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β-Sitosterol stimulates ceramide metabolism in differentiated $Caco_2$ cells

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Abstract

Previous studies from our laboratory on tumor cells suggest that phytosterols stimulate ceramide production, which was associated with cell growth inhibition and stimulation of apoptosis. The objective of the present study was to examine the effect of phytosterols on ceramide metabolism in small intestinal cells that represent the first cells in contact with dietary phytosterols. $Caco_2$ cells, an accepted model for human intestinal epithelial cells, were used in this study. Ceramide and ceramide-containing lipids were examined by labeling the ceramide pool with ^{3}H-serine. Cells were supplemented with 16 μM of sterols (cholesterol, β-sitosterol or campesterol) for 16 days postconfluence and continued to differentiate. Of the two phytosterols, β-sitosterol, but not campesterol, induced more than double the serine labeling when compared with cholesterol. This increase was uniform in sphingomyelin (SM), ceramide and sphingosine labeling. Sterols had no effect on SM concentration in the cells. In addition, sterol had no effect on the activity of SM synthase or sphingomyelinases. There was an inhibition of ceramidases with campesterol supplementation. These data suggest that the observed increases in SM and sphingosine labeling were due to an increase in ceramide turnover. The increase in ceramide turnover with β-sitosterol supplementation was not associated with growth inhibition but was with increases in ceramide glycosylation products such as cerebrosides and gangliosides. It was concluded that β-sitosterol has no effect on differential $Caco_2$, a model of normal small intestinal cells. The increase in the glycosylated ceramide products may offer a means to protect the cells from the harmful effect of ceramide by excreting them with lipoproteins.

Keywords: Intestinal cells; Cholesterol; Phytosterols; β-sitosterol; Glycosylated sphingolipids; Cell growth; Differentiation

1. Introduction

Phytosterols are the plant counterparts of the animal sterol cholesterol. Several forms of phytosterols exist in the diet, the most common being campesterol, β-sitosterol and stigmasterol. The structure of phytosterols varies from that of cholesterol and this is mostly localized to the side chain of the molecule. These variations include the addition of a methyl group (campesterol), an ethyl group (β-sitosterol) or both (stigmasterol) [1,2]. The effect of plant sterols on the absorption of cholesterol from the gut is well documented [3–5]. The mechanism by which phytosterols interfere with cholesterol absorption is not fully understood but could include the difference in sterol solubility and the formation of micelles due to differences in structure [1,2,6]. Phytosterols are poorly absorbed and are quickly eliminated from the body. Due to these differences in absorption and elimination and the lack of the mammalian system to synthesize them, the phytosterol level in the blood is usually in the micromolar range compared with the millimolar range of the cholesterol level [6,7]. Moreover, due to the variation among different phytosterols in rates of their absorption and elimination from the body, the two major phytosterols detected in the blood are campesterol and β-sitosterol [6–8].

It is well accepted that ceramide functions as a second messenger in cells. The increase in its intracellular concentration in response to extracellular signals induces apoptosis in cancer cells, cell arrest and inhibition of growth [9]. We have shown that β-sitosterol induces apoptosis in several tumor cell lines and that this was mediated through the activation of the sphingomyelin (SM) cycle [10,11]. However, the effect of phytosterols on enterocyte ceramide metabolism has not been studied despite the fact that these cells are usually exposed to higher concentrations of phytosterol in the gut at the luminal side compared with those seen in the blood (serosal side). The objective of the

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present study was to examine the effect of phytosterols on ceramide metabolism in differentiated $Caco_2$ cells, a well accepted model for human enterocytes [12,13].

2. Materials and methods

2.1. Materials

β-Sitosterol, cholesterol, campesterol and protease inhibitors were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phospholipids and ceramide were purchased from Avanti Polar Lipids (Alabaster, AL, USA). NBD-labeled lipids [6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoyl) sphingosyl phosphocholine (NBD C_6-SM), 6-((*N*-)7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoyl) sphingosine (NBD C_6-ceramide) and 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoic acid (NBD-hexanoic acid)] were obtained from Molecular Probes (Eugene, OR, USA). Cell culture media [Dulbecco's Modified Eagle Medium (DMEM)], fetal bovine serum (FBS) and MEM nonessential amino acid solution were obtained from Invitrogen (Carlsbad, CA, USA). L-^{3}H-Serine (20 Ci/mmol), SM [choline-methyl-^{14}C] (55 mCi/mmol) and galactose D-[6-^{3}H(N)] (60 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO, USA).

