Carotenoid Transport Is Decreased and Expression of the Lipid Transporters SR-BI, NPC1L1, and ABCA1 Is Downregulated in Caco-2 Cells Treated with Ezetimibe$^{1,2}$

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ABSTRACT Data suggest that intestinal carotenoid absorption is a facilitated process. The present study was conducted to determine whether carotenoids and cholesterol share common pathways (transporters) for their intestinal absorption. Differentiated Caco-2 cells on membranes were incubated (16 h) with a carotenoid (1 $\mu$mol/L) with or without ezetimibe (EZ; Zetia\textsuperscript{®}, an inhibitor of cholesterol transport), and with or without antibodies against the receptors, cluster determinant 36 (CD36) and scavenger receptor class B, type I (SR-BI). Carotenoid transport in Caco-2 cells (cellular uptake + secretion) was decreased by EZ (10 mg/L) as follows: $\beta$-carotene $\approx$ $\alpha$-carotene (50% inhibition) $>$ $\beta$-cryptoxanthin $\approx$ lycopene (20%) $>$ lutein:zeaxanthin (1:1) (7%). EZ reduced cholesterol transport by 31%, but not retinol transport. $\beta$-Carotene transport was also inhibited by anti-SR-BI, but not by anti-CD36. The inhibitory effects of EZ and anti-SR-BI on $\beta$-carotene transport were additive, indicating that they may have different targets. Finally, differentiated Caco-2 cells treated with EZ showed a significant decrease in mRNA expression for the surface receptors SR-BI, Niemann-Pick type C1 Like 1 protein (NPC1L1), and ATP-binding cassette transporter, subfamily A (ABCA1) and for the nuclear receptors retinoid acid receptor (RAR)$\gamma$, sterol-regulatory element binding proteins (SREBP)-1 and -2, and liver X receptor (LXR)$\beta$ as assessed by real-time PCR analysis. The data indicate that 1) EZ is an inhibitor of carotenoid transport, an effect that decreases with increasing polarity of the carotenoid molecule, 2) SR-BI is involved in carotenoid transport, and 3) EZ may act, not only by interacting physically with cholesterol transporters as previously suggested, but also by downregulating expression of these proteins. The cellular uptake and efflux of carotenoids, like that of cholesterol, likely involve more than one transporter. 


KEY WORDS: \ bullet carotenoids \ bullet intestinal absorption \ bullet ezetimibe \ bullet facilitated transport \ Caco-2 cells

The intestinal absorption of carotenoids in vivo involves several crucial steps: 1) release from the food matrix in the lumen, 2) solubilization into mixed micelles, 3) uptake by intestinal mucosal cells, 4) incorporation into chylomicrons, and 5) secretion into the lymph for review, see (1–5). In this process, several aspects are still unclear, such as the mechanisms by which carotenoids are transported across the plasma membrane, transported inside the cell, and incorporated into chylomicrons. To study the molecular basis of the intestinal absorption of carotenoids, an in vitro model was recently developed using the human intestinal cell line Caco-2 (6). Under conditions mimicking the in vivo postprandial state (7), Caco-2 cell monolayers on membranes are able to take up carotenoids and secrete them associated with chylomicrons (6).

Until recently the intestinal absorption of carotenoids was thought to occur via a passive diffusion process. In the mid-1970s, 2 studies in rats (8,9) demonstrated that the amount of $\beta$-carotene transported through the intestinal wall was directly proportional to the dose of $\beta$-carotene given to the rats. By using the above-mentioned in vitro cell culture system, we demonstrated that there was saturation of $\beta$-carotene transport, $\beta$-carotene isomer selectivity, differential transport of individual carotenoids, and carotenoid interactions, all suggesting that the intestinal absorption of carotenoids is a facilitated transport process perhaps mediated by specific transporters (6). At the same time, it was reported that a scavenger receptor with a high homology to mammalian scavenger receptors [i.e., scavenger receptor class B, type I (SR-BI)$^{4}$ and cluster determinant 36 (CD36)] mediated the cellular uptake of carotenoids in Drosophila (10).

