RESEARCH ARTICLE

Phytosterols can impair vitamin D intestinal absorption in vitro and in mice

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Scope: Adequate vitamin D status is necessary and beneficial for health, although deficiency and insufficiency are very common. As cholecalciferol (vitamin D_3) structure is close to cholesterol structure, we hypothesized that phytosterols, frequently used to decrease cholesterol, intestinal absorption and consequently to reduce hypercholesterolemia, may also interact with cholecalciferol absorption.

Methods and results: β-Sitosterol effect on cholecalciferol postprandial response was first assessed in mice. We then evaluated the effect of different sterols on (i) cholecalciferol micellar incorporation, (ii) cholecalciferol apical uptake and (iii) basolateral efflux in vitro or ex vivo. In mice, cholecalciferol bioavailability was 15-fold lower in the presence of β-sitosterol ($p < 0.05$). This can partly be explained by the fact that phytosterols significantly impaired cholecalciferol incorporation into mixed micelles (from −16 to −36% depending on sterol micellar composition). This can also be due to the fact that in Caco-2 cells and mouse intestinal explants, phytosterols significantly lowered cholecalciferol apical uptake (from −13 to −39%). Conversely, phytosterols had no effect on cholecalciferol secretion at the basolateral side of Caco-2 cells.

Conclusion: The present data suggest for the first time that phytosterols can interact with vitamin D_3 intestinal absorption. This interaction can be explained by a competition for micellar incorporation and for apical uptake.

Keywords:
Bioavailability / Cholecalciferol / Cholesterol / Micelles / Phytosterols

1 Introduction

Vitamin D deficiency is now recognized as a major health concern. Sun exposure cannot be sufficient to reach an adequate vitamin D status in most people, so vitamin D should be provided in sufficient amount by the diet [1], i.e. 15 μg per day for healthy adults [2]. Unfortunately, this recommendation is extremely difficult to fulfil [3] and 75% of the US population is vitamin D insufficient [4]. As an adequate vitamin D status appears highly beneficial for bone disease [5] to some cancer [6, 7] prevention, an early diagnosis of insufficiency would be of major interest [8].

It has long been assumed that vitamin D, like other fat-soluble (micro)nutrients, was absorbed by a passive process [9, 10]. However, recent data obtained in our laboratory showed that vitamin D intestinal uptake is not only passive, but also involves cholesterol transporters such as SR-BI (Scavenger Receptor class B type I), CD36 (Cluster Determinant 36) and NPC1L1 (Niemann–Pick C1-Like 1) [11].

Phytosterols are plant sterols classically used as functional ingredients to lower cholesterol absorption [12]. In

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Abbreviations: CD36, Cluster Determinant 36; **DMEM,** Dulbecco's modified Eagle's medium; **NPC1L1,** Niemann–Pick C1-Like 1; **SR-BI,** Scavenger Receptor class B type I

Europe, regular consumers of products with added plant sterols have been estimated to constitute about 10–15% of the population [13]. A meta-analysis of the literature enlightens that a supplementation with 2 g phytosterols per day, corresponding to a 5–25-fold usual daily intake [14], had a significant plasma and LDL cholesterol lowering effect [15]. Phytosterols were shown to reduce cholesterol absorption owing to competition for space in mixed micelles in the lumen. Besides this luminal effect, it was supposed that phytosterols could also act in (i) competing with cholesterol for uptake by the intestinal cell, (ii) activating efflux transporters and/or (iii) interfering with acyl-coenzyme A/cholesterol acyl transferase activity at the enterocyte level [16].

As vitamin D displays a steroid structure and shares common absorption pathways with cholesterol, we hypothesized that phytosterols could also impair cholecalciferol absorption. Thus, the objectives of this study were to test this hypothesis and to identify the mechanisms involved.

2 Materials and methods

2.1 Preparation of vitamin D_3-enriched vehicles for mouse and cell experiments

2.1.1 Preparation of cholecalciferol-rich emulsions supplemented in β-sitosterol

For delivery of vitamin D to mice, emulsions were prepared as follows. An appropriate volume of cholecalciferol and β-sitosterol (Sigma-Aldrich, Saint-Quentin-Fallavier, France) stock solutions was transferred into Eppendorfs to obtain a final amount of 100 μg of vitamin D_3 and of 10 mg of β-sitosterol in each tube. Stock solution solvent was carefully evaporated under nitrogen. Dried residue was solubilized in 100 μL of sunflower vegetable oil (Lesieur, Asnières-sur-Seine, France) and 200 μL of NaCl 0.9% solution was added. The mixture was vigorously sonicated in ice-cold water (Branson 3510 sonication bath, Branson, MO, USA) for 15 min and used for force-feeding within 10 min. Control emulsions were prepared without β-sitosterol.