2.2. $Caco_2$ cell culture

Cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and subcultured weekly in high-glucose (4.5 g/L) DMEM containing 10% FBS, antibiotics (100,000 U/L penicillin and 100 mg/L streptomycin sulfate), 1% nonessential amino acids and 3.7 g/L of sodium bicarbonate at 37°C under a humidified atmosphere of 5% CO_2. For experiments, cells were seeded into 6-well plates at 20,000 cells/cm^2 and allowed to attach for 48 h, after which the medium was changed every other day. At confluence, sterols (16 μM of cholesterol, campesterol or β-sitosterol) were supplied to the cultures dissolved in ethanol. The control group received the same concentration of the ethanol vehicle (0.5%) as did the sterol-treated cultures. Treatments were continued for 16 days postconfluence. Under these conditions, cells were differentiated as assessed by their expression of alkaline phosphatase [14]. Cell growth was measured by counting cells in a hemocytometer after trypsinization.

2.3. Ceramide metabolism

Sterol-induced changes in SM cycle intermediates (i.e., SM, sphingosine and ceramide) were assessed by the method of Jones and Murray [15]. Sterol-treated cells were radiolabeled with ^{3}H-serine (1 μCi/ml) added to the culture medium for 24 h. After two washes (2 ml) with PBS, the confluent cells were incubated with unlabeled serine (5 mM) for 5 h. Cells were washed again, scraped in ice-cold methanol containing 20 μg each of SM, sphingosine and ceramide and extracted by the method of Bligh and Dyer [16]. The organic phase was subjected to mild saponification with 0.1 M of methanolic KOH at 37°C; the lipids were separated on TLC in a solvent system containing chloroform/methanol/ammonia/water (80:20:1:1, vol/vol/vol/vol) up to 9.5 cm on the plate and followed by a solvent system containing diethyl ether/methanol (99:1, vol/vol) up to 19.5 cm on the 20×20-cm plate. The areas corresponding to the sphingolipids were scraped from the plates and quantified by liquid scintillation spectrometry.

2.4. Glycosphingolipid analysis

The effect of sterol treatment on glycosylated sphingolipid synthesis was estimated using the method of Lavie et al. [17]. Briefly, sterol-treated cells were labeled with ^{3}H-galactose (1.0 μCi/ml) in culture for 24 h. Cell monolayers were then rinsed twice with PBS (pH 7.4) and scraped in 2 ml of ice-cold methanol to which 2.5 μg each of glucosylceramide, galactosylceramide, lactosylceramide and ganglioside was added as carriers to improve recovery. The lipids were extracted by the addition of chloroform (4 ml) and water (2 ml) and the lower chloroform phase was taken for lipid separation by TLC on silica gel G using chloroform/methanol/water (60:40:18, vol/vol/vol). After treatment with EN^{3}HANCE spray (Perkin Elmer, Boston, MA, USA), the radiochromatograms were exposed to film for 5 days at −80°C. After alignment of the bands on chromatograms with iodine-stained authentic glycosylated sphingolipids, the radiolabeled lipids were scraped from the plate, 0.5 ml of water was added and the disintegrations per minute (dpm) were quantitated by liquid scintillation spectrometry.

2.5. Sphingolipid mass measurement

Cells supplemented with sterols were lipid extracted by the method of Folch et al. [18], the phospholipids were isolated by TLC [19] and the silica containing the SM was quantitated by measuring inorganic phosphorus content after charing [20]. SM content was expressed in micrograms per milligram of protein. Protein was assayed by the method of Bradford [21].

