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Effect of phytosterols on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells

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Abstract

Epidemiological studies suggest that dietary phytosterols may offer protection form some types of cancer including breast cancer. In an attempt to investigate the mechanism by which phytosterols offer this protection, we investigated the effect of the two most common dietary phytosterols, β-sitosterol and campesterol, on the mevalonate and MAP Kinase (MAPK) pathways in MDA-MB-231 cells. These pathways play a role in cell growth and apoptosis. MDA-MB-231 cell line was used in this study since it is a hormone-insensitive tumor cell line which represents the majority of advanced breast cancer cases. Cells grown in the presence of 16 μM β-sitosterol or campesterol for 3 days exhibited a 70% and 6% reduction in cell growth, respectively, while cholesterol treatment had no effect on growth as compared to the control. Studies investigating the effect of sterol supplementation on the relative and total sterol composition of cells, showed that cells supplemented with cholesterol contained 23% more cholesterol than the control. Cells supplemented with campesterol had almost one-half the cholesterol of controls but accumulated campesterol to account for 40% of the total sterols. In the case of cells supplemented with β-sitosterol, cells had only 25% of their sterols as cholesterol and the rest was in the form of β-sitosterol. All sterols tested equally inhibited de novo cholesterol synthesis using ^{14}C-acetate as substrate. β-Sitosterol supplemented cells had reduced cholesterol synthesis when using ^{3}H-mevalonolactone as substrate, which suggests that the inhibition in this pathway is downstream of mevalonate where processes such as isoprenylation of proteins may take place. Mevalonate supplementation to cells treated with β-sitosterol did not completely correct the observed growth inhibition by β-sitosterol. There was no effect of sterols on the concentrations of both low (21-26 kDa) or high (44-74 kDa) molecular weight isoprenylated proteins in these cells. On the other hand, both the quantity and activity of MAPK was elevated in the cells supplemented with β-sitosterol. These data suggest that the down regulation of cholesterol synthesis from mevalonate and stimulation of the MAPK pathway may play roles in the inhibition of MDA-MB-231 cell growth by β-sitosterol.

Keywords: Breast cancer; Cholesterol; Phytosterols; Signal transduction; Tumor growth

1. Introduction

Phytosterols or plant sterols (PS) are the counterparts of cholesterol in animals. They exist in the diet in many forms, but the two most abundant ones are β-sitosterol and campesterol [1,2]. Several *in vitro* and animal studies suggest that PS offer protection from the most common cancers in the developed countries including colon [3], prostate [4] and breast [5]. It has been shown that dietary consumption of PS is lower in developed countries (80 mg/day) as compared to Asian counties (400 mg/day), where the incidence and/or the death rate from these cancers is at a minimum [6,7].

The exact mechanism by which dietary PS offers this protection is not fully understood. However, some mechanisms have been offered. These include stimulation of apoptosis in breast and prostate cancer cells [7,8], reducing of metastasis in MDA-MB-231 cells implanted in SCID mice [9], inhibition of hormone metabolism in normal tissues [10] and stimulation of the sphingomyelin cycle in HT-29 and LNCaP cells [11].

In the present study, we report on the effect of PS on some aspects of cholesterol metabolism in breast cancer cells as another mechanism for the anticancer properties of PS. The cell line chosen for this study was the metastatic

Abbreviations: MAPK, mitogen activated protein kinase; PS, phytosterols; CD, 2-hydroxpropyl β-cyclodextrin; ERK, extracellular-regulated kinase

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hormone-insensitive MDA-MB-231 cells. The cells represent the majority of advanced breast cancer cases. Cholesterol is essential for the multiplication of all mammalian cells and expected to be in higher demand in fast growing cells such as tumor cells. Most cholesterol is supplied to the tumor usually by the host, however, tumors may also have the machinery to synthesize it. The mevalonate pathway plays an integral role in several signal transduction pathways that regulate cell proliferation and apoptosis such as the MAPK [12,13]. In addition, it has been documented that isoprenylation of certain proteins by byproducts of the mevalonate pathway may play an important role in cell division and apoptosis [14].

2. Materials and methods

2.1. Materials

MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium, Dulbecco's phosphate-buffered saline (PBS), β-sitosterol, campesterol, leupeptin, aprotinin, lovastatin, mevalonic acid lactone, sodium dodecyl sulfate (SDS), Tris-HCl and glycine were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) and antibiotic/antimycotic solution were obtained from GibcoBRL (Grand Island, NY). 2- Hydroxypropyl β-cyclodextrin was obtained from Cerestar USA, Inc. (Hammond, IN). The protein assay kits, molecular weight standards and Trans-blot transfer nitrocellulose membranes were obtained from BioRad Laboratories (Melville, NY). [^{14}C]-Sodium-2-acetate, [^{3}H]-R, S-mevalonolactone were obtained from American Radiolabelled Biochemicals, Inc. (St. Louis, MO). Lipid standards were obtained from Nu-Chek Prep, Inc. (Elysian, MN). Cytoscint ES* liquid scintillation cocktail and EcoLite liquid scintillation cocktail were obtained from ICN Biomedicals, Inc. (Irvine, CA). MAPK Immunoprecipitation kit (Cat. #17-184) and rabbit IgG were obtained from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]-Adenosine 5′-phosphate (3000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). ECL Plus Western Blotting Detection kit was obtained from Amersham Life Science Inc. (Arlington Heights, IL). X-ray Blue Film was obtained from Research Products International Corp. (Mt. Prospect, IL).

