm 1.6 \times 10^{-5}$ cm/s (ezetimibe, 200 μM; $p < 0.05$).

In Vitro Uptake and Inhibition Studies of γ-T3 with Ezetimibe. To confirm the existence of carrier-mediated transport, we investigated saturability in MDCK II cell uptake of γ-T3. MDCK II cells have been reported to express endogenous NPC1L1 (Weinglass et al., 2008a). Consistent with these studies, our Western blotting results showed the expression of NPC1L1 in these cells (Fig. 6A). The uptake of γ-T3 was determined in the presence of the following concentrations: 1, 5, 10, 25, and 50 μM. Figure 6B presents the concentration-dependent cellular uptake of γ-T3. The uptake of γ-T3 by MDCK II cells was saturable at the concentration range examined, and its percentage uptake was significantly reduced from 73 ± 5.8% at 1 μM concentration to 17.8 ± 1.4% at 5 μM ($p < 0.05$; Fig. 6B). To further characterize the NPC1L1-mediated uptake of γ-T3, an uptake assay was performed in the presence of ezetimibe. As shown in Fig. 6C, ezetimibe at the concentration range examined (1, 10, and 100 μM) significantly inhibited γ-T3 uptake by more than 15% compared with its absence ($p < 0.05$).

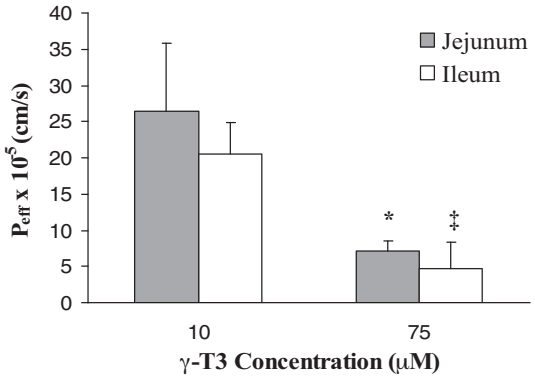

FIG. 4. P_{eff} of γ-T3 obtained from in situ perfused rat jejunum and ileum at 10 and 75 μM luminal concentration. The error bars present the S.E.M. *, $p < 0.05$ compared with the jejunum at 10 μM; ‡, $p < 0.05$ compared with the ileum at 10 μM.

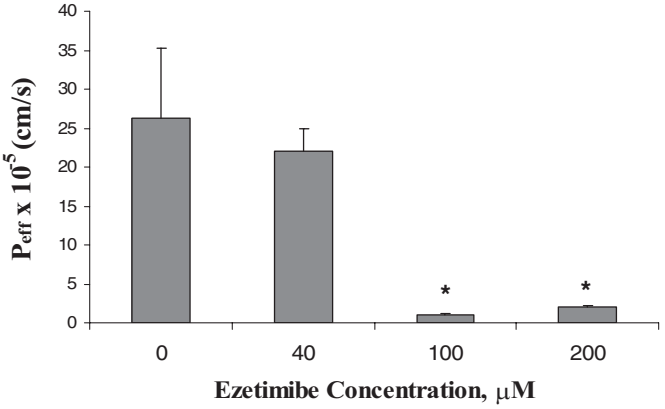

FIG. 5. P_{eff} of γ-T3 (10 μM) obtained from in situ perfused rat jejunum in the absence and presence of the NPC1L1 inhibitor ezetimibe at 40, 100, and 200 μM. The error bars present the S.E.M. *, $p < 0.05$ compared with γ-T3 permeability at 0 and 40 μM ezetimibe concentrations.

Discussion

The results of this study show that intestinal uptake of γ-T3 is inversely proportional to the concentration of γ-T3 present in intestinal lumen. Studies showed that the elevation in the concentration of γ-T3 in the intestinal perfusate resulted in a corresponding reduction in the permeability of γ-T3 into the enterocytes. These findings strongly suggest the transport of γ-T3 across the intestinal membrane involved a carrier-mediated process. These data also indicate that the carrier mediating intestinal absorption of γ-T3 undergoes saturation when exposed to increasingly higher doses of γ-T3 in the intestinal lumen. This hypothesis would explain why it is difficult to obtain elevated levels of γ-T3 in the blood and target tissue after oral administration, and why increasing the oral dose of γ-T3 does not result in a corresponding increase in γ-T3 bioavailability (Hayes et al., 1993; Yap et al., 2001, 2003).