2.1.2 Preparation of cholecalciferol-rich micelles

For delivery of vitamin D_3 to Caco-2 cells or to mouse intestinal explants, mixed micelles were prepared as previously described [17] to obtain the following final concentrations: 40 μM 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine, 160 μM 1-palmitoyl-sn-glycero-3-phosphocholine, 300 μM mono-olein, 500 μM oleic acid, 0.5 μM cholecalciferol (or as indicated) and 5 mM taurocholate (Sigma-Aldrich). These micelles contained either no, 100 or 500 μM cholesterol, and/or no, 100 or 500 μM phytosterols. The phytosterols were either β-sitosterol, campesterol, stigmasterol (Sigma-Aldrich) or a mix of the three molecules at a percentage of 65, 30 and 5% (i.e. 325 μM β-sitosterol, 150 μM campesterol, 25 μM stigmasterol), respectively, according to the dietary ratios [16]. Concentrations of vitamin D_3 and sterols in the micellar solutions were checked before each experiment.

2.2 Mouse experiment

Six-week-old wild-type male C57BL/6 Rj mice were purchased from Janvier (Janvier, Le-Genest-St-Isle, France). 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. The protocol was approved by the ethics committee of Marseilles (agreement ♯4-5032010).

2.2.1 Effect of β-sitosterol on cholecalciferol postprandial response in mice

On the day of the experiment, a blood sample was obtained upon fasting (zero baseline sample) by cutting the extremity of the tail. The mice were then force-fed with a control cholecalciferol-rich emulsion ($n = 4$) or a cholecalciferol-rich emulsion supplemented with β-sitosterol ($n = 5$). At $t = 2$, 4 and 7 h, plasma samples were taken by retro-orbital puncture under forene® anesthesia.

2.2.2 Effect of phytosterols on cholecalciferol uptake by mouse intestinal explants

Intestinal explants were prepared and the experiment was conducted as previously described [11]. Explants were incubated for 3 h in 500 μL of 0.5 μM cholecalciferol-rich mixed micelles containing 100 μM cholesterol and supplemented with either 500 μM β-sitosterol or 500 μM of the phytosterol mix (i.e. 325 μM β-sitosterol, 150 μM campesterol, 25 μM stigmasterol). A control condition was performed without phytosterol. Each condition was performed in triplicate. After incubation, media were harvested and the intestine explants were carefully rinsed twice in PBS. Samples suspended in 0.5 mL PBS were then homogenized with stainless-steel balls using MM301 ball mills (Retsch, Eragny sur Oise, France).

2.3 Cell culture

2.3.1 Caco-2 cell culture

Caco-2 clone TC-7 cells [18] were a gift from Dr. M. Rousset (UMR_S 872, Paris, France). Cells were cultured in the

presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated foetal bovine serum, 1% non-essential amino acid and 1% antibiotics (complete medium) as previously described [17, 19]. For each experiment, cells were seeded and grown during 21 days on transwells to obtain confluent highly differentiated cell monolayers [11, 19]. Twelve hours prior to each experiment, the medium used in apical and basolateral chambers was a serum-free medium.

2.3.2 Effect of cholesterol and phytosterol micellar composition on cholecalciferol uptake by Caco-2 cells

At the beginning of each experiment, cell monolayers were washed with 0.5 mL PBS. The apical side of the cell monolayers received the vitamin D_3-rich micelles containing cholesterol and/or phytosterol(s), whereas the other side received serum-free medium. Cells were incubated for 60 min at 37°C. 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_3, then scraped and collected in 0.5 mL PBS. Absorbed vitamin D_3 was estimated as vitamin D_3 found in the scraped cells plus vitamin D_3 found on the basolateral side of the cell monolayer, if any. Cholecalciferol uptake efficiency was evaluated as the percentage of cholecalciferol taken up by the cells compared with the initial cholecalciferol micellar content. A control experiment was performed using micelles containing a constant cholecalciferol concentration (0.5 μM) and extemporaneously added cholesterol or phytosterol in DMSO.