3. Methods

3.1. Cell culture

MDA-MB-231 cells, a hormone-insensitive metastatic breast cancer cell line, were grown as a monolayer culture at 37°C, 5% CO_2/95% air humidified atmosphere in RPMI 1640 supplemented with 2.0 g/L sodium bicarbonate, 5% FBS and 1% antibiotic/antimycotic (RPMI containing sodium bicarbonate, FBS and antibiotic/antimyotic is herein called complete medium). Cells were routinely seeded in 25 cm^2 vented tissue culture flasks, and left undisturbed for 2 days. Medium was changed every two days until 90-95% confluency was reached. Cells were counted using a Coulter Counter following trypsinization.

3.2. Effect of sterol supplementation on cell growth

MDA-MB-231 cells seeded at 5000 cells/cm^2 were supplemented with 16 μM β-sitosterol, campesterol or cholesterol. To solubilize these sterols, they were complexed with 2-hydroxypropyl-cyclodextrin (CD). Sterol supplemented medium was prepared by the method of Greenberg-Ofrath et al. [15] as modified by Awad et al. [16]. The sterol/CD complex was prepared in the ratio of 16 μM sterol: 5 mM CD. Cells were supplemented with complete media containing 16 μM sterol/5 mM CD or with only 5 mM CD (vehicle) control for 3, 4 or 5 days.

3.3. Incorporation of [^{14}C]-acetate or R, S-[5-^{3}H]-mevalonolactone into cholesterol

This assay was performed as previously described with slight modifications [17]. Briefly, cells were seeded in 6-well plates and treated with CD complete medium in the absence or presence of 16 μM cholesterol, campesterol or β-sitosterol for 3, 4 and 5 days. On the day of the assay, the media were removed from the cell monolayers and replaced with 4.0 μCi/ml Na-[2-^{14}C]-acetate (1 mM sodium acetate) or 15 μCi/ml R, S-[5-^{3}H]-mevalonolactone (60 Ci/mmol) and incubated at 37°C for 2 or 6 h, respectively. Media were removed from each well and the cells were scraped in two 1 ml aliquots of 2 M NaOH. This mixture was incubated at 37°C for 30 min and then 0.5 ml of methanol and 100 μg of non-radioactive cholesterol were added. This mixture was saponified for 1 h at 70°C. Non-saponifiable lipids were extracted 3 times with 1 ml aliquots of hexane. The pooled hexane extract was dried under N_2, reconstituted in chloroform and separated using silica gel G thin layer chromatography and a solvent system made up of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The cholesterol band was scraped from the plate and the radioactivity determined by liquid scintillation counting.

3.4. Incorporation of R, S-[5-^{3}H]-mevalonolactone into [^{3}H] isoprenylated proteins

Isoprenylation of proteins was performed as previously described [18]. Briefly, cells were seeded in 75 cm^2 flasks and treated for 3 days in CD complete medium in the absence or presence of 16 μM cholesterol, campesterol or β-sitosterol. Treated cells were then incubated with 15 μM lovastatin for 16 h to block cholesterol synthesis from endogenous mevalonate. Cells were incubated with 50 μCi/ml

[^{3}H]-RS-mevalonolactone for 18 h, after which they were harvested with trypsin, centrifuged at 3000 x g and washed twice with PBS. Cell pellets were suspended in 150 μl of sample preparation buffer for electrophoresis. Proteins (30-40 μg) were subjected to SDS-PAGE analysis (using 12% polyacrylamide gels). Gels were stained with Coomassie Blue and the bands corresponding to the 21-26 kDa and 44-74 kDa isoprenylated proteins were cut out while the rest of the lane was cut out into five equal fractions. To each fraction, 1 ml of 30% hydrogen peroxide was added and the gels were dissolved by heating at 80°C overnight as described above. Once the solutions cooled to room temperature, 5 ml of Cytoscint ES* was added and the mixture counted in a liquid scintillation counter.

3.5. Effect of sterol supplementation on total sterol content

Cells were grown in 75 cm^2 flasks for 3 days in CD complete medium in the absence or presence of 16 μM cholesterol, campesterol or β-sitosterol at 37°, 5%CO_2 humidified atmosphere. Cell monolayers were washed 3 times with PBS and harvested by scraping with a rubber policeman. Harvested cells were washed 3 times with PBS, the pellet resuspended in 0.5 ml of PBS and sonicated briefly. Aliquots were taken for protein and sterol content determinations. Sterols were analyzed by GLC as previously described with 5 α-cholestane as an internal standard [19].

