RESEARCH ARTICLE

Vitamin D intestinal absorption is not a simple passive diffusion: Evidences for involvement of cholesterol transporters

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Scope: It is assumed that vitamin D is absorbed by passive diffusion. However, since cholecalciferol (vitamin D_3) and cholesterol display similar structures, we hypothesized that common absorption pathways may exist.

Methods and results: Cholecalciferol apical transport was first examined in human Caco-2 and transfected Human embryonic kidney (HEK) cells. Cholecalciferol uptake was then valuated *ex vivo* and *in vivo*, using either wild-type mice, mice overexpressing Scavenger Receptor class B type I (SR-BI) at the intestinal level or mice treated or not with ezetimibe. Cholecalciferol uptake was concentration-, temperature- and direction-dependent, and was significantly impaired by a co-incubation with cholesterol or tocopherol in Caco-2 cells. Moreover Block Lipid Transport-1 (SR-BI inhibitor) and ezetimibe glucuronide (Niemann-Pick C1 Like 1 inhibitor) significantly decreased cholecalciferol transport. Transfection of HEK cells with SR-BI, Cluster Determinant 36 and Niemann-Pick C1 Like 1 significantly enhanced vitamin D uptake, which was significantly decreased by the addition of Block Lipid Transport-1, sulfo-*N*-succinimidyl oleate (Cluster Determinant 36 inhibitor) or ezetimibe glucuronide, respectively. Similar results were obtained in mouse intestinal explants. *In vivo*, cholecalciferol uptake in proximal intestinal fragments was 60% higher in mice overexpressing SR-BI than in wild-type mice ($p < 0.05$), while ezetimibe effect remained non-significant.

Conclusion: These data show for the first time that vitamin D intestinal absorption is not passive only but involves, at least partly, some cholesterol transporters.

Keywords:
Bioavailability / Caco-2 TC-7 cells / Cholecalciferol / Intestinal absorption / Transgenic mice

1 Introduction

Vitamin D plays a key role in skeletal, infectious, inflammatory and metabolic diseases in humans. In particular, vitamin D deficiency in adults can aggravate osteoporosis, cause osteomalacia and increase the risk of fracture [1]. Conversely, a vitamin D supplementation (1100 IU *per* day)

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Abbreviations: BLT1, Block Lipid Transport-1; **CD36,** Cluster Determinant 36; **ED,** electrochemical detector; **FBS,** fetal bovine serum; **HEK,** Human embryonic kidney; **NPC1L1,** Niemann-pick C1-like1; **PDA,** photodiode array detector; **SR-BI,** Scavenger Receptor class B type I; **SR-BI Tg mice,** mice overexpressing SR-BI in the intestine; **SSO,** sulfo-*N*-succinimidyl oleate; **wt,** wild-type

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was shown to reduce the incidence of non-skin cancers. Together with a recent meta-analysis indicating that vitamin D, even in relatively low doses, reduced total mortality, this underlines the growing importance of an early diagnosis of an inadequate vitamin D status [2]. New data from the NHANES study enlighten the fact that more than 75% of the US population is vitamin D insufficient [3]. This alarming conclusion can be extended to many other countries as reports from across the world indicate that hypovitaminosis D is widespread and is re-emerging as a major health problem globally [4].

The human diet mainly provides vitamin D_3 (cholecalciferol) through fatty fish and fortified foods such as dairy products [1]. Nevertheless, only few foods contain vitamin D, making its dietary recommendation particularly difficult to reach [5].

It has long been assumed that vitamin D, like other fat-soluble (micro)nutrients, was absorbed by a passive process [6, 7]. However, cholesterol, which exhibits a chemical structure very close to vitamin D structure, is not absorbed through a simple diffusion. Indeed, several transporters, *i.e.* Scavenger Receptor class B type I (SR-BI) [8], Cluster Determinant 36 (CD36) [9], Niemann-Pick C1 Like 1 (NPC1L1) [10] or ABC transporters [11], have been implicated in its trafficking across enterocyte membranes. We thus hypothesized that cholecalciferol and cholesterol could share common absorption pathways, especially through transporters displaying broad substrate specificity. The objectives of this study were to assess whether intestinal absorption of vitamin D is protein-mediated, and to specify the involvement of three main cholesterol transporters in this phenomenon: SR-BI, CD36 and NPC1L1.

2 Materials and methods

2.1 Chemicals

Cholecalciferol (≥96% pure), ergocalciferol (≥96% pure), 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium taurocholate, dicyclohexylcarbodiimide, hydroxysulfosuccinimide sodium salt, DMF, DMSO, methylthiazoletetrazolium, mouse monoclonal anti-human CD36 IgM, anti-mouse IgM and anti-mouse IgG conjugated to alkaline phosphatase were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Mouse monoclonal IgG raised against the external domain (amino acids 104–294) of human SR-BI, also known as CLA-1, was purchased from BD Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-human NPC1L1 IgG was purchased from Abcam (Paris, France). BLT1 (Block Lipid Transport-1, *i.e.* 2-hexyl-1-cyclopentanone thiosemicarbazone, a chemical inhibitor of lipid transport mediated by SR-BI) was purchased from Chembridge (San Diego, CA, USA). Ezetimibe (1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone, a chemical inhibitor of cholesterol transport mediated by NPC1L1) was purchased from Sequoia Research Products (Pangbourne, UK). Ezetimibe-glucuronide (the active metabolite of ezetimibe) was a generous gift from E. Levy (CHU Ste Justine, Montréal, Canada). DMEM containing 4.5 g/L glucose and trypsin-EDTA (500 and 200 mg/L, respectively) was purchased from BioWhittaker (Fontenay-sous-Bois, France), fetal bovine serum (FBS) came from Biomedia (Issy-les-Moulineaux, France), and non-essential amino acids, penicillin/streptomycin and PBS were purchased from Gibco BRL (Cergy-Pontoise, France). The protease inhibitor cocktail tablets came from Roche (Fontenay, France). Human SR-BI cDNA cloned in pCDNA3.1 plasmid was a generous gift of Gerald Gaibelet (INSERM U563, Toulouse, France) and empty pCDNA3.1 plasmid was a gift from Franck Peiretti (INSERM U626, Marseille, France). Human CD36 in pIRES plasmid and empty pIRES plasmid were provided by Jean-François Landrier. Human NPC1L1 cDNA cloned in pCR-XL-TOPO plasmid was purchased from Open Biosystems (Thermo Scientific Abgene, Surrey, UK) and subcloned into pCEP4 plasmid, which was a gift from R. S. Molday (UBC, Vancouver B.C, Canada).