The oral absorption of drugs is determined by several processes, and intestinal permeability is considered one of the major parameters governing this process. Enhanced intestinal absorption displays a direct correlation with increased bioavailability (Lennernäs, 2007). In addition, intestinal metabolism plays an important role in determining drug bioavailability (Thelen and Dressman, 2009). Micellar solubilization is important for γ-T3 absorption, but it is not the only factor attributed to its absorption into the circulation. It is unfortunate that very little is known about intestinal uptake of γ-T3, and it is widely believed that all forms of vitamin E are absorbed by passive diffusion (Kayden and Traber, 1993; Kamran et al., 2007). The aim of the

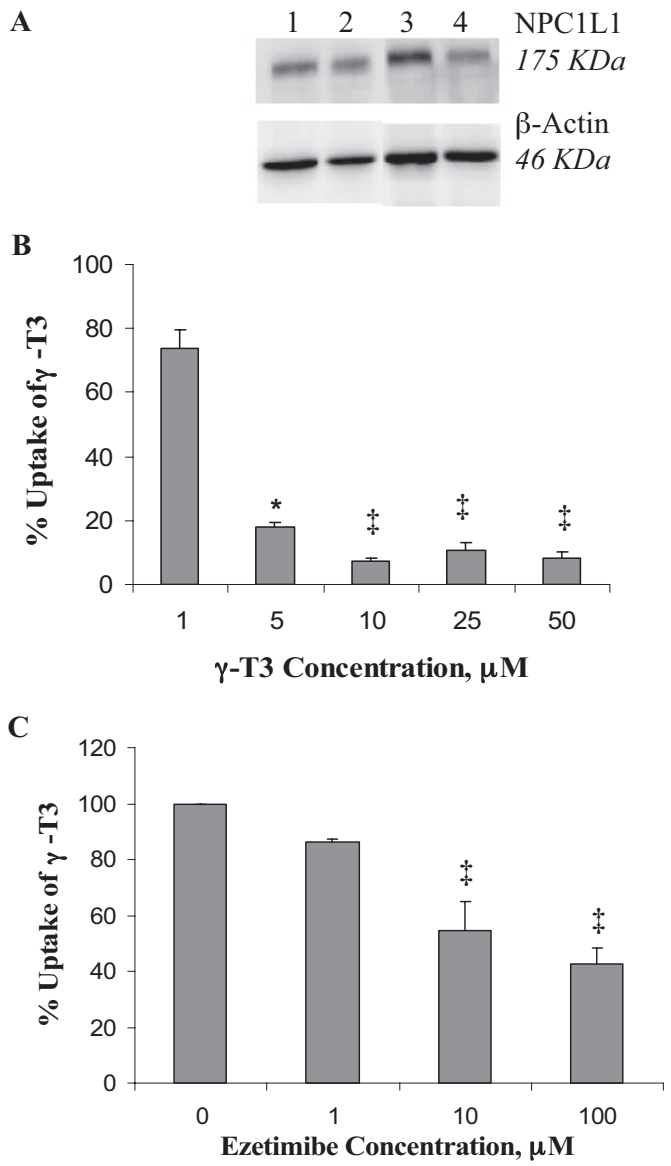

FIG. 6. A, Western blot analysis of endogenous NPC1L1 in MDCK II cells. Lanes 1 through 4 represent NPC1L1 analyses from four different preparations. B, effect of increasing concentrations of γ-T3 on its percentage uptake. The uptake of γ-T3 by MDCK II cells was examined at 37°C for 60 min. The error bars represent the mean ± S.D. of three determinations. *, significantly different from 1 μM γ-T3; ‡, significantly different from 5 μM γ-T3 ($p < 0.05$). C, inhibitory effect of ezetimibe on the uptake of 1 μM γ-T3. The uptake of γ-T3 by MDCK II cells was determined at 37°C for 60 min in medium containing increasing concentration of ezetimibe (1, 10, and 100 μM). The uptake of γ-T3 with increasing concentrations of ezetimibe is presented as percentage reduction in its uptake relative to 100% uptake in the absence of ezetimibe. *, significantly different from 0 (no ezetimibe); ‡, significantly different from 1 μM ezetimibe ($p < 0.05$).

present investigation was to unravel the factors governing the absorption and transport of γ-T3 by the enterocytes.

There are several models that have been used to study the intestinal absorption of drugs. One of the most reliable models is the in situ single-pass intestinal perfusion in rats (Singhal et al., 1998; Kaddoumi et al., 2006). In situ intestinal perfusion provides a valuable tool to assess the role of regional differences in the coupled and separate contributions of intestinal drug transport and metabolism to drug absorption variability and dose-dependent pharmacokinetics. Moreover, intestinal permeability determined in rat perfusion studies provides excellent correlations with human absorption data (Amidon et al., 1988; Fagerholm et al., 1996). Using this model and to investigate its uptake by the enterocyte, γ-T3 was prepared as a mixed micelle solution containing, in addition to γ-T3, sodium taurocholate and phosphatidylcholine (Kimura et al., 1985; Ehehalt et al., 2004).