4. MAPK assay

Cells were grown for 4 days in CD complete medium in the absence or presence of sterols. Cell monolayers were washed twice with PBS and the residual PBS was completely removed. Ice cold lysis Buffer A (500 μl) was added to each monolayer and cells were scraped. Buffer A was made up of 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 0.1% 2-mercaptoethanol, 0.1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin and 1 μM microcystin. Cell suspensions were transferred to prechilled microcentrifuge tubes and placed on an orbital shaker with a gentle rocking motion for 15 min at 4°C. The cell suspension was then snap frozen at -70°C and stored overnight at -80°C. When ready for use, cell lysates were quickly thawed and the mixture was centrifuged at 14,000 X g for 15 min. The cell lysate protein [20] content and supernatants containing the active MAPK were then immediately assayed for MAPK activity. The MAPK assay was performed using a MAP Kinase IP kit. Briefly, an immunocomplex between the protein A agarose beads coated with anti-ERK 1/2 antibody and sample ERK 1/2 was formed. After the immunocomplex coated agarose beads were pelleted and washed, sample MAP kinase activity was assessed by its ability to phosphorylate myelin basic protein using [γ-^{32}P]-ATP as the phosphate source. After a 20 min incubation at 20°C, ^{32}P-labeled myelin basic proteins were isolated on P81 paper and counted in liquid scintillation cocktail.

Samples treated with rabbit IgG-protein A agarose coated beads served as a non-specific control for MAP kinase activity. Sample MAP kinase activity was corrected with the non-specific controls.

4.1. Western blot of MAPK

Western blots were performed on whole cell lysates (20 μg protein/lane) as prepared above using a 12% resolving gel and transferred onto nitrocellulose membranes. After blocking with 3% non-fat milk for 30 min, the membrane was incubated with rabbit anti-ERK 1/2 antibody at 4°C overnight. After washing and incubation with the secondary goat anti-rabbit horseradish peroxidase antibody for 90 min at room temperature, the membrane was again washed. ERK 1/2 protein bands were detected using ECL and documented on x-ray film.

4.2. Statistical analysis

Values are expressed as the mean $\pm$ standard error of the mean. All data were analyzed using a one-way analysis of variance. The differences between the means were tested for significance using the Student's Newman-Keul post hoc test. The differences between means were considered statistically significant at a P value <0.05. The statistical software package used was ProStat (Jandel) and curves were made using Sigma Plot (SPSS Inc.).

5. Results

5.1. Sterol supplementation and the growth of tumor

Fig. 1 depicts the effect of sterol supplementation (16 μM) for 3 days on the growth of MDA-MB-231 cells. Phytosterol treatment significantly inhibited the growth of cells. However, the inhibitory effect was significantly more pronounced with β-sitosterol as compared to campesterol, accounting for a 70% and 6% decrease in growth, respectively. Cholesterol treatment did not affect the cell number relative to the vehicle (CD) controls.

5.2. Effects of sterol supplementation on sterol incorporation into MDA-NB-231 cells

In order to confirm that the effect observed with the phytosterols and cholesterol were due to the presence of the sterols inside the cell as opposed to only acting indirectly outside the cell, the sterol content of the cells was measured. β-sitosterol and campesterol supplementation accounted for 70% and 40% of the cell's total sterol content, respectively

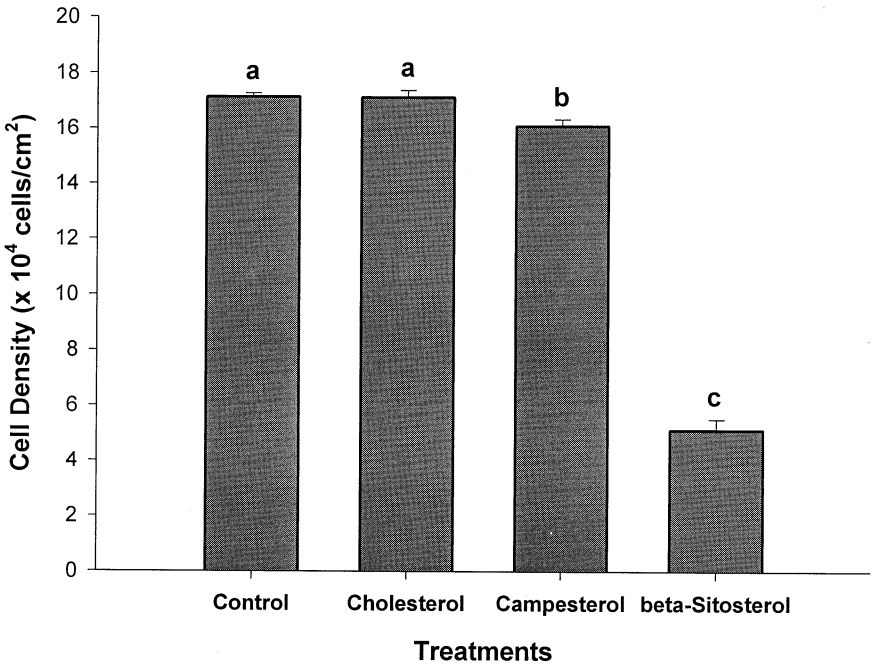

Fig. 1. Effect of 3 day sterol supplementation on growth of MDA-MB-231 cells *in vitro.* Cells (5000 cells/cm^2) were seeded in 6-well plates and after 24 h, cells were supplemented with 16 μM sterols for 3 days. Cell growth was quantitated by counting with a Coulter counter after harvesting by trypsinization. Values (mean ± S.E.M., n = 6) with different letters are significantly different ($P < 0.05$) using the Student's Newman-Keuls test. Experiments were performed 3 times and typical data are represented.