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$^4$ Abbreviations used: ABCA1, ATP-binding cassette transporter, subfamily A; CD36, cluster determinant 36; EZ, ezetimibe; IC$_{50}$, 50% inhibitory concentration; LXR, liver X receptor; NPC1L1, Niemann-Pick type C1 Like 1 protein; RAR, retinoid acid receptor; RXR, retinoic X receptor; SR-BI, scavenger receptor class B, type I; SREBP, sterol-regulatory element binding protein.
Several observations suggest that intestinal absorption of carotenoids and cholesterol may follow common mechanistic pathways. First, cholesterol absorption was also reported to be a facilitated process that would involve the participation of the scavenger receptors SR-BI and CD36 (11). Second, incorporation of cholesterol into chylomicrons is also an important step in the overall process of cholesterol absorption. Finally, it was reported that plant sterols reduced cholesterol absorption by 60% and β-carotene bioavailability by ~50% in normocholesterolemic men (12), and a diet containing carrots rich in α- and β-carotene decreased cholesterol absorption in rats (13).

Ezetimibe [EZ; Zetia® or 1-(4-fluorophenyl)-3(R)-3-(4-fluoro-phenyl)-3(S)-hydroxypropyl]-4-(S)-(4-hydroxyphenyl)-2-azetidinone] is a potent inhibitor of the intestinal absorption of cholesterol and phytosterols in rats and monkeys (14) and humans (15). EZ does not affect the absorption of triglycerides, ethinylestradiol, progesterone, vitamins A and D, and taurocholic acid in rats (16). Although it was demonstrated by autoradiographic analysis that 3H-EZ preferentially accumulated at the apical surface of the enterocytes of the villous tips (17), the exact mechanism by which EZ inhibits cholesterol transport remains to be defined. The present study was conducted to investigate the effect of EZ on the cellular uptake and secretion of carotenoids in Caco-2 cells.

### MATERIALS AND MEHTODS

#### Chemicals

Carotenoids, retinoids, cholesterol, and other chemicals were purchased from Sigma-Aldrich. All-trans β-carotene (type IV, >95% purity), α-carotene (type V, >95% purity), lycopene (from tomato: 90–95% purity), cholesterol, and other chemicals were purchased from Sigma Chemical. Lutein, zeaxanthin, and β-carotanthin were from Indofine Chemical. The rabbit polyclonal antibody against SR-BI (anti-SR-BI) and the mouse monoclonal antibody against human CD36 (anti-CD36) were purchased from Novus Biologicals. Nonimmunized rabbit serum was provided by DAKO. 14C-cholesterol (55 mCi/mmol, 99% purity) was purchased from American Radiolabeled Chemicals.

#### Cell culture

Caco-2 cells were obtained from the American Type Culture Collection and grown as described previously (6). For experiments, cells were grown on transwells (6-well plate, 24 mm diameter, 3-μm pore size, Corning Costar) in the presence of complete medium [DMEM plus 20% heat-inactivated fetal bovine serum, 1% nonessential amino acids, and 1% antibiotics (Gibco, Life Technologies)] for 3 wk (6). Before performing an experiment, the integrity of the cell monolayer was tested either by determining the diffusion of phenol red from the apical side to the basolateral side for 2–3 h (a diffusion close to 0% is found for an intact cell monolayer) or by measuring the resistance of the cell monolayer with a Milli-cell® Electrical Resistance System (Millipore; a typical resistance of 250 Ω is found for an intact cell monolayer). At the zero-time of each experiment, the apical side received 2 mL of serum-free medium (DMEM with 1% nonessential amino acids) supplemented with 0.5 mmol/L taurocholate, 1.6 mmol/L oleic acid, and 43 μmol/L glycerol, plus other compounds to be tested (carotenoids, retinol, cholesterol, and EZ) (see below for their mode of delivery to cells), and the basolateral side received 2 mL of serum-free medium. After 16-h incubation, media from each side of the membrane were harvested and the cell monolayer was washed 3 times with 2 mL of HBSS.