2.6. Enzyme activities

SM synthase was measured by the method of Nikolova-Karkashian [22] with modification using NBD-ceramide as a substrate. Cell monolayers were washed and scraped in PBS and homogenized in ice-cold lysis buffer by forcing them through a 27-gauge needle. The lysis buffer was made of 10 mM of HEPES (pH 7.4), 0.25 M of sucrose, 5 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride and 20 μg/ml each of chymostatin, leupeptin, antipain and pepstatin. The cell lysates were centrifuged at 1000×g for 10 min and the supernatants (50 μg protein) were incubated with the substrate (20 μM NBD-ceramide). The substrate was prepared using 60 μM of phosphatidylcholine (final concentration), which was previously sonicated for 5 min in 10 mM of HEPES (pH 7.4), 0.25 M of sucrose and 0.15 mM

of KCl. After incubation (60 min) at 37°C, the reaction was terminated with chloroform/methanol (1:1, vol/vol) and the lower phase, containing the NBD-SM formed, was dried under N_2 and separated by TLC using a solvent system made of chloroform/methanol/H_2O (80:35:5, vol/vol/vol). The NBD-SM spots were analyzed for relative fluorescence using a Fuji Image Analyzer LAS-1000plus (Stamford, CT, USA) with excitation at 455 nm and emission at 530 nm. Values were corrected for background using appropriate blanks.

Sphingomyelinase (SMase) activities (neutral and acidic) were measured according to the method described by Liu and Hannun [23] with some modifications. Briefly, cells were washed in ice-cold PBS and lysed in a buffer containing 50 mM of Tris–HCl, pH 7.4, 5 mM of EDTA, 2 mM of EGTA, 5 mM of β-glycerophosphate, 1 mM of sodium fluoride, 1 mM of sodium molybdate, 1 mM of DTT, 1 mM of phenylmethylsulfonyl fluoride, 1 μg/ml each of chymostatin, leupeptin, antipain and pepstatin A for measuring the neutral SMase or 50 mM of sodium acetate, pH 5.0 for measuring the acidic SMase. Lysates (25–50 μg protein) were incubated in an assay mixture that contained 100 mM of Tris–HCl, pH 7.4, 5 nmol of ^{14}C-SM (100,000 dpm) substrate, 5 nmol of phosphatidyl serine, 5 mM of DTT, 0.1% Triton X-100 and 5 mM of magnesium chloride in a total volume of 100 μl for the neutral SMase assay. The assay mixture for the acidic SMase contained 100 mM of sodium acetate, pH 5.0, 5 nmol of ^{14}C-SM (100,000 dpm) substrate and 0.1% Triton in 100 μl. The reaction was allowed to proceed at 37°C for 2 h and then stopped by adding 1.5 chloroform/methanol (2:1, vol/vol) and 0.2 ml of H_2O. Aliquots of the upper phase were assayed for radioactivity by liquid scintillation counting. Activity of the enzymes was expressed as nanomoles of substrate hydrolyzed per milligram of protein per hour.

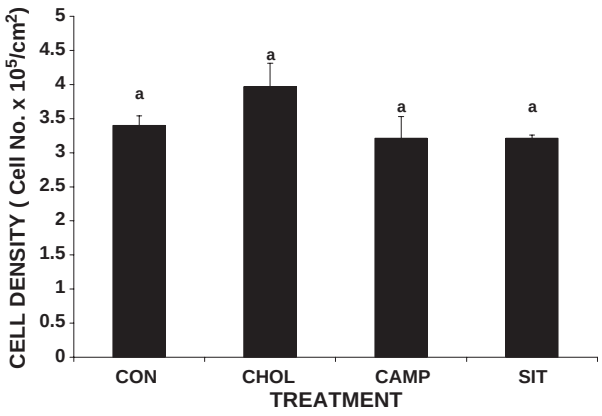

Fig. 1. Effect of sterols on differentiated $Caco_2$ cell growth. Cells were treated with either the vehicle (CON, control) or the sterols (CHOL, cholesterol; CAMP, campesterol; SIT, sitosterol) for 16 days postconfluence. Cell density is expressed as the mean of four experiments ± S.E.M. Bars with the same superscript letter indicate no significant difference (P > .05) in cell density after sterol treatment.

Table 1
Effect of sterol treatment on incorporation of ^{3}H-serine into $Caco_2$ cell sphingolipids*

Treatment	SM	Ceramide	Sphingosine	Total
Control	12,321±665^a	291±11^a	854±46^a	13,466±653^a
Cholesterol	7456±159^b	196±7^b	617±72^a	8266±119^b
Campesterol	8206±318^b	204±12^b	728±66^a	9136±374^b
β-Sitosterol	18,242±800^c	436±21^c	1531±163^b	20,210±769^c

* Values (dpm/10^6 cells) are expressed as the mean ± S.E.M. of four to six samples. Values in the same column with different superscript letters are significantly different (P < .05; Neuman–Keuls test).