(Table 1). This supplementation resulted in a concomitant decrease in the cholesterol content of the cell. The total sterol content of the cells was also affected by sterol supplementation. While β-sitosterol supplementation did not change the total sterol content, campesterol supplementation significantly decreased it by 20%. As expected, cholesterol treatment resulted in an increase in both the cholesterol and total sterol content of the cells.

5.3. Effect of sterols on de novo cholesterol synthesis

Since it was observed that total cholesterol content was inhibited by phytosterol supplementation, the effect of the phytosterols on *de novo* cholesterol synthesis was investigated. To evaluate the effect on the cholesterol biosynthetic pathway, [^{14}C]-acetate and [^{3}H]-mevalonolactone incorporation into cholesterol was studied to determine whether the effect of the phytosterols occurred before or after the rate limiting step catalyzed by HMG-CoA reductase.

All the sterols tested resulted in a decreased incorporation of ^{14}C-acetate into cholesterol (Fig. 2). There was no difference among the sterols in their ability to inhibit the de novo synthesis of cholesterol from [^{14}C]-acetate. Similar to formation of [^{14}C]-cholesterol studies from acetate, all the sterols inhibited the synthesis of cholesterol from [^{3}H]-mevalonolactone (Fig. 3). However, β-sitosterol treatment resulted in a significantly lower incorporation of [^{3}H]-mevalonolactone into cholesterol when compared with the other two sterol-treatment groups.

5.4. Effect of sterol supplementation in the absence and presence of exogenous mevalonate on cell growth

To determine whether mevalonate supplementation could reverse the inhibitory effect of β-sitosterol treatment on cell growth, thereby suggesting that regulation of the rate-limiting step is one mechanism used by phytosterols to exert their effects, the effect of exogenous mevalonate supplementation on the growth of sterol treated cells was investigated (Fig. 4). When 500 μM mevalonate was supplied together with β-sitosterol to the cells, no change in cell growth was observed. However, increasing the mevalonate concentration to 1 mM resulted in only a modest increase in cell growth, but failed to reach the level of the control. In contrast, cholesterol supplemented cells responded to added mevalonate with an increased cell growth that was almost

Table 1
Cellular sterol content of MDA-MB-231 cells as influenced by 16 μM sterol supplemented*

Sterol Treatments	μg sterol/mg protein			
	Cholesterol	Campesterol	β-Sitosterol	Total
Control**	24.5 ± 0.9^{a}	0	0	24.5 ± 0.9^{a}
Cholesterol	30.2 ± 0.8^{b}	0	0	30.2 ± 0.8^{b}
Campesterol	11.9 ± 0.6^{c}	7.8 ± 0.4	0	19.7 ± 1.0^{c}
β-Sitosterol	6.4 ± 0.6^{d}	0	17.3 ± 0.7	24.7 ± 1.3^{a}

* Values (mean ± S.E.M., n = 3) with different letters in a column are significantly different ($P < 0.05$) using the Student's Newman-Keuls test.
**Controls received the vehicle alone.