#### Addition of ezetimibe (EZ) to the cell culture medium

Each tablet of Zetia® (Merck/Schering-Plough) contains 10 mg of the active compound (EZ) and the following inactive ingredients: croscarmellose sodium, lactose monohydrate, magnesium stearate, microcrystalline cellulose, providone, and sodium lauryl sulfate. For each experiment in which the effect of EZ was tested, one tablet was crushed and the required amount was weighed (to obtain a final concentration of 2–20 μg in 2 mL of cell culture medium). EZ was solubilized with the taurocholate/oleic acid (10:32 mmol/L) mixture by agitation for 1 h at ~30–40°C. The resulting solution was then passed through a 0.22-μm filter.

#### Delivery of carotenoids, retinol, and cholesterol to cells

For delivering carotenoids, retinol, and cholesterol to cells, the “Tween” method was used (18). In a sterilized glass tube, the required amounts of the carotenoid in hexane (for a final concentration of 1 μmol/L), of retinol in hexane (for 5 μmol/L), of cholesterol (for 1–100 μmol/L) or 14C-cholesterol in ethanol, and of Tween 40 (for 0.1%) in acetone were introduced, solvents evaporated, and the dried residue solubilized in serum-free medium. The resultant clear micellar solution was then added to the apical side of cell monolayers.

#### Lipid extraction from cell monolayer

Total lipids from the cell monolayer were extracted 3 times with 2 mL of 2-propanol: dichloromethane (2:1, v/v) at 20°C for 30–60 min. The 3 extracts were combined, solvents evaporated, and lipids redissolved in 1 mL of methanol. Aliquots of the lipid extract were used to analyze carotenoids and retinol by HPLC and to count 14C derived from 14C-cholesterol.

#### Extraction and analysis of carotenoids and retinoids

Extraction and analysis of carotenoids and retinoids from apical and basolateral media was carried out as previously described (19,20). Carotenoids and retinoids were analyzed using a Waters HPLC system equipped with a Model 717 Plus autosampler, Model 996 photodiode array detector, and a Millenium32 chromatography manager (Waters T system). A TSK gel ODS 120-A C18 reverse-phase column, 4.6 × 250 mm (Toso- haas) was used. Carotenoids were eluted with methanol:dichloromethane (84:16, v/v) as the mobile phase at a flow rate of 1 mL/min. For retinol and retinyl esters, a linear gradient from methanol:water (90:10, v/v) containing 0.1% ammonium acetate (100%) to methanol:dichloromethane (84:16, v/v) (100%) at a flow rate of 1 mL/min was applied for 12 min, followed by an isocratic elution with methanol:dichloromethane (84:16, v/v) at a flow rate of 1.2 mL/min. Carotenoids and retinoids were quantified from their peak area using external standard reference curves established with each compound (0.5–500 pmol) at 450 or 325 nm, respectively.

#### RNA isolation from cells

Cells on T25 flasks were washed twice with 10 mL of Hank’s medium (without Ca2+ or Mg2+), and 10 mL of Trizol reagent (Invitrogen) was added to lyse the cells. The resulting mixture was rapidly transferred into a 15-mL Falcon tube and stored at ~80°C until use. Total cellular RNA was isolated according to the manufacturer’s instruction.

#### Real-time PCR analysis

All probes and primers were designed using Primer Express software (Applied Biosystems) with sequences obtained from Genbank (see Table 1). Extracted RNA was treated with DNase in the presence of an RNAse inhibitor using a commercially available kit (TurboDNase Ambion). RNA integrity, quantity, and genomic DNA contamination were assessed using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies). cDNA was synthesized using random priming and a commercially available kit (Impromtu, Promega); 30 ng/well of this cDNA in a final volume of 15 μL was used for PCR amplification. PCR was performed using the Absolute QPCR Rox Mix (Abgene) on an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. We normalized gene expression based upon the constant amount of RNA/cDNA amplified. This method was proposed recently as the most reliable means of standardization of quantitative measurement of mRNA expression by real-time PCR provided an accurate estimation of total RNA can be made, e.g., via use of the Agilent Bioanalyzer (21,22).