2.2 Sulfo-*N*-succinimidyl oleate synthesis

Sulfo-*N*-succinimidyl oleate (SSO) (a chemical inhibitor of CD36) synthesis was adapted from the procedure of Harmon *et al.* [12]. Briefly, 1.26 mmol dicyclohexylcarbodiimide and 1.15 mmol hydroxysulfosuccinimide sodium salt were added to a solution of 1.15 nmol oleic acid dissolved in 10 mL DMF dried over 3 Å molecular sieves. After stirring overnight at room temperature, the dicyclohexylurea precipitate was removed by filtration (Whatman n°1). The filtrate was cooled at 4°C, eight volumes of ethyl acetate were added and the solution was left to precipitate overnight. SSO was then collected by filtration (Whatman n°1) and dried under pump vacuum (54% yield). Purity (>99%) and mass were confirmed through UPLC/ESI(-)MSn and 1H-NMR analyses.

2.3 Preparation of vitamin D-enriched vehicles for cell and mouse experiments

2.3.1 Vitamin D-rich micelles

For delivery of vitamin D to Caco-2 cells or to mouse intestinal explants, mixed micelles with similar lipid composition as those found *in vivo* [13] were prepared as previously published [14] to obtain the following final concentrations: 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.3 mM monoolein, 0.1 mM free cholesterol, 0.5 mM oleic acid, 0.01–10 μM vitamin D [15]

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and 5 mM taurocholate. Concentration of vitamin D in the micellar solutions was checked before each experiment.

2.3.2 Cholecalciferol-rich complete medium

For delivery of vitamin D to Human embryonic kidney (HEK) cells, an appropriate volume of vitamin D stock-solution in ethanol was added to a glass tube to obtain a final concentration of 0.5 μM. Stock solution solvent was carefully evaporated under nitrogen. Dried residue was solubilized into FBS overnight and DMEM was then added to reach a final FBS concentration of 10%. Concentration of cholecalciferol in the medium was checked before each experiment.

2.3.3 Preparation of cholecalciferol-rich emulsions

For delivery of vitamin D to mice, emulsions were prepared as follows. An appropriate volume of cholecalciferol stock solution was transferred to eppendorf tubes to obtain a final amount of 300 μg in each tube. Stock solution solvent was carefully evaporated under nitrogen. Dried residue was solubilized in 100 μL of corn oil (Lesieur, Asnières-sur-Seine, France), and 200 μL of NaCl 0.9% solution were added. The mixture was vigorously mixed in ice-cold water in a sonication bath (Branson 3510, Branson) for 15 min and used for force-feeding within 10 min.

2.4 Cell culture

2.4.1 Caco-2 cell culture

Caco-2 clone TC-7 cells [16, 17] were a gift from Dr. M. Rousset (UMR_S 872, Villejuif, France). Cells were cultured in the presence of DMEM supplemented with 20% heat-inactivated FBS, 1% non-essential amino acid and 1% antibiotics (complete medium), as previously described [14, 18]. For each experiment, cells were seeded and grown on transwells for 21 days as previously described [14, 18] to obtain confluent and highly differentiated cell monolayers. Twelve hours prior to each experiment, the medium used in apical and basolateral chambers was a serum-free complete medium.

2.4.2 HEK cell culture and transfection

HEK 293-T cells were purchased from American Type Culture Collection (Manassas VA, USA). Cells were cultured in 10% FBS complete medium at 37°C in a humidified atmosphere of air/carbon dioxide (90/10, v/v) and the medium was changed every 48 h. Monolayers were subcultured with a 4-day passage frequency when they reached a confluence of about 80% by treatment with 0.25% trypsin-EDTA. For each experiment, cells were seeded at 1:10 dilution in six-well plates, and transfected 24 h after with 3 μg DNA and 6 μL Jet PeI *per* well in NaCl 150 mM according to the manufacturer. The medium was then changed 10–12 h after and cells were grown for an additional 24 h. Transfection efficiency was checked by Western blotting as previously published [19].

2.5 Characterization of vitamin D apical transport in cells

2.5.1 Uptake

At the beginning of each experiment, cell monolayers were washed with 0.5 mL PBS. For uptake experiments, the apical or basolateral side of the cell monolayers received the vitamin D-rich medium (whereas the other side received the serum-free complete medium if applicable). Cells were incubated for 30–60 min at either 37°C or 4°C, depending on the experiment. At the end of each experiment, media were harvested. Cells were washed twice in 0.5 mL ice-cold PBS to eliminate adsorbed vitamin D, then scraped and collected in 0.5 mL PBS. Absorbed vitamin D was estimated as vitamin D found in scraped cells plus vitamin D found on the opposite side of the cell monolayer (basolateral side when micellar vitamin D was added to the apical side, and *vice versa*) if applicable.

2.5.2 Apical efflux

The cells first received the cholecalciferol-rich micelles at the apical side for 4 h. They were then washed two times with PBS and equilibrated in serum-free complete medium for 1 h. The cells received then apical medium containing vitamin D acceptors, *i.e.* vitamin-D-free mixed micelles, for 3 h.