2.3.3 Effect of phytosterols on cholecalciferol basolateral efflux by Caco-2 cells

At the beginning of each experiment, cell monolayers were washed with 0.5 mL PBS. The apical side of the cell monolayers received a vitamin D_3-rich micellar solution containing β-sitosterol, whereas the other side received serum-free medium (vitamin D_3 was extemporaneously added in ethanol to micelles containing β-sitosterol to standardize cholecalciferol concentration). The control condition was performed without β-sitosterol. A concentration of vitamin D_3 around 5 μM was selected to have an appropriate cellular amount of newly absorbed vitamin D_3 and to accurately measure its basolateral efflux. Cells were incubated for up to 12 h at 37°C. Aliquots of the basolateral medium were taken at different times, and replaced by the same volume of new medium. 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_3, then scraped and collected in 0.5 mL PBS.

All samples were stored at −80°C under nitrogen before vitamin D_3 extraction and HPLC analysis. Aliquots of cell samples supplemented with an appropriate volume of protease inhibitor cocktail (Roche, Fontenay, France) were used to assess protein concentrations using a bicinchoninic acid kit (Pierce, Montluçon, France).

2.4 Vitamin D and sterol extraction

To simultaneously extract cholecalciferol, cholesterol and phytosterols from 400 μL aqueous samples, we used the Bligh and Dyer method [20] with minor changes. Briefly, ergocalciferol (internal standard) was added to the samples in 100 μL ethanol. About 1.5 mL of chloroform/methanol (1:2, v/v) was first added and vigorously mixed. 500 μL of distilled water was then added, as well as 500 μL of chloroform and the mixture was vigorously mixed again. The lower chloroform phase obtained after centrifugation (500 × *g*, 10 min, 4°C) was evaporated to dryness under nitrogen, and the last step was repeated again. Dried extract was dissolved in 200 μL of mobile phase. A volume of 100–180 μL was used for the HPLC analysis. Preliminary experiments showed that this extraction method allowed a recovery of more than 80% of the different sterols.

2.5 HPLC analysis

The HPLC system comprised a Shimadzu separation module (LC-20ADSP HPLC Pumps and SIL-20CHT Autosampler, Shimadzu, Champs-sur-Marne, France), an SPD-M20A Shimadzu photodiode array detector (detection at 265 nm for vitamin D, detection at 205 nm for cholesterol/phytosterols [21], spectral analysis between 190 and 300 nm) and an electrochemical Coulchem III detector (Esa-Dionex, Aix-en-Provence, France, applied potential of 850 mV) specifically used for the quantification of low amounts of vitamin D [11].

Ergocalciferol (Sigma-Aldrich), cholecalciferol, cholesterol, campesterol/stigmasterol and β-sitosterol were separated using a 250 × 4.6 nm RP C_{18}, 5 μm Zorbax column (Interchim, Montluçon, France) and a guard column as previously described [11] with minor changes. Briefly, the mobile phase was 60% acetonitrile, 38% methanol and 2% water, containing 20 mM sodium perchlorate and 10 mM perchloric acid, the flow rate was 1.2 mL/min and the column was kept at a constant temperature (40°C). Vitamin D and sterols were identified by spectral analysis and/or retention time and co-injection compared with pure standards (Fig. 1). Neither vitamin D nor phytosterol was detected in cells or in mouse plasma in basal conditions (data not shown). Quantification was performed using the