Fluorescence signals measured during amplification were processed digitally and were regarded as positive if the fluorescence intensity was >20-fold the SD of the baseline fluorescence. This level is defined as the Ct. Ct values obtained for treated cells were compared with those obtained with untreated cells. The relative fold increase or decrease was calculated as:

\[
\text{Fold change} = 2^{-\Delta\Delta CT}
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where ΔΔCT is defined as the Ct. Ct values obtained for treated cells were compared with those obtained with untreated cells. The relative fold increase or decrease was calculated as:

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**RESULTS**

*Effect of EZ on β-carotene transport.*  EZ treatment significantly increased β-carotene in the apical medium and significantly reduced β-carotene concentrations in cells and in the basolateral medium (Table 2). The inhibitory effect of EZ on both cellular uptake and secretion of β-carotene was concentration dependent. Indeed, β-carotene concentrations in cells and basolateral medium were decreased by 27% and 57%, respectively, with EZ at 1 mg/L and by 43% and 67% with EZ at 10 mg/L (Table 1). Thus, the data indicate that EZ at 10 mg/L is a strong inhibitor of the transport of β-carotene through Caco-2 cells. EZ concentrations used in this study (1 mg/L (2.4 μmol/L) and 10 mg/L (24 μmol/L)) are within the range of EZ concentrations (up to 30 μmol/L) used to show an effect on cholesterol transport in vitro (23) and are close to the calculated pharmacologically active concentration (2.5 mg/L) that would be found in the small intestine of a human adult after the intake of the recommended dose (10 mg EZ/d).

**Effect of EZ on cholesterol and retinol transport.** 14C-cholesterol transport (sum of cellular uptake and secretion) was decreased by EZ in a concentration-dependent fashion (reduction by 15% (P < 0.05) and 31% (P < 0.05) for 1 and 10 mg EZ/L, respectively [data not shown]). The overall inhibition observed with EZ was somewhat lower than the marked inhibitory effect of EZ (54%) reported in vivo (14,15). Note that under standard cell culture conditions (without EZ), 14C-cholesterol was well taken up by Caco-2 cells (14,15). Indeed, the marked inhibitory effect of EZ on cholesterol transport (sum of cellular uptake and secretion) was decreased by EZ in a concentration-dependent fashion (reduction by 15% (P < 0.05) and 31% (P < 0.05) for 1 and 10 mg EZ/L, respectively [data not shown]). The overall inhibition observed with EZ was somewhat lower than the marked inhibitory effect of EZ (54%) reported in vivo (14,15).

**Effect of EZ on the transport of other carotenoids.**  The following experiment was conducted to determine whether EZ inhibits the intestinal transport (sum of cellular uptake and secretion) of carotenoids other than β-carotene (Fig. 1). EZ had a strong inhibitory effect on the transport of the 2 hydrocarbon carotenoids with 2 ionone rings, β-carotene and α-carotene, (~50%, P < 0.0001), an intermediate inhibitory effect on the transport of the oxycarotenoid with 1 hydroxyl group, cryptoxanthin, and on the transport of the hydrocarbon carotenoid without an ionone ring, lycopene (~20%, P < 0.002), and a small effect on the transport of the 2 oxygenated carotenoids with 2 hydroxyl groups, lutein and zeaxanthin (~10%, P < 0.05) (Fig. 1). No differential effect of EZ was observed on the transports of lutein and zeaxanthin when examined separately.
Effects of anti-SR-BI alone, anti-SR-BI × EZ combination, and anti-CD36 on β-carotene transport. It was suggested that at least in vitro, EZ would interact with the scavenger receptor SR-BI, resulting in the blockage of cholesterol transport (23). Thus, the following experiment was conducted to determine whether anti-SR-BI affects β-carotene transport (Fig. 2). Anti-SR-BI treatment reduced β-carotene transport by ~20% (P < 0.05) compared with nonimmunized rabbit serum control. EZ treatment alone reduced β-transport by ~40%. A greater inhibition was observed when anti-SR-BI and EZ were added together; β-carotene transport was decreased by almost 60%. In addition to SR-BI, it was suggested that CD36 could play a role in the selective uptake of sterols in the small intestine (11). However, anti-CD36 did not affect β-carotene transport (Fig. 2). It was suggested recently that EZ could have another protein target, the Niemann-Pick type C1 (NPC1L1) protein, a newly discovered protein thought to have a critical role in the absorption of cholesterol (24). Because no antibody against NPC1L1 was available and because the NPC1L1 protein shares extensive homology with NPC1 protein (~50% amino acid homology) (25), the mouse monoclonal antibody against human NPC1 (anti-NPC1) was also tested at 8 mg/L. Anti-NPC1 did not affect β-carotene transport through Caco-2 cells (data not shown).