2.5.3 Competition with cholesterol

Cholecalciferol uptake was measured after incubation of cholecalciferol-rich mixed-micelles (0.5 μM) containing either no cholesterol, 0.1 mM cholesterol (control) or 0.5 mM cholesterol.

2.5.4 Competition with *R,R,R*-α-tocopherol

Cholecalciferol uptake was measured after incubation of cholecalciferol-rich mixed-micelles (0.5 μM) containing either no tocopherol, 10 μM tocopherol or 100 μM tocopherol.

2.5.5 Vitamin D transport inhibition by BLT1

For the uptake experiment, BLT1 was used as previously described [18, 19]. Briefly, cell monolayers were pretreated

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with either DMSO (control) or BLT1 at 10 μM for 1 h. The cells then received cholecalciferol-rich medium with 10 μM of BLT1, and uptake was measured as described above. For efflux experiments, cholecalciferol-enriched cells received apical medium supplemented with either DMSO or BLT1 at 10 μM and efflux was measured as described above.

2.5.6 Vitamin D uptake inhibition by SSO

SSO can be used in concentration ranges up to 1.25 mM [20], but it is classically used between 200 and 500 μM. During experiments, cell monolayers were thus pretreated with either DMSO (control) or SSO at 400 μM for 1 h. Then, cells received cholecalciferol-rich medium with the same concentration of SSO, and uptake was measured as described above.

2.5.7 Vitamin D uptake inhibition by ezetimibe-glucuronide

For the uptake experiment, differentiated cell monolayers were pretreated with either DMSO (control) or ezetimibe glucuronide at 100 μM for 1 h [21]. The cells then received cholecalciferol-rich medium supplemented with either DMSO or 100 μM of ezetimibe glucuronide, and uptake was measured as described above.

2.6 Characterization of vitamin D uptake in mouse intestine

2.6.1 Animals

Six-week-old wild-type (wt) male C57BL/6 Rj mice were purchased from Janvier (Janvier, Le-Genest-St-Isle, France). Detailed information on the characteristics of mice overexpressing SR-BI in the intestine (SR-BI Tg mice) has been described previously and immunocytochemistry confirmed the intestinal overexpression of SR-BI in Tg mice at the same sites than in wt mice, *i.e.* in the proximal intestine [22]. The mice were housed in a temperature-, humidity- and light-controlled room. They were given a standard chow diet and water *ad libitum*. Mice were fasted overnight before each experiment. All the protocols were approved by a local ethics committee.

2.6.2 Cholecalciferol uptake inhibition in mouse intestinal explants

Mice were fasted overnight and intestinal explants were prepared as previously described [23]. Briefly, on the day of the experiment, the 4 cm after the first 3 cm of one mouse intestine were rapidly removed under forene® inhalation narcosis before animals were euthanized by an excess of CO_2. The intestine samples were carefully rinsed with a sterile NaCl solution (0.9%), sliced into strips on ice as previously described [23] and homogeneously distributed in 12-well plates (Falcon plates, Becton Dickinson, le Pont-de-Chaix, France). Explants were incubated for 3 h in 500 μL of 0.5 μM vitamin D-enriched mixed micelles supplemented or not with either 10 μM BLT1, 400 μM SSO or 100 μM ezetimibe glucuronide. Each condition was performed in triplicate. After incubation, media were harvested and the intestine explants were carefully rinsed twice in PBS containing 5 mM sodium taurocholate. Samples were then suspended in 500 μL PBS, homogenized with 2 3-mm-diameter stainless-steel balls in 2 mL eppendorf tubes using a MM301 ball mill (Retsch, Eragny sur Oise, France) and stored until analysis. This experiment was reproduced with three different animals.

2.6.3 Vitamin D uptake in mice overexpressing SR-BI in the intestine

On the day of the experiment, the mice were force-fed with a cholecalciferol-enriched emulsion. After 2 h of digestion, the intestine of each animal was quickly harvested under forene® inhalation narcosis before euthanasia by an excess of CO_2. The intestines were carefully rinsed with PBS and cut in 2 cm segments on a total length of 20 cm. All the samples were suspended in 500 μL PBS and homogenized as described above before storage and analysis.

2.6.4 Effect of ezetimibe on vitamin D uptake in mice

The mice received or not 10 mg/kg ezetimibe by intra-peritoneal injection 27 and 4 h before the beginning of the experiment. Mice were then force-fed with a cholecalciferol-enriched emulsion and intestinal segments were taken as described above.

All the samples were sealed under nitrogen and stored at −80°C until vitamin D analysis. Aliquots of cell samples were used to assess protein concentrations using a bicinchoninic acid kit (Pierce, Montluçon, France).

2.7 Vitamin D extraction

Cholecalciferol was extracted from 500 μL aqueous samples using the following method. Distillated water was added to sample volumes below 500 μL to reach a final volume of 500 μL. Ergocalciferol, which was usually used as internal standard, was added to the samples in 500 μL ethanol. For experiments aiming at evaluating ergocalciferol uptake, cholecalciferol was used as the internal standard. The mixture was extracted once with two volumes of hexane. The

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hexane phase obtained after centrifugation (500 *g*, 10 min, 4°C) was evaporated to dryness under nitrogen, and the dried residue was dissolved in 200 μL methanol. A volume of 100–180 μL was used for HPLC analysis. Preliminary experiments showed that this extraction method allowed a recovery of vitamin D of more than 80%.