The method of Nikolova-Karakashian and Merrill [24] was used to measure the ceramidase activity in cell lysates using NBD-hexanoic ceramide as a substrate. Sterol-treated cells were washed twice with ice-cold PBS, lysed in 0.2% Triton X-100 in 10 mM of Tris, pH 7.4, containing 10 μg/ml of leupeptin and aprotinin, 1 mM of sodium vanadate, 1 mM of 2-mercaptoethanol, 1 mM of EDTA and 15 mM of NaCl. Cell lysate (0.1 mg protein) and 10 μM (final) of NBD-substrate were added to a reaction buffer containing 0.2% Triton X-100 and 10 mM of Tris, pH 7.4 (neutral), or 0.5 M of acetate buffer, pH 4.5 (acidic), or 10 mM of HEPES, pH 9.0 (alkaline), in a total volume of 0.3 ml. After 1 h of incubation at 37°C, the reactions were stopped with chloroform/methanol (1:1, vol/vol) and samples were analyzed for NBD-hexanoate as previously described for NBD-SM in the SM synthetase assay. Blank values were subtracted from total fluorescence values to yield the relative NBD-hexanoate formed.

2.7. Statistical methods

Data are presented as means ± S.E.M. Data were analyzed using analysis of variance followed by the Student–Newman–Keuls post hoc test to test the significance (P < .05) between groups. The ProStat software package (Poly Software, Pearl River, NY, USA) was used for the analysis.

3. Results

3.1. Sterol effect on growth of differentiated $Caco_2$ cells

Supplementation of cells for 16 days prior to differentiation with the different sterols had no effect on cell density (Fig. 1).

Table 2
Effect of sterol treatment on $Caco_2$ SM content*

Treatment	SM (μg/mg protein)
Control	15.6±0.6^a
Cholesterol	16.0±1.2^a
Campesterol	16.1±0.7^a
β-Sitosterol	16.5±0.6^a

* $Caco_2$ cells were treated with the sterols for 16 days postconfluence or with the ethanol vehicle (control). Data are expressed as the mean of five samples ± S.E.M. Values with the same superscript letter are not significantly different (P > .05).

Table 3
Effect of sterol treatment on $Caco_2$ cell SM synthase activity*

Treatment	Arbitrary fluorescence (U/μg protein/30 min)
Control	14.6±0.6[a]
Cholesterol	17.0±1.1[a]
Campesterol	15.0±1.2[a]
β-Sitosterol	18.3±0.9[a]

* Activity is expressed as the arbitrary fluorescence units quantitated in NBD C_6-sphingomyelin/μg protein/30 min. Values (mean±S.E.M. of five samples) with the same superscript letter indicate nonsignificant difference ($P>.05$).

3.2. Effect of sterol supplementation on ^{3}H-serine incorporation into sphingolipids

To examine the effect of sterols on ceramide metabolism, sphingolipids were labeled using ^{3}H-serine in cells treated with sterols for 16 days postconfluence (Table 1). β-Sitosterol-supplemented cells had a 232% increase in serine incorporation into sphingolipids compared with cholesterol-treated and campesterol-treated cells. There was no difference in serine incorporation into ceramide-containing lipids between cholesterol-treated and campesterol-treated cells. The increase in individual lipids among the sterol-supplemented cells was proportionate.

3.3. Effect of sterol supplementation on SM concentration

Table 2 presents the data on the concentration of SM in cells supplemented with different sterols. These data suggest that the increase in ceramide production by β-sitosterol supplementation was not from SM degradation.

3.4. Effect of sterol supplementation on major enzymes involved in ceramide metabolism

The data presented in Tables 3 and 4 indicate that neither the SM synthase nor the neutral and acidic SMases were affected by sterol supplementation to $Caco_2$ cells. Table 5 presents data obtained from the activities of the three known ceramidases in $Caco_2$ cells supplemented with different sterols. Cells supplemented with campesterol had almost half the activities of those supplemented with either cholesterol or β-sitosterol. There was no significant difference in these enzyme activities in cells supplemented with cholesterol or sitosterol.