Effect of cholesterol on β-carotene transport. The transport of β-carotene through Caco-2 cell monolayers was reduced with increasing concentrations of cholesterol according to a logarithmic regression (γ = −1.56 Ln(x) + 12.4, R^2 = 0.912) and a significant effect of cholesterol was observed at cholesterol concentrations ≥ 25 μmol/L (Fig. 3). A 50% inhibition (IC50) of β-carotene absorption occurred at a cholesterol concentration of 53 μmol/L, supporting the idea that β-carotene could utilize some of the cholesterol transporters. The data indicate also that a relatively high amount of cholesterol would be required to reach saturation of those transporters in Caco-2 cells. Note that the ratio of β-carotene:cholesterol concentrations (i.e., 1:50 μmol/L) at which the IC50 was observed corresponds roughly to the ratio of dietary intake of these compounds.

Comparison of the expression of various genes in Caco-2 cells with human small intestine samples. mRNA levels for proteins involved in vitamin A metabolism [lecithin:retinol acyltransferase (LRAT), β-carotene 15,15′-monooxygenase (BCMO), and cellular retinol-binding protein (CRBP)I] were significantly lower in Caco-2 cells (~10% of the levels found in human small intestinal samples) (Fig. 4). More marked differences were noted in the mRNA levels for proteins involved in lipid transport [SR-BI, NPC1L1, ATP-binding cassette transporter, subfamily A (ABCA1) and CD36]. For instance, SR-BI mRNA level was ~500% and CD36 mRNA level 4% of the respective mRNA levels found in human small intestinal samples (Fig. 4). These differences in gene expression of cholesterol transporters, e.g., SR-BI and CD36, may account for the lower absorption rate of cholesterol observed in Caco-2 cells, compared with that in humans. Finally, in Caco-2 cells, mRNA levels for proteins involved in the transcriptional regulation [retinoid acid receptors (RARs), retinoid X receptors (RXRs), sterol-regulatory element binding proteins (SREBPs), liver X receptors (LXRs), and peroxisome proliferator-activated receptor (PPAR)γ] did not differ from

FIGURE 1  Effect of EZ (expressed as % inhibition) on the transport [cellular uptake + secretion into the basolateral medium (BM)] of β-carotene (β-C), α-carotene (α-C), lycopene (LYC), β-cryptoxanthin (β-crypto), and lutein + zeaxanthin (LUT/ZEA) through Caco-2 cells. The carotenoid concentration was 1 μmol/L (0.5:0.5 μmol/L for lutein + zeaxanthin mixture) in the presence of EZ at 0 or 10 mg/L. Values are means ± SD, n = 3 independent experiments. P-values shown are for the difference between carotenoid transport in the presence of EZ versus the absence of EZ. Experimental group means without a common letter differ. a:b: P < 0.05; a:c: P < 0.02; b: c: P < 0.05.