2.8 Vitamin D HPLC analysis

The HPLC system comprised a Shimazu separation module (LC-20ADSP HPLC Pumps and SIL-20CHT Autosampler, Shimazu, Champs-sur-Marne, France), an SPD-M20A Shimadzu photodiode array detector (PDA, detection at 265 nm, spectral analysis between 190 and 300 nm) and an electrochemical Coulchem III detector (ED, Esa-Dionex, Aix-en-Provence, France, applied potential of 850 mV). The ED was specifically used for the quantification of low amounts of vitamin D (the limit of quantification was 1 ng *per* injection with the PDA and 0.1 ng with the ED). Cholecalciferol and ergocalciferol were separated using a 250 × 4.6 nm RP C_{18}, 5 μm Zorbax column (Interchim, Montluçon, France) and a guard column according to Esa application note ♯70–4935P with the following changes. The mobile phase was 60% acetonitrile, 38% methanol and 2% water, containing 20 mM sodium perchlorate and 10 mM perchloric acid. Flow rate was 1.5 mL/min, and the column was kept at a constant temperature (40°C). Vitamin D was identified by spectral analysis and/or retention time and co-injection compared with pure standards (Fig. 1). No vitamin D was detected in cells or in mouse intestinal samples in basal conditions (data not shown). Quantification was performed using Chromeleon software (version 6.50 SP4 Build 1000, Dionex) comparing peak area with standard reference curves. All solvents used were HPLC grade from SDS (Peypin, France).

2.9 Statistical analysis

Results are expressed as means ± SD. Differences between two groups of unpaired data were tested using the nonparametric Mann–Whitney *U* test. Values of $p < 0.05$ were considered significant. All statistical analyses were

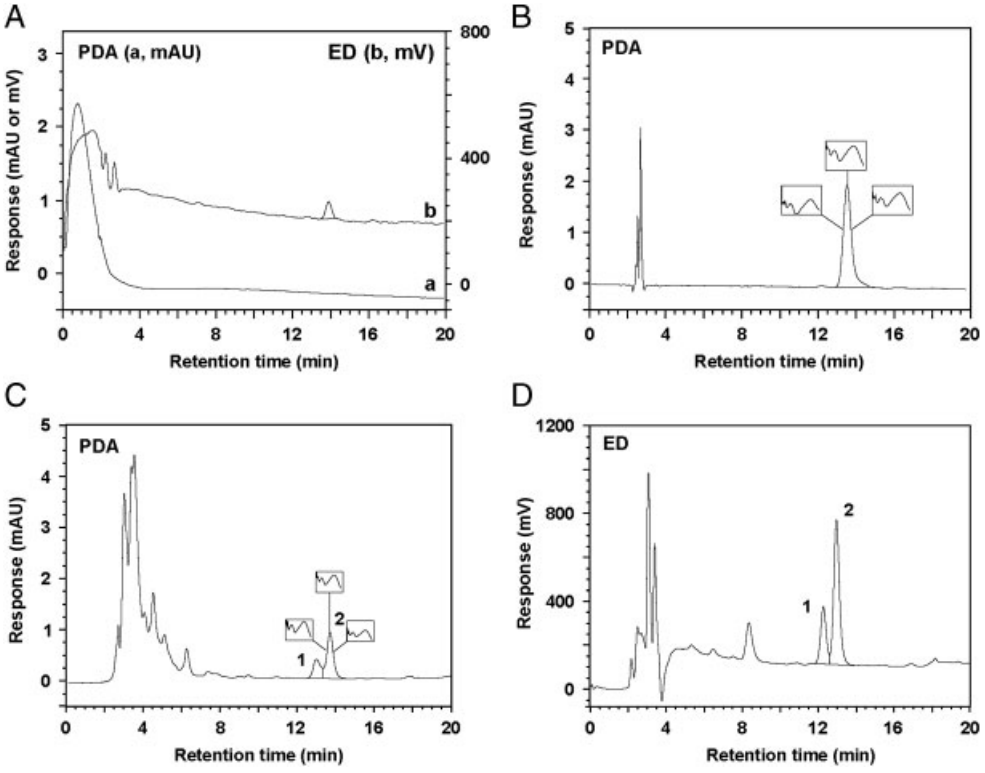

Figure 1. Chromatograms of vitamin D_3 standards and cell samples. Analytical conditions: the mobile phase was 60% acetonitrile, 38% methanol and 2% water, containing 20 mM sodium perchlorate and 10 mM perchloric acid. Flow rate was 1.5 mL/min, and the column was kept at 40°C. Detection was performed with a PDA at 265 nm or with an ED with an applied potential of 850 mV. Spectral analysis was performed with the PDA between 190 and 300 nm. (A) PDA and ED detection of 0.1 ng pure vitamin D_3 standard. (B) PDA detection and spectral analysis of 10 ng pure vitamin D_3 standard. (C and D) Chromatograms of a Caco-2 TC7 cell sample after 60 min incubation with FBS-free medium containing cholecalciferol-enriched mixed micelles at 0.5 μM obtained using PDA and ED, respectively. No vitamin D was detected in the cell samples in basal conditions. 1 = vitamin D_2, 2 = vitamin D_3. Although vitamin D_3 could be identified with PDA, ED allowed a better quantification. Similar chromatogram profiles were obtained with *ex vivo* and *in vivo* samples.

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performed using Statview software, version 5.0 (SAS Institute, Cary, NC, USA). Relationships between two variables were examined by regression analysis on SigmaPlot software (Systat Sotfware, Chicago IL, USA).

3 Results

3.1 Characterization of cholecalciferol apical uptake by differentiated Caco-2 cell monolayers

The uptake curve of cholecalciferol was not linear and similar to ergocalciferol uptake curve at low micellar concentrations (Fig. 2A). The best fits for the two vitamin D forms were hyperbolic curves $y = ax/(x+b)$, $R > 0.99$. Q_{max} represents maximal amount of vitamin D absorbed, and K represents the concentration of micellar vitamin D required to reach $Q_{max}/2$ (Table 1). It is noteworthy that at high micellar concentrations, cholecalciferol uptake was not saturable anymore and became linear (Fig. 2B).

Figure 2C shows that there were drastic falls of cholecalciferol uptake (i) at 4°C compared to 37°C (−67%) and (ii) when vitamin-D-rich micelles were delivered at the basolateral side compared with the apical side (−79%).