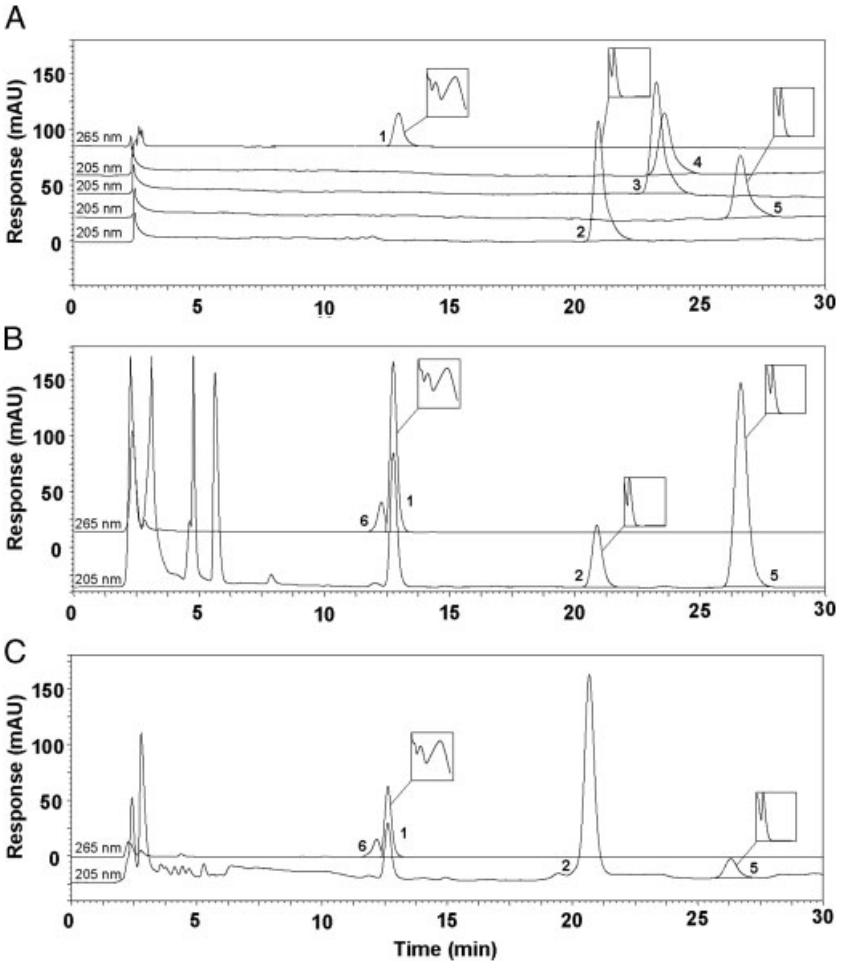

Figure 1. Chromatograms of vitamin D standards and cell samples. Analytical conditions: the mobile phase was 60% acetonitrile, 38% methanol and 2% water, containing 20 mM sodium perchlorate and 10 mM perchloric acid. Flow rate was 1.2 mL/min, and the column was kept at 40°C. Detection was performed with a photodiode array detector at 265 and 205 nm. Spectral analysis was performed between 190 and 300 nm: 1, vitamin D_3, 2, cholesterol, 3, stigmasterol, 4, campesterol, 5, β-sitosterol, 6, vitamin D_2 (internal standard). (A) Detection of cholecalciferol, cholesterol and phytosterol standards. (B) Chromatogram of a mixed micelle sample containing cholecalciferol, cholesterol and β-sitosterol. (C) Chromatogram of a Caco-2 TC7 cell sample after 60 min incubation with serum-free medium containing cholecalciferol-, cholesterol- and β-sitosterol-enriched mixed micelles. Neither vitamin D nor phytosterol was detected in the cell samples in basal conditions. Similar chromatogram profiles were obtained with in vivo samples.

Chromeleon software (version 6.50 SP4 Build 1000, Dionex) by comparing peak areas with the standard reference curves. All solvents used were of HPLC grade from SDS (Peypin, France).

2.6 Statistical analysis

The results are expressed as mean ± SEM. Differences between more than two groups of unpaired data underwent the non-parametric Kruskal–Wallis test. The non-parametric Mann–Whitney *U* test was used as a post hoc test when the Kruskal–Wallis test showed significant differences between groups. Differences between only two groups of unpaired data were tested by the Mann–Whitney *U* test. Correlations between two groups of paired data were tested using the non-parametric Spearman test. Multiple linear regression was used to assess the association between cholecalciferol micellar content and sterol micellar content with cholecalciferol uptake efficiency by Caco-2 cells (as dependent variable). The values of $p < 0.05$ were considered significant. All statistical analyses were performed using the Statview software, version 5.0 (SAS Institute, Cary, NC, USA).

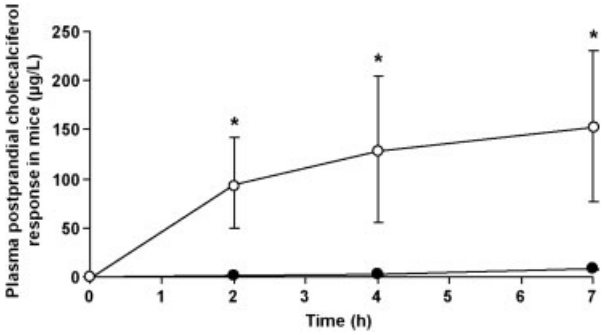

Figure 2. Effect of β-sitosterol on postprandial cholecalciferol response in mice. Mice were force-fed with cholecalciferol-rich emulsions. Data are means ± SEM, $n = 5$ for control mice (○) and $n = 4$ for β-sitosterol supplemented mice (●). An asterisk indicates a significant ($p < 0.05$) difference between two means obtained at the same time.

3 Results

3.1 Effect of β-sitosterol on postprandial plasma cholecalciferol response in mice

β-Sitosterol was selected as a phytosterol model. The presence of this phytosterol in the cholecalciferol-rich gavage emulsion significantly decreased postprandial plasma cholecalciferol response (0.32 ± 0.19 μg/L versus 0.02 ± 0.00 μg/L after 7 h, Fig. 2).