FIGURE 2  Effects of nonimmunized rabbit serum, anti-SR-BI, EZ, anti-SR-BI × EZ, and anti-CD36 treatments (expressed as % inhibition) on the transport [cellular uptake + secretion] of β-carotene by Caco-2 cells. β-Carotene was at 1 μmol/L, nonimmunized rabbit serum, anti-CD36, and anti-SR-BI at 8 mg/L, and EZ at 10 mg/L. When an antibody or rabbit serum was used, cells were preincubated for 2 h with the antibody at the concentration indicated above and remained in the medium for the following 16-h incubation in the presence of the carotenoid. The “absolute” control experiment consisted of the cells incubated with β-carotene at 1 μmol/L only (no treatment) and the “antibody” control corresponded to nonimmunized rabbit serum. Values are means ± SD, n = 3 independent experiments. *Different from the “absolute” control (Dunnett’s test), *P < 0.05. Experimental group means without a common letter differ, P < 0.05.
The absorption of carotenoids was a facilitated process (6). The levels found in human intestinal samples, except for RARγ and LXRα, which had mRNA levels 200–260% of the respective levels found in human samples.

**Effect of EZ on the expression of genes involved in vitamin A metabolism, lipid transport, and transcriptional regulation.** Among the various genes involved in vitamin A metabolism, only the LRAT mRNA level was significantly higher in cells treated with EZ (197% of the level found in control cells; Table 3). The mRNA levels for cytochrome P450, family 26 (CYP26A), BCMOI, and CRPBII were not different in cells treated with or without EZ. Among the genes implicated in lipid transport, the mRNA levels for SR-BI, NPC1L1, and ABCA1 were significantly lower in cells treated with EZ than in control cells (Table 3). The magnitude of that reduction in gene expression of SR-BI, NPC1L1, and ABCA1 was identical (~67% reduction), suggesting that these 3 genes could be regulated by EZ via a common mechanism of action. The mRNA levels for CD36 and SR-BII were unchanged in cells treated with or without EZ. Finally, among the various genes encoding for different nuclear receptors, the mRNA levels for RARγ, RXRα, SREBP-1, SREBP-2, and LXRβ were significantly lower in cells incubated with EZ (18–44% of the levels found in control cells). The mRNA levels for RARα, RARβ, RXRβ, RXRγ, LXRβ, and PPARY were not affected by EZ. These results indicate that EZ could affect the transcription of the surface receptors SR-BI, NPC1L1, and ABCA1 via the downregulation of the gene expression of the nuclear receptors RARγ, RXRα, SREBP-1, SREBP-2, and LXRβ.

When the experiment was repeated using 5 flasks of control cells and 5 flasks of EZ-treated cells, a similar trend was observed for the various genes analyzed, confirming the data shown in Table 3. For instance, in cells treated with EZ, the mRNA levels for SR-BI, NPC1L1, and ABCA1 were significantly lower (26, 24, and 14%, respectively, of the levels found in control cells), whereas the mRNA level of CD36 was unchanged (79% of the level found in control cells) (data not shown).

**DISCUSSION**

Our previous in vitro data suggested that the intestinal absorption of carotenoids was a facilitated process (6). The main objective of the present study was to further understand that process. Several lines of evidence suggest that carotenoids and cholesterol could share common mechanistic pathways to cross the intestinal cell. A specific inhibitor of cholesterol absorption, EZ, was chosen to investigate its potential effect on carotenoid transport through Caco-2 cells. In contrast to other cholesterol-lowering agents that reduce plasma cholesterol by decreasing cholesterol biosynthesis, EZ and its analogs have no effect on 3-hydroxy-3-methyl-glutaryl coenzyme A reductase or on acyl coenzyme A cholesterol acyltransferase (26). Instead, it was proposed that EZ could interact physically with 1 or several specific transporter(s) of cholesterol on the apical surface of the enterocyte.

In Caco-2 cells, EZ inhibited cholesterol and β-carotene transport (cellular uptake + secretion) in a dose-responsive manner, but did not affect retinol transport. The present in vitro cell culture system was thus a good model with which to study the effects of EZ on the intestinal absorption of carotenoids because both the positive-control (EZ effect on cholesterol) and the negative-control (EZ effect on retinol) worked as predicted and in agreement with previous in vivo data (15,16). The degree of inhibition of carotenoid transport by EZ was determined by the carotenoid structure and decreased with increasing polarity of the carotenoid molecule. The data suggest that nonpolar carotenoids and polar carotenoids could use different pathways (or transporters) more or less sensitive to EZ. Reinforcing that idea, our previous observations showed carotenoid interactions between 2 nonpolar carotenoids, but not between a nonpolar carotenoid and a polar carotenoid during their passage through Caco-2 cells (6).