At last, micellar cholecalciferol uptake was significantly impaired by increasing micellar cholesterol (Table 2) and tocopherol (Table 3) concentrations.

3.2 Effect of BLT1 and ezetimibe glucuronide on cholecaciferol apical transport in Caco-2 cells

Figures 3A and B show that the addition of 10 μM of the specific chemical inhibitor of SR-BI (BLT1) significantly decreased both cholecalciferol uptake from cholecalciferol-enriched mixed-micelles (about 50%, panel A) and cellular efflux to cholecalciferol-free mixed-micelles (about 20%, panel B) in Caco-2 cells.

Similarly, the addition of 100 μM of the specific chemical inhibitor of NPC1L1, *i.e.* ezetimibe glucuronide, significantly decreased cholecalciferol uptake (about 30% Fig. 3C).

3.3 Effect of SR-BI, CD36 and NPC1L1 transfection on cholecalciferol uptake in HEK cells

The transfection with human SR-BI led to a significant twofold increase of cholecalciferol uptake compared to control (cells transfected with an empty plasmid), and this increase was significantly impaired by 10 μM BLT1 (Fig. 4A). Figure 4B shows that HEK cell transfection with human CD36 significantly increased cholecalciferol uptake and that this increase was totally suppressed by the addition of a chemical inhibitor of CD36: SSO (400 μM). At last, the transfection with human NPC1L1 led to a significant threefold increase of cholecalciferol uptake compared to control, and this increase was significantly impaired by ezetimibe glucuronide 100 μM (Fig. 4C).

3.4 Effect of BLT1, SSO and ezetimibe glucuronide on cholecalciferol uptake by mouse intestinal explants

BLT1, SSO and ezetimibe glucuronide significantly impaired cholecalciferol uptake by mouse intestinal explants (50, 40 and 65%, respectively, Fig. 5).

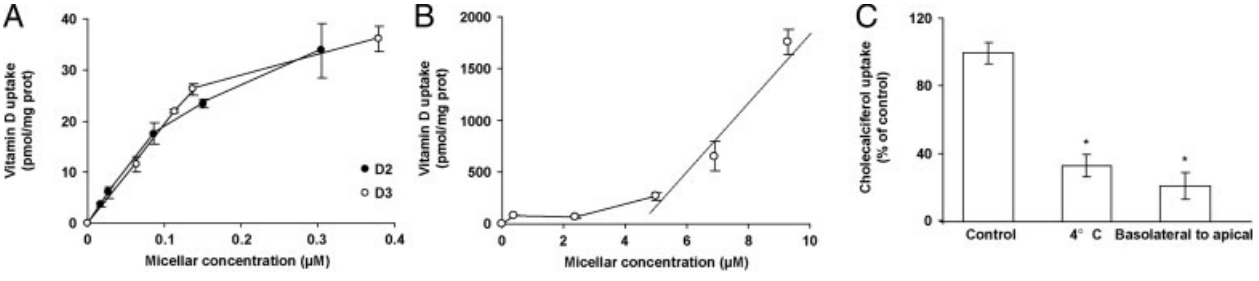

Figure 2. Characterization of vitamin D uptake in Caco-2 TC7 cells. (A) Comparison of vitamin D_3 and vitamin D_2 uptake by differentiated Caco-2 TC-7 cell monolayers at 37°C at low concentrations. The apical side received FBS-free medium containing either cholecalciferol (D_3, ○) or ergocalciferol (D_2, ●) -rich mixed micelles, and the basolateral side received FBS-free medium. Incubation time was 30 min. Data are means ± SEM of three assays. (B) Cholecalciferol uptake by differentiated Caco-2 TC-7 cell monolayers at 37°C at high concentrations. The apical side received FBS-free medium containing cholecalciferol (D3, ○)-rich mixed micelles, and the basolateral side received FBS-free medium. Incubation time was 30 min. Data are means ± SEM of three assays. (C) Effect of temperature and transport direction on cholecalciferol uptake by differentiated Caco-2 TC-7 monolayers. Cholecalciferol absorption (pmol/mg of protein) was measured at 37°C after apical micellar cholecalciferol delivery (control = 100%). Cholecalciferol uptake at 4°C and cholecalciferol uptake following addition of micellar cholecalciferol in the basolateral chamber were compared to absorption measured with vitamin D-rich micelles at the same micellar concentration at 37°C and in the apical to basolateral direction. Incubation time was 30 min. Data are means ± SEM of three assays. An asterisk indicates that the value was significantly different compared to the control.

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Table 1. Parameters of vitamin D absorption as a function of micellar vitamin D concentrations in differentiated Caco-2 TC-7 cell monolayers

Vitamin D forms	Apparent Q_{max} (pmol vitamin D absorbed/mg of protein)	Apparent K (μM)	R
Cholecalciferol (D_3)	53.14 ± 4.21	0.17 ± 0.03	0.99
Ergocalciferol (D_2)	57.27 ± 1.79	0.21 ± 0.01	0.99

Vitamin D uptake was measured at 37°C after apical micellar vitamin D delivery in increasing concentrations. Incubation time was 30 min. Best fitting curves were hyperbolic curves $y = ax/(b+x)$. Q_{max} (a) represents the maximal amount of vitamin D absorbed and K (b) represents the concentration of micellar vitamin D required to reach $Q_{max}/2$. Data are means ± SEM of three assays.

Table 2. Effect of micellar cholesterol concentration on micellar cholecalciferol absorption by differentiated Caco-2 TC-7 cell monolayers

Micellar cholesterol concentration (mM)	Cholecalciferol absorption (% of control)
0.0	140.3 ± 7.7*
0.1	100.0 ± 7.4
0.2	93.3 ± 6.3

The apical side received FBS-free medium containing cholecalciferol-rich mixed micelles (0.5 μM) with either no cholesterol, 0.1 mM cholesterol (control) or 0.2 mM cholesterol. The basolateral side received FBS-free medium. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control.