3.2 Effect of sterols on cholecalciferol micellar incorporation

We first compared the solubility of β-sitosterol with those of other phytosterols and cholesterol in mixed micelles (Fig. 3A). The order of solubility was as follows: cholesterol > campesterol ≥ β-sitosterol > stigmasterol. As expected, phytosterols significantly impaired cholesterol incorporation into mixed micelles when added at 500 μM, but not at 100 μM. Overall, the total amount of sterols solubilized into the micelles reached a plateau around 300 μM.

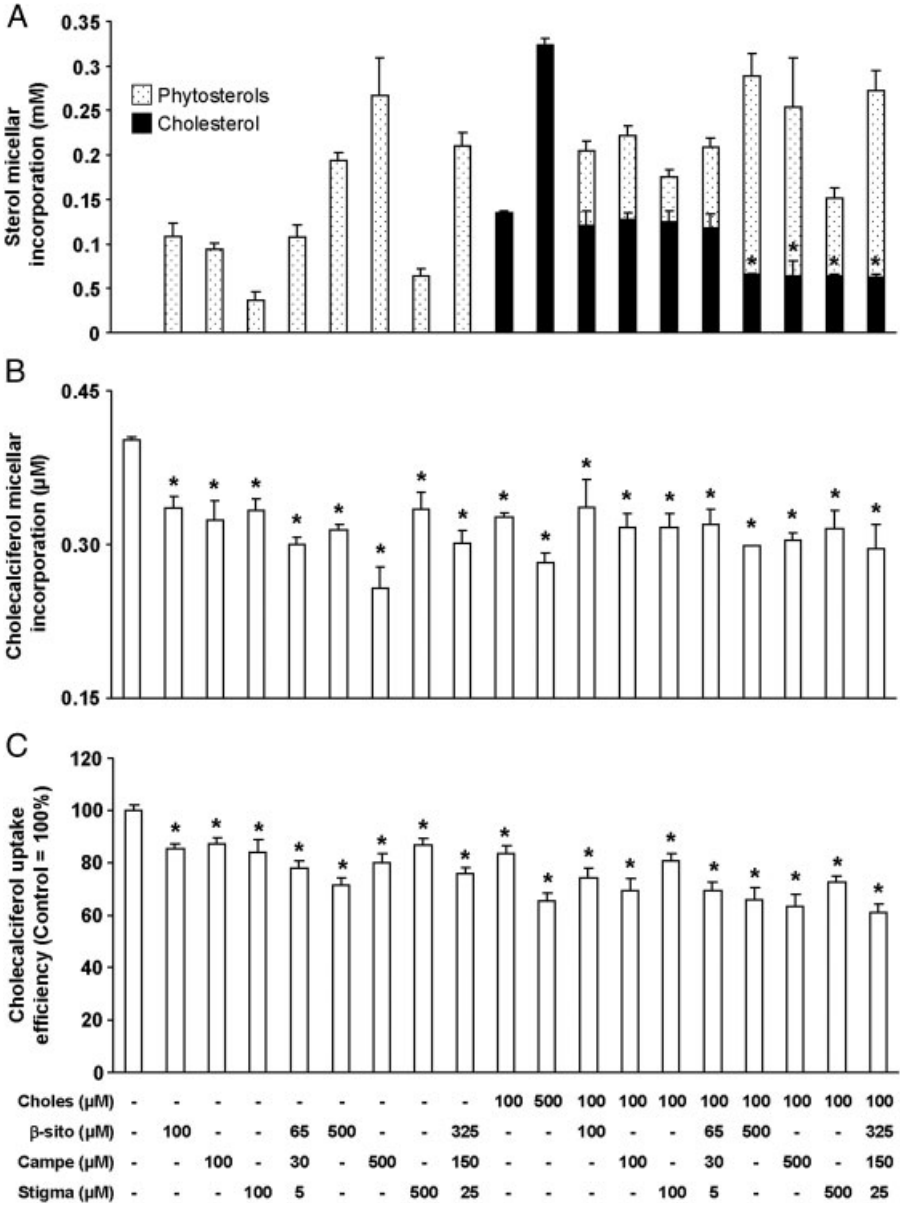

Figure 3. Solubility of cholesterol, phytosterols and cholecalciferol into mixed micelles and effect of sterols on cholecalciferol uptake by differentiated Caco-2 TC-7 cell monolayers. Choles: cholesterol, β-sito: β-sitosterol, Campe: campesterol, Stigma: stigmasterol. (A) Phytosterols and cholesterol micellar incorporation. An asterisk indicates a significant difference of cholesterol incorporation with the control (micelles containing cholesterol 100 μM and no phytosterols). Data are means ± SEM of three assays. (B) Vitamin D_3 micellar incorporation. An asterisk indicates a significant difference of cholecalciferol incorporation with the control (micelles containing cholecalciferol but neither cholesterol nor phytosterols). Data are means ± SEM of three assays. (C) Effects of sterols on vitamin D_3 uptake efficiency by Caco-2 TC-7 cells. The apical side received mixed micelles containing cholecalciferol and different sterols at either 100 or 500 μM. The basolateral side received serum-free medium. Incubation time was 60 min. An asterisk indicates a significant difference of cholecalciferol uptake efficiency with the control (uptake efficiency in cells that received micelles containing cholecalciferol but neither cholesterol nor phytosterols). Data are means ± SEM of six assays.

Cholecalciferol incorporation was significantly decreased up to 25.4% when 100 μM of sterol (cholesterol, β-sitosterol, campesterol, stigmasterol or a mix of the three phytosterols) was added to the mixture (Fig. 3B). This decrease was higher with higher sterol concentrations, especially with 500 μM campesterol (−36.1%), 500 μM cholesterol (−29.7%) or the mix of 100 μM cholesterol/325 μM β-sitosterol/150 μM campesterol/25 μM stigmasterol (−26.4%), enlightening a dose–response association. Indeed, there was a significant negative correlation between total micellar sterol and micellar cholecalciferol (ρ: −0.760, $p = 0.001$, Fig. 4A).