Table 4
Effect of sterol treatment on $Caco_2$ SMase activity*

Treatment	Neutral SMase (pH 7.4)	Acidic SMase (pH 5.0)
Control	12.0±0.5[a]	28.7±1.1[a]
Cholesterol	11.2±0.6[a]	28.7±1.6[a]
Campesterol	11.3±0.7[a]	25.8±1.3[a]
β-Sitosterol	11.7±0.8[a]	28.0±1.4[a]

* SMase is expressed as nmol phosphocholine/mg protein/h. Values (mean±S.E.M. of three samples) with the same superscript letter in a column are not significantly different ($P<.05$).

Table 5
Effect of sterol treatment on $Caco_2$ ceramidase activity*

Treatment	Ceramidase activity		
	Acidic	Neutral	Alkaline
Control	1.93±0.3[a]	2.61±0.5[a]	2.36±0.5[a]
Cholesterol	3.24±0.2[b,c]	4.93±0.6[b]	4.64±0.3[b]
Campesterol	0.83±0.3[d]	2.06±0.3[a]	2.57±0.8[a]
β-Sitosterol	2.75±0.1[c]	4.11±0.4[b]	4.37±0.4[b]

* Activity is expressed in arbitrary fluorescence units/μg protein/2 h as NBD-hexanoic acid (C_6) produced from NBD-C_6-ceramide substrate. $Caco_2$ cell lysates (100 μg) were incubated for 2 h at pH 4.5 (acid), pH 7.4 (neutral) or pH 9.5 (alkaline). All incubation buffers contained 0.2% Triton X-100. Values (mean of four to five samples±S.E.M.) with different superscript letters in a column are significantly different ($P<.05$).

3.5. Effect of sterol supplementation on glycosphingolipid synthesis in $Caco_2$ cells

Supplementation of β-sitosterol and campesterol was found to increase total glycosphingolipids by 20% compared with that of cholesterol (Fig. 2). These increases were detected in both cerebrosides and gangliosides without affecting lactosylceramide.

4. Discussion

The objective of the present project was to examine the effect of two common plant sterols, β-sitosterol and campesterol, on ceramide metabolism in human intestinal cells. Ceramide is considered as a second messenger and its increase is associated with inhibition of growth [8,25], cell proliferation [25] and stimulation of apoptosis [26,27] in several types of tumor cells. However, the effect of phytosterols on the growth of normal intestinal cells has not been investigated. In this study, we used differentiated $Caco_2$ cells, an accepted model for human intestinal mucosa [12,13]. These cells maintain all the characteristics of the different transport systems known in human intestinal cells [28].

The most important finding of this study is that cell supplementation with β-sitosterol, but not with campesterol, significantly increased the incorporation of labeled serine into ceramide, SM and sphingosine above the levels observed in cells supplemented with cholesterol. This suggests that β-sitosterol activated the de novo ceramide synthesis pathway as demonstrated by the lack of effect of β-sitosterol treatment on cellular SM concentration. In addition, the present study demonstrated no changes in the activities of SM synthase and SMases with sterol supplementation. The difference between the results of the present study and those of studies from this laboratory on tumor cells [12,13] is the source of intracellular ceramide. In HT-29 tumor cells, the source of ceramide was the degradation of cellular SM, which was reduced by 50% in cells supplemented with β-sitosterol [12]. This is in agreement with several published reports suggesting that the increase in ceramide was a result of activation of neutral SMase in