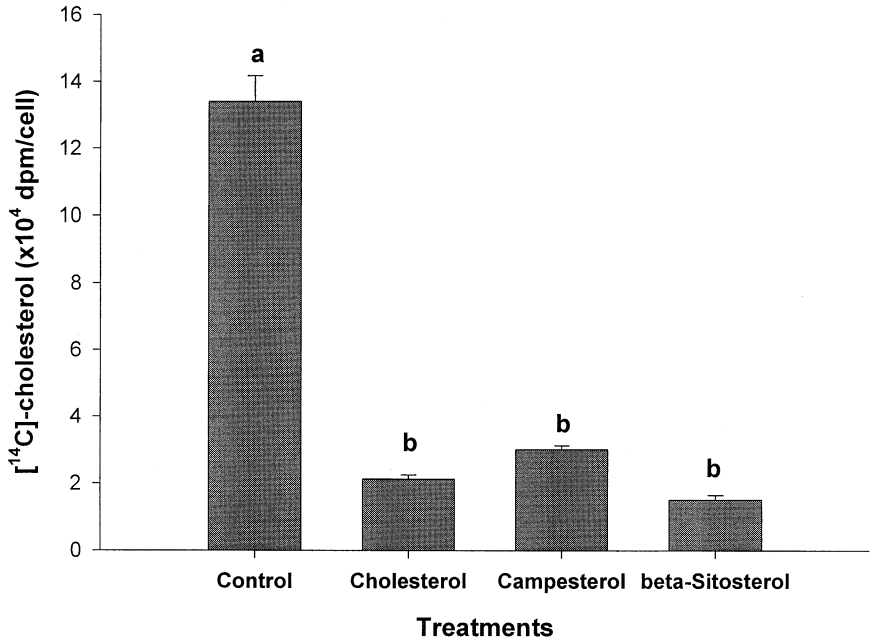

Fig. 2. Effect of 3 day sterol supplementation on the incorporation of [^{14}C]-acetate into cholesterol. Pretreated cells with 16 μM sterols were incubated with [^{14}C]-acetate (4 μCi/ml) for 2 h at 37°C. Cells were harvested by scraping and lipids were extracted and saponified as described in the Methods. Non-saponifiable lipids were detected by thin layer chromatography and radioactivity (band scrapings) was quantitated by liquid scintillation counting. Values (mean ± S.E.M., n = 3) with different letters are significantly different ($P < 0.05$) using the Student's Newman-Keuls test. Experiments were performed 3 times and typical data are represented.

2-fold higher than the growth with sterol supplementation alone, thereby matching the control growth. Mevalonate supplementation did not increase cell growth of campesterol treated cells.

5.5. Effect of sterol treatments on protein isoprenylation

Another possible step in the cholesterol biosynthetic pathway, where the effects of cell growth and metastasis

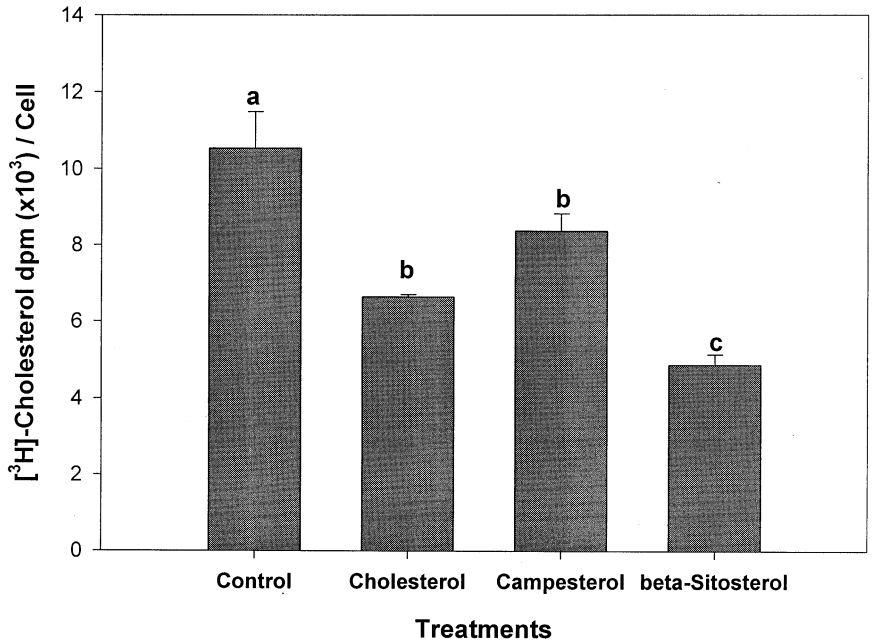

Fig. 3. Effect of 3 day sterol supplementation on the incorporation of [^{3}H]-mevalonolactone into cholesterol. Pretreated cells with 16 μM sterols were incubated with [^{3}H]-mevalonolactone (15 μCi/ml) for 6 h at 37°C. Cells were harvested by scraping and lipids were extracted and saponified as described in the Methods. Non-saponifiable lipids were detected by thin layer chromatography and radioactivity (band scrapings) was quantitated by liquid scintillation counting. Values (mean ± S.E.M., n = 3) with different letters are significantly different ($P < 0.05$) using the Student's Newman-Keuls test. Experiments were performed 3 times and typical data are represented.