3.3 Effect of sterols on cholesterol uptake by Caco-2 TC-7 cells and mouse intestinal explants

We used the mixed micelle solutions presented in the previous paragraph to perform this experiment. Figure 3C shows that the presence of sterols (cholesterol or phytosterols) in the mixed micelles significantly decreased the efficiency of cholecalciferol uptake by Caco-2 cells (from −12.9 to −39.2%, depending on the amount of sterol solubilized into the micelles) in a dose-dependent manner. Multiple linear regression showed that the efficiency of cholecalciferol uptake was negatively correlated with the sterol micellar content ($p < 0.05$, Fig. 4B and Table 1). Conversely, the effect of the initial cholecalciferol micellar content on the efficiency of cholecalciferol uptake remained negligible in the range of concentrations considered (Table 1). A similar significant decrease in cholecalciferol uptake efficiency was observed when cholesterol or phytosterol was added extemporaneously in DMSO as well (data not shown).

Both β-sitosterol and the mix of the three phytosterols at 500 μM also impaired cholecalciferol uptake by mouse intestinal explants: −66.4 ± 17.1% and −42.5 ± 14.5%, respectively ($p < 0.05$).

3.4 Effect of β-sitosterol on cholecalciferol basolateral secretion by the enterocyte

We selected β-sitosterol as a phytosterol model. Figure 5A shows that β-sitosterol addition into micelles at either 100 or 500 μM significantly decreased by up to 25% the amount of basolateral cholecalciferol secreted by differentiated Caco-2 cells after 4–12 h incubation (67.7 ± 3.6 pmol/mg versus 50.4 ± 2.7 pmol/mg protein with 100 μM β-sitosterol and 51.2 ± 2.8 pmol/mg with 500 μM β-sitosterol after 12 h). However, no significant decrease in vitamin D_3 secretion was observed when the output was expressed as a percentage of vitamin D absorbed (i.e. vitamin D_3 found in scraped cells plus vitamin D_3 found in the basolateral media, Fig. 5B).

4 Discussion

During the last decade, phytosterols have increasingly been used as a part of functional foods to lower plasma and LDL cholesterol in humans [15]. Because vitamin D status is generally not optimal in humans and considering their structural homologies, we aimed to evaluate the possible competition occurring between phytosterols and vitamin D at the intestinal level.