Table 3. Effect of *RRR*-α-tocopherol on cholecalciferol absorption by differentiated Caco-2 TC-7 cell monolayers

Micellar α-tocopherol concentration (μM)	Cholecalciferol absorption (% of control)
0.0	100.0 ± 7.9
10	93.4 ± 4.7
100	73.2 ± 2.9*

The apical side received FBS-free medium containing cholecalciferol-rich mixed micelles (0.25 μM) plus mixed micelles containing either no other microconstituent or *RRR*-α-tocopherol (10 or 100 μM). The basolateral side received FBS-free medium. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control (cholecalciferol-rich micelles plus vitamin-free mixed micelles).

3.5 Effect of intestinal SR-BI overexpression on cholecalciferol uptake in mice

After gavage, the cholecalciferol content of the first four fragments of SR-BI tg mice was significantly higher than the vitamin D content of the first four fragments of wt mice (+70.8%, $-p = 0.0090$, +77.0% $-p = 0.0176$, +67.1% $-p = 0.0106$, +39.9% $-p = 0.0446$, respectively, Fig. 6A).

3.6 Effect of ezetimibe treatment on cholecalciferol uptake in mice

Cholecalciferol content of mouse intestinal fragments was decreased in the medium and the distal part of the intestine (−14 to −27%), but these differences remained non-significant (Fig. 6B).

4 Discussion

To reinvestigate in details vitamin D transport across the enterocyte, we first used the human Caco-2 TC-7 cell model, which usually gives reproducible figures that correlate closely with human *in vivo* data [24]. This model has frequently been employed to evaluate the intestinal transport of lipid nutrients such as fatty acids [25], cholesterol [9, 26, 27], carotenoids [19, 28–30] or vitamin E [18, 31, 32]. Caco-2 cells spontaneously differentiate into polarized absorptive cell monolayers and display morphological and biochemical characteristics similar to human enterocytes after differentiation. The clone TC-7 was specifically chosen because it is more homogenous than the parent Caco-2 cell line [33]. We then performed transfection experiments using human HEK 293-T cells, which have already been validated as an overexpression model for SR-BI [34], CD36 [35] and NPC1L1 [21], which are not expressed in these cells in basal conditions. It is one of the most commonly used cell lines because of its high transfection efficiency and because the human glycosylation pattern is conserved.

Vitamin D dietary recommendation has recently been updated to 15 μg (600 UI) *per* day for healthy adults [36]. Considering the fat-soluble vitamin concentrations recovered in mixed micelles after digestion as compared to the amount initially ingested [37], we estimated that *in vivo* duodenal vitamin D micellar concentration should be close to 0.01 μM. However, to allow an accurate quantification, we used micellar concentrations ranging from 0.1 to 0.5 μM to mimic a dietary situation, and up to 10 μM to mimic pharmacological conditions. Our first result confirms that cholecalciferol and ergocalciferol show similar uptake patterns [38]. The saturable uptake of cholecalciferol, when delivered to cells at concentrations close to physiology, as well as both the temperature and the direction dependencies

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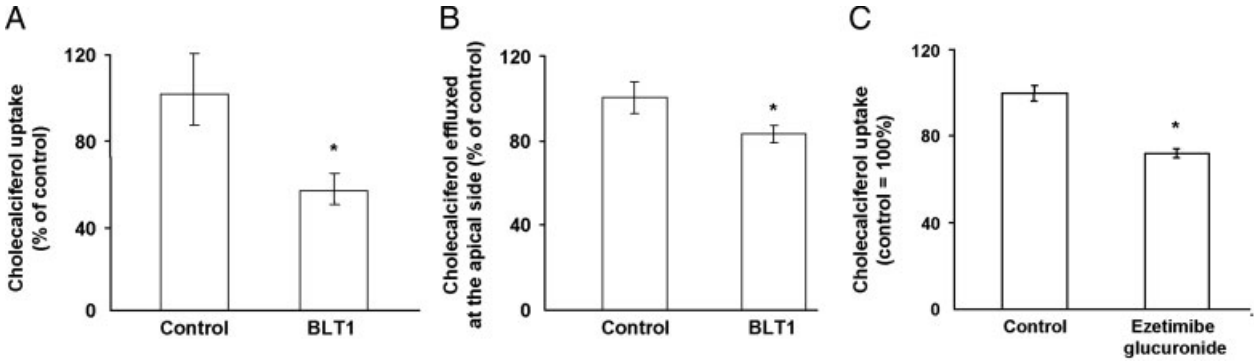

Figure 3. Effect of cholesterol transporter inhibitors on cholecalciferol apical transport by differentiated Caco-2 TC-7 monolayers. (A) Effect of BLT1 on cholecalciferol uptake. The apical sides of the cell monolayers were preincubated for 60 min with BLT1 at 10 μM, and they then received FBS-free medium containing cholecalciferol-enriched mixed micelles at 0.5 μM. The basolateral sides received FBS-free medium. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control (assay performed without BLT1). (B) Effect of BLT1 on cholecalciferol apical efflux. Cell monolayers were first enriched in cholecalciferol. The apical sides of the monolayers were then carefully rinsed and received either FBS-free medium containing vitamin D-free-mixed micelles or the same mixture plus BLT1. The basolateral side received FBS-free medium. Efflux time was 180 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control (assay performed without BLT1). (C) Effect of ezetimibe glucuronide on cholecalciferol uptake The apical sides of the cell monolayers were preincubated for 60 min with ezetimibe glucuronide at 100 μM, and then received FBS-free medium containing cholecalciferol-enriched mixed micelles at 0.5 μM. The basolateral sides received FBS-free medium. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control (assay performed without ezetimibe glucuronide).