To test for the existence of a carrier-mediated system for the uptake of γ-T3 in rats, the permeability of γ-T3 as a function of concentration was evaluated. Data from these studies showed that the uptake of γ-T3 by the enterocytes is concentration-dependent and involved both saturable and nonsaturable processes. Furthermore, regional studies as a function of perfusing concentration did not display regional differences in γ-T3 uptake. In both the jejunal and ileal regions of the small intestine, the P_{eff} of γ-T3 decreased with increased perfusate concentration. Whereas the intestinal permeability exhibited a nonlinear uptake behavior, the jejunal tissue concentration was directly related to the perfusate concentration in the range of 10 to 150 μM. Such consistent pattern between γ-T3 permeability and tissue concentration actually was expected in the range of 25 to 150 μM but not at 10 μM. However, the disappearance of γ-T3 from the lumen is not necessarily to reflect its appearance in the tissue. Several, combined or independent, factors could attribute to this observation at 10 μM, including enterocyte metabolism, drug transport at the basolateral side to the mesenteric blood, and the rate of its lymphatic uptake.

Although the vitamin E family of compounds is composed of eight naturally occurring isoforms (α, β, γ, and δ tocopherols and α, β, γ, and δ tocotrienols), most available studies have focused on α-tocopherol absorption. In vitro analysis of α-tocopherol cellular uptake by Caco-2 cells revealed that uptake was concentration-dependent (Reboul et al., 2006; Brisson et al., 2008; Narushima et al., 2008). Two carriers have been investigated for their possible role in α-tocopherol transport: NPC1L1 (Narushima et al., 2008) and scavenger receptor class B type 1 (SR-B1; Reboul et al., 2006). Results in the present study showed that the permeability of γ-T3 and α-tocopherol displayed reduced permeability at perfusate concentrations of 50 μM compared with 10 μM, but the permeability of α-tocopherol at the 10 μM concentration was significantly higher than γ-T3. The concentration-dependent permeability results of α-tocopherol in the current study is consistent with the in vitro studies supporting the participation of a carrier-mediated process in its intestinal uptake. Like α-tocopherol, our results support a carrier-mediated process associated with γ-T3 intestinal uptake.

Inhibition studies using ezetimibe (inhibitor of NPC1L1) and BLT1 (a chemical inhibitor of SR-B1) provided valuable information regarding the transporter-mediated γ-T3 uptake. Initial studies with BLT1 indicated that SR-B1 does not play a major role in the transport of γ-T3 (data not shown). On the other hand, inhibition studies with ezetimibe showed that γ-T3 is a substrate for NPC1L1. NPC1L1 is a polytopic protein present on the enterocyte brush-border membrane that facilitates cholesterol absorption (Yu, 2008). Ezetimibe is a cholesterol-lowering agent that works by selective and direct inhibition of NPC1L1. The majority of animal, genetic, and biochemical findings support NPC1L1 as being the target for ezetimibe (Altmann et al., 2004; Davis et al., 2004; Garcia-Calvo et al., 2005).

Intestinal perfusion of 10 μM γ-T3 with ezetimibe (100 and 200 μM) caused a significant decrease in the permeability of γ-T3, suggesting γ-T3 as a substrate for NPC1L1. Moreover, this reduction in the permeability of γ-T3 caused by ezetimibe was comparable with γ-T3 permeability when perfused at high concentrations, i.e., 100 and 150 μM, indicating that NPC1L1 is the primary carrier that contributes to the transport of γ-T3 across the intestinal membrane. Compared with γ-T3, α-tocopherol transport was inhibited only at higher concentration of ezetimibe, suggesting the possible contribution of another transport system in addition to NPC1L1 (e.g., SR-B1) (Reboul

et al., 2006) and/or α-tocopherol has lower affinity to NPC1L1 compared with γ-T3. Further studies are required to explain these data. Consistent with the in situ intestinal perfusion data, results obtained from the in vitro studies using MDCK II cells showed that γ-T3 uptake is saturable, and its uptake was inhibited by ezetimibe in a dose-dependent manner.

NADPH-dependent synthesis of γ-CEHC was shown in human and rat liver microsomes, and functional analysis of several recombinant human liver cytochrome P450 enzymes revealed that γ-T3 biotransformation is catalyzed by the enzyme CYP4F (Sontag and Parker, 2002; Tsuzuki et al., 2007). In addition, Abe et al. (2007) could detect CYP4F mRNA in the rat jejunum and suggested that γ-T3 is metabolized to γ-CEHC in the intestine. However, the present study showed that γ-T3 intestinal metabolism to γ-CEHC is minimal and does not contribute to its reported low bioavailability.

In conclusion, in the present study, a saturable transport mechanism of γ-T3 on the luminal side of the rat intestine was identified. Using an in situ rat intestinal perfusion model showed that the intestinal uptake of γ-T3 is concentration-dependent and a saturable process. Results from the in situ and in vitro inhibition studies revealed the significant contribution of NPC1L1 to the uptake and transport of γ-T3 across the cell membrane. Although the current experimental model does not investigate whether the permeability reduction would trigger a significant reduction in γ-T3 absorption, revealing and understanding the mechanism of its intestinal uptake is vital to developing delivery systems that are able to improve γ-T3 bioavailability and consequently its therapeutic effect. Further mechanistic studies are currently being conducted in our laboratory to characterize the kinetics of γ-T3 intestinal transport by NPC1L1.

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