Thus, we assessed the effect of β-sitosterol, i.e. the main dietary phytosterol, on postprandial cholecalciferol response in vivo in mice. The amounts of β-sitosterol (10 mg/mouse) and vitamin D_3 (100 μg/mouse) used in this experiment were a balance between (i) the amounts necessary with to respect the dietary vitamin D/phytosterol ratio (15 μg vitamin D according to the updated recommendations [2] versus 80–400 mg phytosterols [14]), (ii) the minimal amount of vitamin D necessary for accurate HPLC quantification from mouse plasma and (iii) the maximal amount of phytosterols able to be solubilized in our emulsion without modifying its

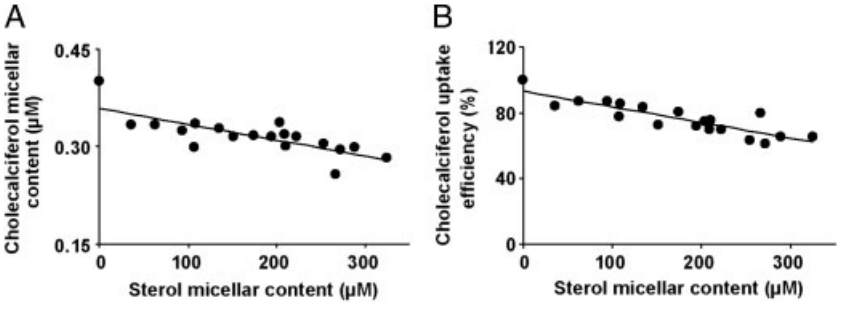

Figure 4. Effect of sterol micellar content on cholecalciferol micellar content or cholecalciferol uptake efficiency. (A) Correlation between sterol micellar content and cholecalciferol micellar content. (B) Correlation between sterol micellar content and cholecalciferol uptake efficiency.

Table 1. Multivariate linear regression of the associations between cholecalciferol uptake efficiency (%) in Caco-2 cells (as the dependent variable), and cholecalciferol micellar content and sterol micellar content (independent variables)

Independent variables	β-Coefficient	Standardized error	*p*
Sterol micellar content (μM)	−0.10	0.02	0.001
Cholecalciferol micellar content (μM)	−0.17	68.47	0.998

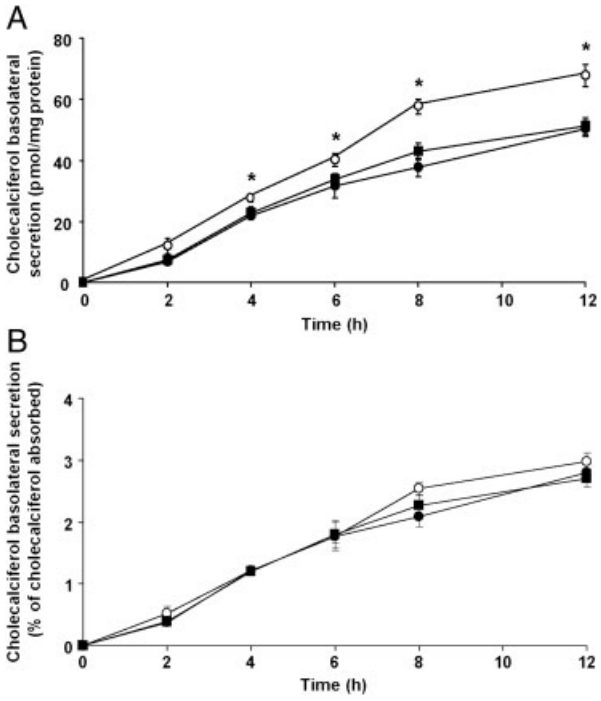

Figure 5. Effect of phytosterols on cholecalciferol basolateral efflux by differentiated Caco-2 TC-7 cell monolayers. Cell monolayers differentiated on filters received at the apical side mixed micelles containing 5 μM cholecalciferol (○), mixed micelles containing 5 μM cholecalciferol and enriched with 100 μM β-sitosterol (●) or mixed micelles containing with 5 μM cholecalciferol and enriched with 500 μM β-sitosterol (■). The basolateral side received serum-free medium. Incubation time was 12 h. Data are means ± SEM of three assays. An asterisk indicates a significant ($p<0.05$) difference between two means obtained at the same time. (A) Cholecalciferol secretion in the basolateral medium. (B) Cholecalciferol secretion as a percentage of cholecalciferol absorbed.

viscosity. This quantity of phytosterols was checked to be non-toxic for rodents [22]. This in vivo β-sitosterol supplementation led to a drastic decrease in vitamin D_3 postprandial plasma response as soon as 2 h after gavage (about 65-fold), and until the end of the follow-up at 7 h (about 15-fold).