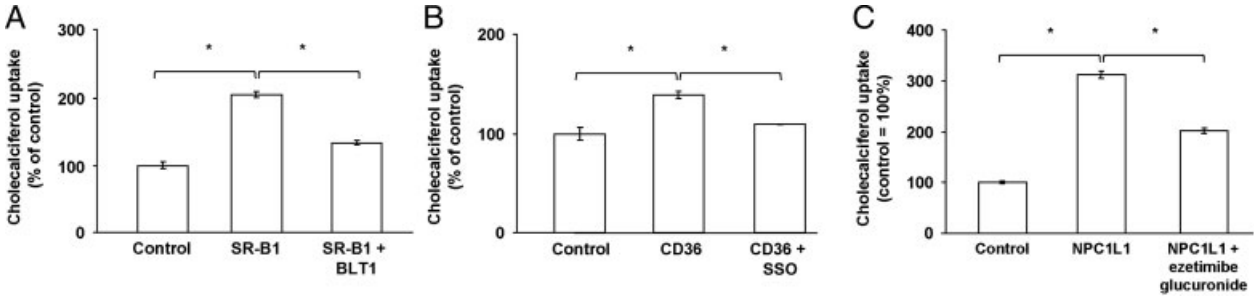

Figure 4. Effect of cholesterol transporter inhibitors on cholecalciferol uptake by 293-T HEK cells overexpressing cholesterol transporters. (A) Effect of BLT1 on cholecalciferol uptake by 293-T HEK cells overexpressing SR-BI Cells were transfected with either an empty pCDNA3.1 plasmid (control) or with a pCDNA3.1 plasmid containing SR-BI (SR-BI). The cell monolayers then received complete medium containing cholecalciferol at 0.5 μM supplemented or not with BLT1 at 10 μM. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference between groups. (B) Effect of SSO on cholecalciferol uptake by 293-T HEK cells overexpressing CD36 Cells were transfected with either an empty pIRES plasmid (control) or with a pIRES plasmid containing CD36 (CD36). The cell monolayers then received complete medium containing cholecalciferol at 0.5 μM supplemented or not with SSO at 400 μM. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference between groups. (C) Effect of ezetimibe glucuronide on cholecalciferol uptake by 293-T HEK cells overexpressing NPC1L1. Cells were transfected with either an empty $pCEP_4$ plasmid or with a $pCEP_4$ plasmid containing NPC1L1. The cell monolayers received then complete medium containing cholecalciferol at 0.5 μM supplemented or not with ezetimibe glucuronide at 100 μM. Incubation time was 60 min. Data are means ± SEM of three assays. An asterisk indicates a significant difference with the control (cells transfected with an empty $pCEP_4$ plasmid and without ezetimibe glucuronide).

[39], were first arguments in favor of a protein-mediated uptake. However, at pharmacological concentrations, cholecalciferol uptake was linear, showing that a passive diffusion can occur as well. We then performed competition studies. Both cholesterol and tocopherol decreased cholecalciferol uptake, suggesting common absorption pathways for the three molecules. As we previously showed that the cholesterol transporter SR-BI was also involved in vitamin E transport by the enterocyte [18], this scavenger receptor was a first possible candidate for vitamin D uptake. Another candidate was NPC1L1, which has been described as the main cholesterol transporter at the intestinal level [10, 40], and which is also able to transport tocopherol [41].

The finding that cholecalciferol apical transport in Caco-2 cells was inhibited by BLT1, a specific chemical inhibitor of SR-BI [42, 43], is taken as initial evidence that SR-BI is involved in vitamin D absorption. The fact that BLT1

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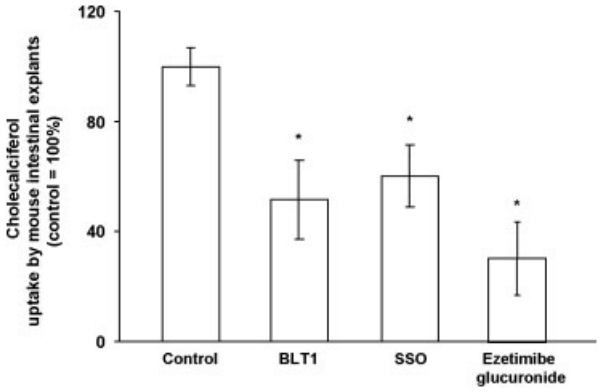

Figure 5. Effect of cholesterol transporter inhibitors on cholecalciferol uptake by *ex vivo* mouse intestinal explants. The mouse intestinal explants received FBS-free medium containing cholecalciferol-enriched mixed micelles at 0.5 μM. supplemented or not with BLT1 at 10 μM, SSO at 400 μM or ezetimibe glucuronide at 100 μM. Incubation time was 3 h. Data are means ± SEM of nine assays (three different assays in three different mice). An asterisk indicates a significant difference between groups.