EZ is thus a potent inhibitor of β-carotene and α-carotene transport through Caco-2 cells; it reduced their absorption by almost 65%, a value close to the inhibitory effect of EZ observed on the in vivo intestinal absorption of cholesterol.
mRNA expression of various genes involved in vitamin A metabolism, lipid transport, and transcriptional regulation in Caco-2 cells treated with EZ compared with control cells

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1 After treatment (EZ vs. Control) for 16 h, total RNA was isolated from cells and gene expression analyzed by RT-PCR. Data of each group are means of 3 T25 flasks of Caco-2 cells treated and analyzed independently. For each gene analyzed, its expression in control cells was normalized to 100%.

2 The present data were extracted from a set of 4 different treatments.

3 NS, P ≥ 0.05.

[54% reduction in humans (15) and up to 96% in animals (14)]. In addition, β-carotene transport was decreased by cholesterol in a dose-dependent manner, suggesting that β-carotene and cholesterol interact during their transport through Caco-2 cells. Therefore, nonpolar carotenoids and cholesterol could share 1 (or more) common transporter(s) targeted by EZ.

SR-BI and NPC1L1 are the 2 proteins that were investigated to date as potential targets of EZ. Both proteins play a role in the intestinal transport of cholesterol and are expressed in the brush border membrane of the enterocyte (24,27,28). The study of Altmann et al. (23) showed that in vitro, EZ bound to SR-BI with a high affinity and functionally blocked SR-BI-mediated cholesterol absorption, whereas in vivo, by using SR-BI knockout mice, SR-BI was not required for cholesterol absorption or for the inhibitory activity of EZ to occur. Recently, by using NPC1L1 knockout mice (24), the same authors demonstrated the importance of the expression of NPC1L1 for both cholesterol absorption and EZ activity in mice, but did not show a direct binding of EZ to NPC1L1. It is clear that more investigations are required to understand the molecular mechanism of action of EZ.

We focused on the role(s) of the scavenger receptor SR-BI and its homolog CD36 as potential carotenoid transporters for several reasons. First, both SR-BI and CD36 were shown to facilitate the uptake of cholesterol in the small intestine with a contribution of 25 and 35%, respectively (11). Second, Kiefer et al. (10) demonstrated that a scavenger receptor with a high homology to the mammalian scavenger receptors SR-BI and CD36 mediated the cellular uptake of carotenoids in Drosophila. Third, as mentioned above, SR-BI was bound by EZ in vitro (23) and was also the only potential target of EZ known at the beginning of the present study. Both SR-BI and CD36 are expressed in Caco-2 cells. β-Carotene transport was inhibited (up to 23%) by anti-SR-BI in a dose-responsive manner (for 2, 4, and 8 mg anti-SR-BI/L) and the best fitting curve of that inhibition was a polynomial regression: \( y = -0.25x^2 + 5.2x, R^2 = 1 \) (data not shown). In support, similar results were shown recently for the absorption of lutein through TC7 cells, which was reduced by 32% in the presence of SR-BI antibody applied at 5 mg/L (29). In contrast, CD36 antibody did not affect β-carotene transport through Caco-2 cells. The lack of effect of anti-CD36 could be related to the relatively low mRNA level for CD36 found in those cells (4% of the level found in human small intestine). Together, the data suggest the partial participation of SR-BI, but probably not CD36, in the intestinal transport of polar and nonpolar carotenoids.