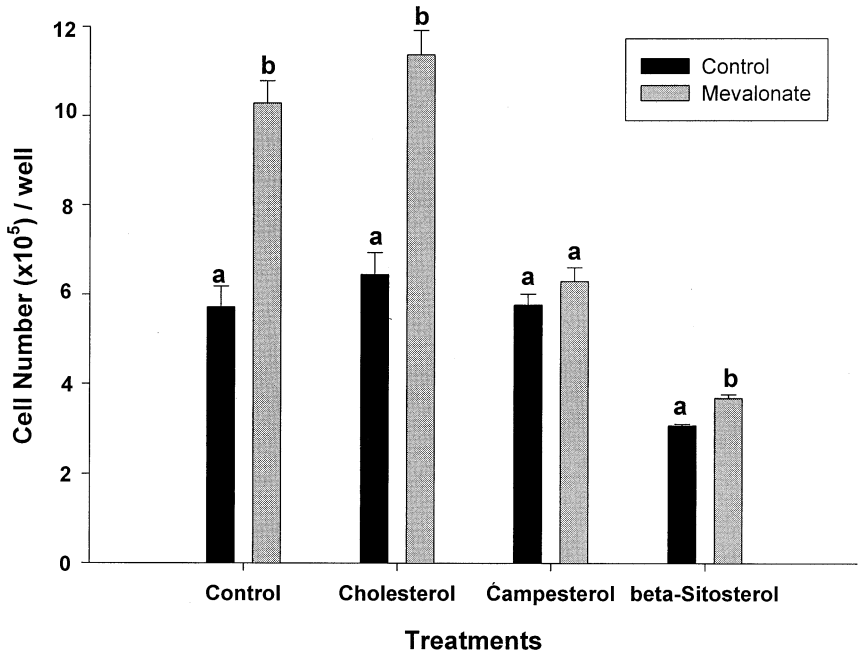

Fig. 4. Comparison of the effect of 3 day sterol supplementation in the absence and presence of exogenous mevalonate on growth of MDA-MB-231 cells. Cells (5000 cells/cm^2) were seeded in 12-well plates and treated with 16 μM sterols in the absence or presence of 1 mM mevalonate. Cell growth was determined by counting with a Coulter counter. Values (mean $\pm$ S.E.M., n = 4) with different letters within each group are significantly different ($P < 0.05$) using the Student's Newman-Keuls test. Experiments were performed 3 times and typical data are represented.

may be controlled, is at the level of protein isoprenylation. Therefore, the effect of sterol supplementation on isoprenylation of low and high molecular weight proteins was evaluated (Fig. 5). There was no effect of sterol treatments on the prenylation of either the low or high molecular weight proteins.

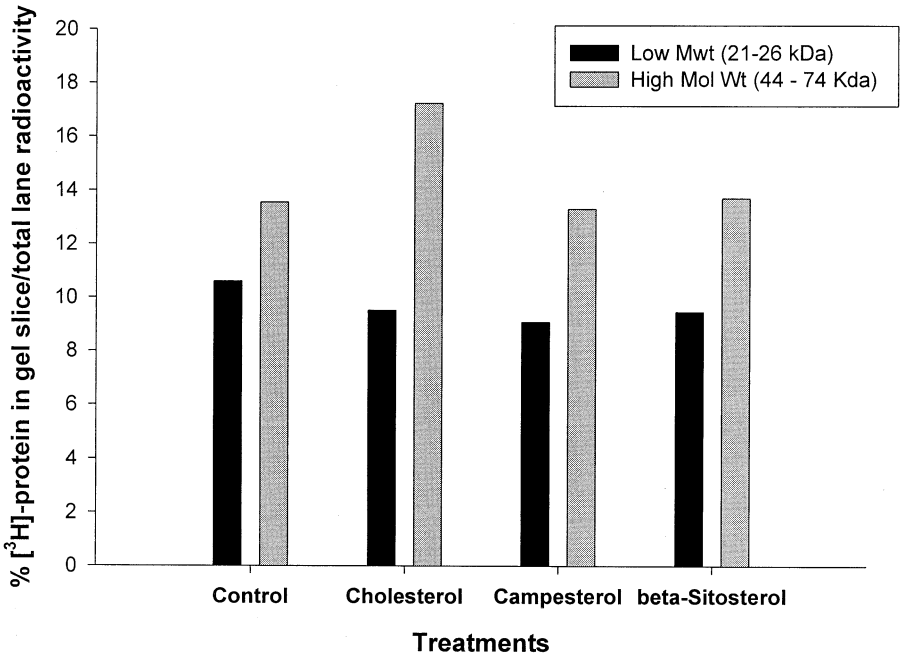

Fig. 5. Effect of 3 day sterol supplementation on isoprenylation of low and high molecular weight proteins. Cells were treated for 3 days with 16 μM sterols, then incubated with 15 μM lovastatin for 16 h. The lovastatin mixture was removed from the monolayer and cells were then incubated with [^{3}H]-mevalonolactone (50 μCi/ml) for 24 h at 37°C. Cells were harvested by scraping and dissolved in SDS sample buffer. Protein (30 μg) was loaded on SDS-PAGE. Protein bands corresponding to 21-26 kDa and 44-74 kDa proteins were cut out from the gel and digested overnight with 30% H_2O_2 at 70°C. Values (average of two gels) are expressed as % [^{3}H]-protein in each gel slice/total radioactivity in each lane.