To understand the mechanisms behind this novel observation, we then performed in vitro experiments. In 1983, Ikeda and Sugano showed that phytosterols could compete with cholesterol for inclusion into mixed micelles [23]. Thus, we first investigated whether phytosterols, as well as cholesterol, could compete with cholecalciferol for micellar incorporation. Again, a particular care was given with respect to a vitamin D_3/sterol ratio close to the one observed in the human diet. The vitamin D_3 content was kept as low as possible close to physiological conditions, but sufficient to ensure an accurate quantification [11]. Phytosterols were added either in a similar concentration than cholesterol to mimic habitual dietary conditions, or at a 5-fold higher concentration to mimic a supplementation with functional foods [12, 24]. Mixed micelles were prepared with lipids and products of their hydrolysis in proportions similar to those found in human duodenum during digestion [17]. Under these conditions, cholesterol, β-sitosterol and campesterol were more soluble in mixed micelles than stigmasterol, which is consistent with the previous data on sterol solubilization into taurodeoxycholate micelles [25]. Moreover, we confirmed that adding phytosterols at high concentrations into mixed micelles induced a reduction of cholesterol incorporation, probably due to a dynamic competition [26], while the low concentrations mimicking dietary conditions were without effect. Our results show for the first time that both cholesterol and phytosterols can impair cholecalciferol micellar solubility. This negative effect was proportional to the total amount of sterols solubilized in the mixed micelles. As diet naturally provides cholesterol and phytosterols, and as biliary micelles contain a substantial amount of cholesterol, the micellar incorporation of vitamin D_3 is probably not optimal under standard dietary conditions, and even much lower after ingestion of high amounts of phytosterols as provided by enriched foods.

We then tested the effect of cholesterol and phytosterols on vitamin D_3 uptake efficiency by Caco-2 cells, which are commonly employed as a reproducible and convenient human intestinal epithelia model [11]. Both micellar cholesterol and phytosterols impaired vitamin D_3 apical uptake by Caco-2 cells in a similar manner and this effect was proportional to the total sterol micellar content. These data are strengthened by the fact that phytosterols also significantly decreased vitamin D_3 uptake in mouse intestinal fragments. The first hypothesis to explain such an interaction at the cellular level is that the presence of sterols in the mixed micelles led to a different micellar structure that was less efficiently absorbed. Another possibility is a competition for uptake via a common membrane transporter. NPC1L1 has clearly been described as the main cholesterol and phytosterol transporter in the small intestine [27, 28], and it has also recently been involved in cholecalciferol uptake [11]. Thus, the hypothesis that NPC1L1 is involved in this competition seems very likely. Conversely, the hypothesis of an activation of the efflux transporter ABCG5/G8 appears to be unlikely, as previously reported [29].

Finally, we tested the effect of β-sitosterol on vitamin D_3 basolateral secretion by differentiated Caco-2 cell monolayers. Lower amounts of vitamin D_3 were secreted in the basolateral medium in the presence of β-sitosterol in mixed micelles at apical side. However, after normalization of vitamin D_3 basolateral efflux by the amount of vitamin D_3 actually absorbed by the cells, no significant difference was observed in the presence of phytosterol as compared with the control condition. This suggests that β-sitosterol interferes with the uptake of micellar vitamin D_3 causing less vitamin D_3 to be secreted, and that it does not interact directly with

the vitamin D_3 basolateral secretion, according to what has previously been observed for cholesterol in Caco-2 cells [30]. Moreover, the potential effect of phytosterols on cholesterol secretion is supposed to occur by interfering with acyl-coenzyme A/cholesterol acyl transferase activity, which is responsible for cholesterol esterification [16]. Such esterification is a key step for cholesterol incorporation into chylomicrons but has never been described for vitamin D. As phytosterols were shown without effect on the postprandial triacylglycerol-rich lipoprotein response [31], it is thus not surprising that no effect of β-sitosterol was found regarding cholecalciferol basolateral secretion rate.

Surprisingly, phytosterols were claimed to have no effect on vitamin D status in different clinical trials [32, 33], despite the postprandial cholecalciferol response has never been measured under such conditions. However, vitamin D status was evaluated as plasma 25(OH)-vitamin D, which is highly variable depending on factors such as season and sun exposure. As a consequence, 25(OH)-vitamin D levels can display a high variability between the beginning and the end of the study or between the different groups [34]. In addition, the intervention periods may have been too short to enlighten a significant effect on vitamin D homeostasis. Finally, it is worth mentioning that a close look at one study revealed a significant lowering effect of a sitostanol ester enriched-margarine on vitamin D plasma level after a long-term treatment in humans [35], in agreement with another study in rats [36].

In conclusion, our results show for the first time that vitamin D intestinal absorption can be impaired by phytosterols using complementary in vivo and in vitro models. Thus, we suggest that avoiding long-term dietary interaction with phytosterols, or consuming appropriate vitamin D supplement when using phytosterols, would be of major interest for people at high risk of vitamin D deficiency such as the elderly [37].

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The authors have declared no conflict of interest.

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