When added together, EZ and anti-SR-BI had combined inhibitory effects on β-carotene transport through Caco-2 cells, indicating that the 2 did not act by an identical mechanism. To further understand the molecular mechanism of action of EZ, we investigated the potential effects of EZ on the mRNA expression of several proteins involved in the transport of lipids. We found that EZ decreased SR-BI, NPC1L1, and ABCA1 mRNA levels by 67%, but did not affect SR-BI and CD36 mRNA levels. SR-BI and NPC1L1 are expressed on the apical surface of Caco-2 cells and play a role in cellular cholesterol uptake (24,27,28), whereas ABCA1, which is present on the basolateral surface of Caco-2 cells, mediates cholesterol efflux (30,31). Thus, EZ downregulates the gene expression of 3 surface receptors, SR-BI, NPC1L1, and ABCA1, implicated in the intestinal trafficking of cholesterol. On the other hand, Repa et al. (32) stated that the inhibition of cholesterol absorption by an analog of EZ (SCH58053) was not mediated via changes in mRNA expression for ABCA1, ABCG5, or ABCG8 in the enterocyte (ABCG3 and ABCG8,
located at the apical surface of the enterocyte, form a heterodimer and preferentially transport plant sterols out of the intestinal cells into the gut lumen). However, in that study (32), mRNA levels for all these 3 proteins were consistently lower in mice fed the basal diet (0.02% cholesterol) supplemented with EZ than in mice fed the basal diet alone. In an experiment repeated for a 3- vs. 12-d feeding period with the same diets, the authors observed the same results (32). Thus, these data and our findings converge to indicate that EZ and its analogs could modulate the mRNA levels of sterol transporters in the enterocyte. Instead, the authors (32) hypothesized that SCH58053 blocked luminal cholesterol uptake and that the resulting reduction in intracellular cholesterol content would result in decreasing mRNA levels for ABCA1, ABCG5, or ABCG8. This hypothesis was based on previous observations showing that dietary cholesterol upregulates the intestinal mRNA expression of ABCA1, ABCG5, or ABCG8 in mice (33,34). However, in the present study, the effects of EZ on the expression of various genes were examined without cholesterol being delivered to Caco-2 cells (the serum-free cell culture medium did not also contain any cholesterol). Thus, the effects of EZ on the gene expression of lipid transporters were independent of cholesterol absorption in cells.

The magnitude of inhibition in the expression of SR-BI, NPC1L1, and ABCA1 mRNAs was identical (67% reduction), suggesting that these 3 genes could be regulated by EZ via similar mechanisms of action. In agreement with this idea, the present data showed that EZ downregulated the gene expression of several nuclear receptors (SREBP-1, SREBP-2, RARγ, RXRα, and LXRβ). The presence of specific DNA sequences (or response elements) for these different nuclear receptors was reported in the promoters of SR-BI, NPC1L1, and ABCA1 genes (25,35–39), suggesting that EZ could effectively affect the transcription of these surface receptors via the downregulation of the expression of RARγ, RXRs, SREBP-1, SREBP-2, and LXRβ. The mechanism by which EZ affects the transcription of these nuclear receptors is another question that remains to be resolved in the future.

Thus, in Caco-2 cells, EZ caused a paralleled diminution of β-carotene and α-carotene absorption rates and of the SR-BI, NPC1L1, and ABCA1 mRNA levels, suggesting that these 3 cholesterol transporters could play a role in the intestinal transport of β-carotene and α-carotene. It is also possible that other unidentified proteins sensitive to EZ as well as proteins not sensitive to EZ are involved in intestinal carotenoid transport. This latter possibility is particularly true for the intestinal transport of polar carotenoids such as lutein (and zeaxanthin), which was diminished by SR-BI blocking antibody (29) but was poorly decreased by EZ. A full understanding of both cholesterol and carotenoid absorption in the intestine will require further investigation.

In summary, the present data clearly indicated the following: 1) EZ is an inhibitor of the intestinal absorption of carotenoids, an effect that decreased with increasing polarity of the carotenoid molecule; 2) SR-BI is involved in intestinal carotenoid transport; and 3) EZ acts not only by interacting physically with cholesterol transporters as previously suggested, but also by downregulating the gene expression of 3 proteins involved in cholesterol transport in the enterocyte, the transporters SR-BI, NPC1L1, and ABCA1. The intestinal transport of carotenoid is thus a facilitated process resembling that of cholesterol; therefore, carotenoid trafficking in intestinal cells may also involve more than one transporter.

LITERATURE CITED

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