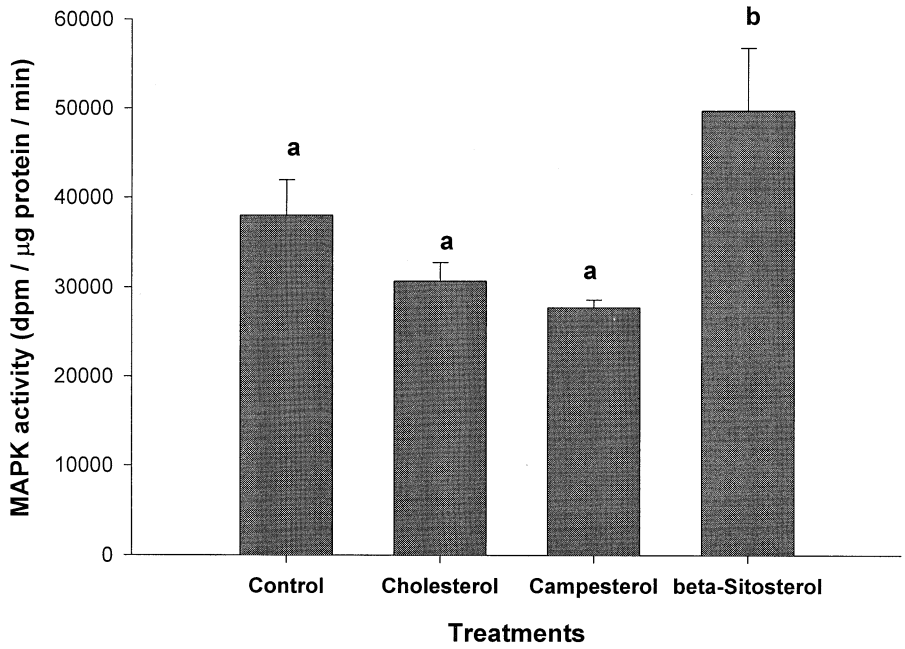

Fig. 6. Effect of sterol supplementation on MAPK activity of MDA-MB-231 cells. Cell lysates (100 μg protein) from sterol treated cells were analyzed for MAPK activity as described in the methods. Values (mean ± SEM, n = 4) with different letters (a, b) are significantly different ($P < 0.05$) by the Newman-Keul test.

5.6. Effect of sterol treatments on MAP kinase

Since no direct sterol effect on protein isoprenylation was observed, one of their target signal transduction pathways, the MAPK pathway, which is involved in cell growth was investigated. The effect of sterol supplementation on both the activity and the amount of MAPK enzyme was determined (Figs. 6 and 7). When the activity of the enzyme was investigated, there was a concomitant 62% increase with β-sitosterol treatment when compared with cholesterol treatment (Fig. 6). No significant difference was observed among the other treatments. Among the sterols tested, only β-sitosterol was found to increase the concentration of p42/p44 MAP kinase protein (the arbitrary units based on densitometry are 0.98, 0.99, 1.00, and 1.54 for control, cholesterol, campesterol and β-sitosterol treatments, respectively) (Fig. 7).

6. Discussion

Dietary factors are thought to contribute to as much as one-third of the factors influencing the development of cancer [21]. There is now considerable evidence from *in vitro* and animal studies to suggest a protective role of plant based diets on tumorigenesis [1,3–5,22]. One of the components of a plant-based diet with anticancer effects is the dietary phytosterols [1]. It was previously shown in this lab that a common dietary phytosterol, β-sitosterol, could inhibit the growth of colon, prostate and breast cancer cell lines in culture [3–5]. More recently, dietary phytosterols were shown to inhibit both the growth of MDA-MB-231 cells and their metastasis *in vivo* and *in vitro* [9]. However, the exact mechanism by which phytosterols offer this protection is not completely understood. In the present studies, we offer an additional mechanism to those previously proposed [9–11].

Studies using cholesterol-lowering drugs, such as lovastatin, have implicated cholesterol and intermediates of the mevalonate pathway in promoting the growth and metastasis of tumor cells in animals [23,24]. Cholesterol is essential for cell viability and growth [25]. It is a critical component of the cell membranes where it serves several functions including regulation of the membrane fluidity and activity of membrane bound proteins such as integrins, membrane

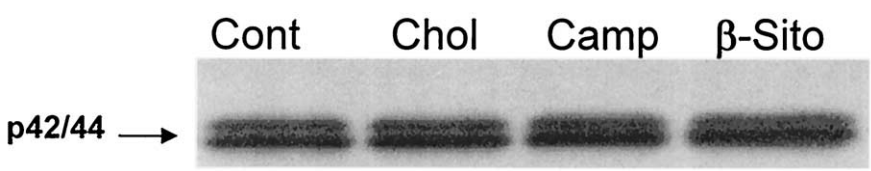

Fig. 7. Western blot of MAPK expression in MDA-MB-231 supplemented with 16 μM sterols. Cell lysates (20 μg protein) from sterol treated cells (control, cont; cholesterol, chol; campesterol, camp and β-sitosterol, β-sito) were analyzed by SDS-PAGE. Protein bands were transferred to a nitrocellulose membrane and p42/44 MAPK was detected by chemiluminescence as described in the Methods.

bound enzymes, and several signal transduction pathways. It has been recently shown that cholesterol is also required for cell cycle progression from G2 to M phase [12]. In addition, these drugs inhibit the mevalonate pathway at two points, the rate limiting HMGCoA reductase step and the isoprenylation of members of the Ras superfamily of proteins [26]. The integrity of these steps is essential for tumor growth [26]. Moreover, isoprenylation is critical for several signal transduction pathways that are involved in the metastatic process [27]. One of the important pathways, the Ras/Raf-1/MEK1/2/MAPK1/2 cascade, occupies a central point in the integration of many extracellular and intracellular signals [28]. This pathway has been implicated in the promotion of cell proliferation, and the expression of genes that promotes the metastatic cascade and moreover, its role as a negative modulator of the cell cycle is now being appreciated [28–31].