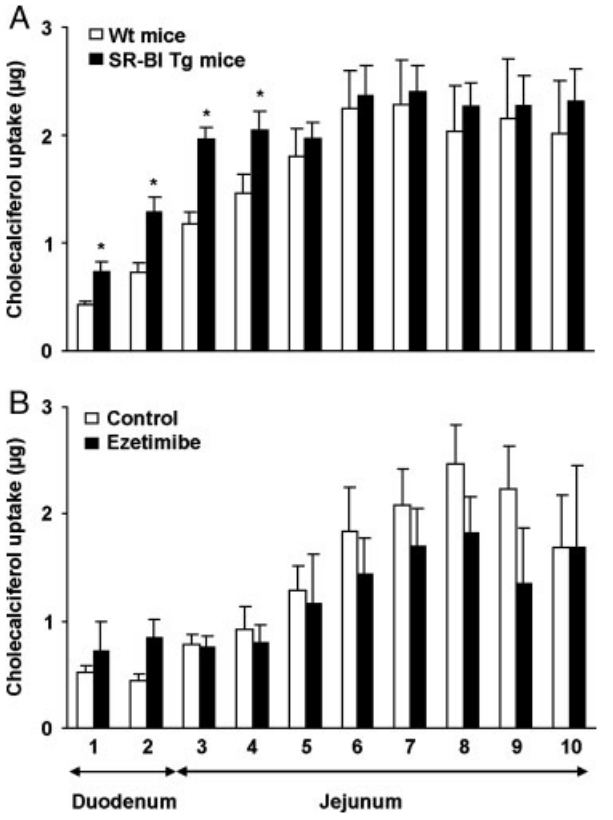

Figure 6. Cholecalciferol content of mouse intestinal fragments in mice after force-feeding with a cholecalciferol-enriched emulsion. (A) Effect of SR-BI overexpression at the intestinal level. Two hours after force-feeding, mouse intestines were harvested and cut in ten fragments. Data are means ± SEM, $n = 6$ for control mice and 6 for SR-BI tg mice. (B) Effect of ezetimibe treatment. Mice were treated or not with a 10 mg/kg ezetimibe solution by intraperitoneal injections 27 and 4 h before force-feeding. Mice were then force-fed with a cholecalciferol-enriched emulsion. Two hours after force-feeding, the first 20 cm after the biliary canal of the mice intestine were harvested, rinsed and cut in ten fragments. Data are means ± SEM, $n = 5$ for control mice and 5 for mice treated with ezetimibe.

inhibited cholecalciferol efflux from Caco-2 cells was not surprising as this transporter is involved in both lipid uptake and efflux [44]. This result was confirmed in HEK cells transfected with SR-BI, which displayed an increased BLT1-sensitive cholecalciferol uptake. Similar experiments with CD36, another scavenger receptor involved in cholesterol intestinal transport [45, 46], could not be performed in Caco-2 TC7 cells as this cell line does not express this transporter [47]. Nevertheless, HEK cells transfected with CD36 exhibited a SSO-sensitive uptake of cholecalciferol that suggests the involvement of CD36 in cholecalciferol absorption [48]. Similarly, cholecalciferol uptake in Caco-2 cells was also inhibited by ezetimibe glucuronide, which is a specific chemical inhibitor of NPC1L1. HEK cells transfected with NPC1L1 exhibited an increased uptake of cholecalciferol, and this enhancement was impaired by ezetimibe glucuronide, which supported our previous results.

In order to further examine the involvement of these transporters in cholecalciferol uptake, we performed *ex vivo* experiment using mouse intestinal explants. We obtained a significant inhibition of vitamin D uptake by BLT1 (about 50%). Moreover, we confirmed the result obtained in HEK cells as SSO inhibited up to 40% of cholecalciferol uptake in mouse intestinal explants. A 65% inhibition of vitamin D uptake by ezetimibe glucuronide was also observed. This last inhibition was more important than the inhibition obtained in Caco-2 cells (20%). This is likely due to different transporter expression profiles *in vivo* and in cells.

Finally, we performed *in vivo* uptake experiments. We first compared cholecalciferol bioavailability between wt mice and mice overexpressing SR-BI in the intestine. Cholecalciferol contents of the intestinal fragments where the SR-BI overexpression occurs were significantly higher in tg than in wt mice. This is another evidence that SR-BI is able to facilitate vitamin D uptake. We then evaluated the effect of a treatment with ezetimibe on vitamin D bioavailability in wt mice. Ezetimibe decreased both medium and distal intestinal fragment cholecalciferol contents, but these differences were not significant. Note that a control performed with radiolabelled cholesterol in similar conditions showed a significant negative effect of ezetimibe on cholesterol uptake by mouse intestine (data not shown). This result indicates that even if NPC1L1 can transport vitamin D, its *in vivo* involvement may be moderate. Indeed, NPC1L1-preferred substrate is probably cholesterol, which is present in very high concentrations compared to vitamin D in dietary conditions, leading thus to a

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competitive effect in favor of cholesterol absorption. However, in accordance with our results, a careful check of a previous study aiming at evaluating ezetimibe effect on vitamin D absorption in rats revealed that vitamin D absorption was lower in the ezetimibe group, although the difference also remained non-significant [49]. Additionally, in line with this moderate effect of ezetimibe, a recent paper enlightened a non-significant but negative effect on bone mineral density in patients treated for a year with this drug [50]. Further research is needed to test whether a longer treatment would lead to a significant effect due to a chronically reduced vitamin D absorption in these patients.

Altogether, these results are pioneering as they show for the first time that vitamin D intestinal absorption is not occurring by a simple passive diffusion process only, but that some membrane transporters such as SR-BI, CD36 or NPC1L1 can be involved in this phenomenon. These transporters have already been described to transport cholesterol, which displays a similar sterol structure, at the intestinal level. Moreover, SR-B transporters (*i.e.* SR-BI, SR-BII and CD36) have recently been described as transporting estradiol, a sterol hormone, from lipoprotein to osteoblasts [51]. It is thus very likely that class B scavenger receptors are able to transport sterols in general with broad ligand specificity.

Nevertheless, we cannot rule out the possibility of other membrane protein contributions. Indeed, in wt mice, the uptake of cholecalciferol was interestingly higher in the jejunum than in the duodenum, suggesting that another transporter specifically expressed in the jejunum may play an important role in vitamin D uptake. At last, it cannot be excluded that a fraction of vitamin D is absorbed by passive diffusion.

While the physiopathological consequences of our results remain to be characterized, we hypothesize that variations in SR-BI, CD36 or NPC1L1 expression or efficiency may affect vitamin D intestinal absorption and status in humans. Indeed, as we previously showed that genetic variation in SCARB1 gene, encoding for SR-BI, could affect human plasma level of vitamin E [52], we suggest that it could be the case regarding vitamin D as well. Identifying the people bearing unfavorable genetic variants and thus at risk of low vitamin D status would be of a great interest in terms of osteoporosis management [53], especially in subgroups of the population at a higher risk of deficiency such as the elderly [54].

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The authors have declared no conflict of interest.

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