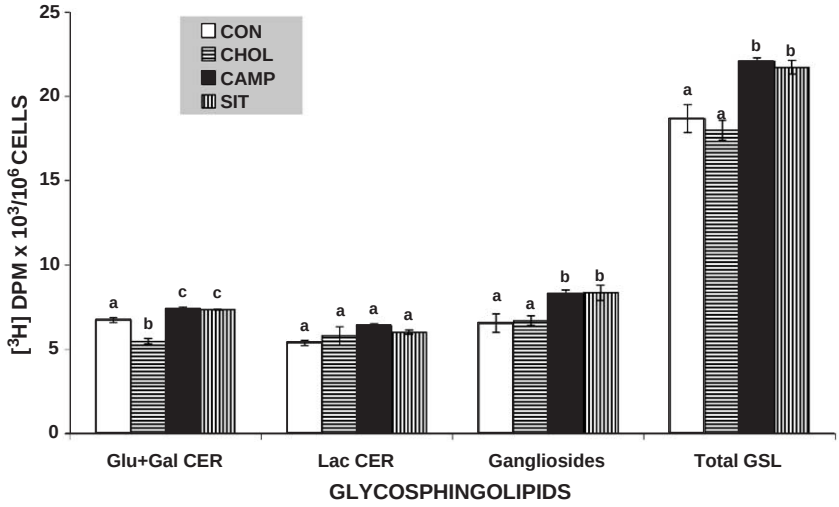

Fig. 2. Effect of sterol supplementation on ^{3}H-galactose incorporation into glycosphingolipids (GSLs) of differentiated $Caco_2$ cells. Cells were treated for 16 days postconfluence with either the vehicle (CON) or the sterols (CHOL; CAMP; SIT). Total GSL dpm=[Glu+Cal CER] (cerebrosides) dpm+Lac CER dpm+Gangliosides dpm. Bars (mean of three experiments±S.E.M.) with different letters are significantly different ($P<.05$) within each class of GSL.

several tumor cells [29,30]. Recently, O'Donnell et al. [31] found that fenretinide, a synthetic retinoid, induces an increase in de novo ceramide synthesis in tumor cells but minimally affects this pathway in nonmalignant lymphoid cells. Thus, it appears that the source of ceramide varies with the type of cells and the agonist used [32].

How β-sitosterol, but not campesterol, stimulates ceramide synthesis in $Caco_2$ cells is not clear from these studies. A possible explanation is that some enzymes of the ceramide synthesis pathway may be sensitive to alterations in membrane fluidity induced by incorporation of sterols with a structure different from that of the mammalian sterol (cholesterol). Leikin and Brenner [33] reported a decrease in the fluidity of hepatic membranes upon feeding rats a diet containing 3% β-sitosterol plus 2% campesterol for 21 days. This resulted in increases in the activities of delta-9, delta-6 and delta-5 fatty acid desaturases in these membranes. On the other hand, work from our laboratory reported decreases in the activities of hepatic 5α-reductase and aromatase in male rats fed a diet rich in phytosterols [34]. Thus, it appears that different membrane-bound enzymes may require specific degrees of membrane fluidity for their optimum function [35]. There are no studies available in the literature to compare the effect of different phytosterols on fluidity of membranes.

Ceramide can be also converted to sphingosine or glycoceramide. These compounds may regulate several physiological parameters of cells including apoptosis and cell growth [36,37] and the ABC transporters [38,39]. In the case of sphingosine, the present study indicates the inhibition of ceramidases by campesterol compared with β-sitosterol supplementation, which would theoretically allow an accumulation of ceramide in these cells. On the contrary, campesterol supplementation did not increase ceramide labeling when compared with β-sitosterol supplementation. The reason for the inhibition of ceramidases by campesterol supplementation is not clear from this study. It may be suggested that these membrane-bound enzymes may be sensitive to changes in membrane fluidity induced by incorporation of different sterols [39].

The increase in the synthesis of glycosphingolipids by β-sitosterol and campesterol supplementation in $Caco_2$ cells is of interest. Since these molecules are more soluble in aqueous solution than in ceramide, it has been suggested that they could provide a means by which the cells reduce the harmful effect of ceramide [40,41]. Accumulation of these glycolipid molecules inside the cell has been shown to reduce apoptosis and increase cell proliferation in several transformed cell lines [42,43]. In the present study, the sterol tested had no effect on cell density. Thus, it appears that differentiated $Caco_2$ cells may reduce the harmful effect of ceramide by glycosylation and efflux of ceramide lipids. $Caco_2$ cells actively synthesize lipoproteins, which have been shown to be the carrier for ceramide-containing lipids [44].

In conclusion, it has been suggested that phytosterol supplementation to differentiated $Caco_2$ cells may influence the activities of some enzymes involved in ceramide metabolism. The present study indicates that β-sitosterol at a physiological level (16 μM) stimulates the de novo synthesis of ceramide in differentiated $Caco_2$ cells. Both phytosterols studied, β-sitosterol and campesterol, stimulate the synthesis of cerebrosides and gangliosides.

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