Results from this study indicate that phytosterols are effective in lowering the cholesterol content of the MDA-MB-231 cells. This decrease in cholesterol content may have been due to a decrease in the *de novo* synthesis of cholesterol from acetate or mevalonate. Inhibition of mevalonate synthesis has been shown to be a potent anticancer mechanism. Previous studies indicated that β-sitosterol can inhibit the activity of HMGCoA reductase [32]. However, this was achieved using micelles containing 200 μM β-sitosterol in a CaCo-2 cell model. Even though the expression and activity of HMGCoA reductase was not measured in this study, the inhibition of cholesterol synthesis from [^{14}C]-acetate coupled with the failure to reverse this inhibition with mevalonate suggests that HMGCoA reductase is not being regulated at the concentration of β-sitosterol used in this study. However, further studies are needed to support this conclusion.

Interestingly, in the MDA-MB-231 cell line, cholesterol also caused a decrease in *de novo* cholesterol synthesis suggesting that the cholesterol feedback mechanism is intact in these cells as it is in other tumor cells [33]. While both phytosterols inhibited cholesterol synthesis, it appears that there is a minimal cholesterol level, that is required for cells to maintain their cellular functions, as campesterol was found to have very little effect on growth. On the other hand, dramatic cholesterol reduction, as observed with β-sitosterol treatment, is consistent with growth inhibition and a reduced metastatic potential of BN472 monomuclear cells [34].

Experiments with HMGCoA reductase inhibitors, such as lovastatin, indicate that mevalonate is crucial for mouse mammary tumor growth [23]. When tumor cells were treated with lovastatin, their growth was impaired. However, this effect was reversed when cells were co-supplemented with mevalonate at 20 times the concentration of lovastatin. Results from this study found that the inhibition of cell growth with 16 μM β-sitosterol was only reversed at exogenous mevalonate at concentrations exceeding 1 mM, albeit modestly. Additionally, even 10 mM mevalonate did not completely reverse the growth inhibitory effect of β-sitosterol when compared with the controls (data are not shown). Taken together, these results suggest that β-sitosterol is a potent inhibitor of cholesterol synthesis and, hence, total cholesterol content. This inhibition is likely to be downstream from the HMGCoA reductase rate limiting step.

The use of farnesyl pyrophosphate transferase and geranylgeranyl pyrophosphate transferase inhibitors have implicated a role for these transferases in the promotion of colon tumor and melanoma growth [35,36]. In the present study, sterol supplementation did not appear to affect the action of these enzymes. However, this did not preclude a modulatory role of the sterols on down stream signaling cascades such as the MAPK pathway. Here, it was found that while campesterol and cholesterol did not affect the concentration and activity of p42/44 MAPK, β-sitosterol treatment resulted in an increased concentration and activity of the enzyme. While, this observation seems paradoxical in light of the role of this cascade in the promotion of tumor growth, a role for increased activation of MAPK in growth arrest is not unprecedented [28,30,31]. In support of our present observations, Ahn and Schroeder [37] recently reported that other sphingolipids such as sphinganine, which also inhibits cell proliferation and induces apoptosis, increases the phosphorylation of ERK 1/2, JNK 1/2 and p 38 nitrogen activated protein kinases in HT-29 cells [37].

We have shown recently that β-sitosterol dramatically decreases the growth of MDA-MB-231 cells by inducing G2/M arrest [38]. The cyclin B/p34cdc2 complex or M-phase promoting factor (MPF) plays a critical role in the G2/M transition. Its activation is required for entry into the M phase and its inactivation is required for exit out of the M-phase [39]. While cholesterol starvation has been shown to reduce the activity of the P34cdc2 kinase, sustained activation of p42 MAPK has been shown to maintain high levels of MPF activity and to stabilize cyclin B thereby preventing the entry of the cell into M phase and preventing the exit out of M phase, respectively. The relative importance of these mechanisms is not known, but they both result in the arrest of cells at G2/M. p 42/44 MAPK belongs to a group of MAPK signaling pathway components that include SAPK and p 38. The latter two are regulated by extra cellular stressors [39]. It is interesting to note that activated MEK1, a precursor of p42/44 MAPK, can stimulate the SAPK pathway and induce cell apoptosis [39]. The elevation of MAPK activity in cells treated with β-sitosterol in the present study supports previous findings from this laboratory which demonstrated that β-sitosterol induces apoptosis in this cell line [5].

In summary, this study demonstrates that the main phytosterol with antitumor effects is β-sitosterol. One of the processes by which β-sitosterol mediates decreased growth appears to be related to its ability to decrease the total cholesterol content of the cells, possibly by decreased de novo synthesis, and to increased activation of MAPK (ERK

1/2). Taken together, results from this study, as well as others [9], suggest that β-sitosterol may play a role in the prevention of metastatic breast cancer. Enrichment of the American diet with β-sitosterol can be easily achieved by the inclusion of rich sources such as plant oils, nuts and legumes such as peanuts [40].

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