

## $\beta$ -Sitosterol activates Fas signaling in human breast cancer cells

A.B. Awad<sup>a,\*</sup>, M. Chinnam<sup>a</sup>, C.S. Fink<sup>a</sup>, P.G. Bradford<sup>b</sup>

<sup>a</sup>Department of Exercise and Nutrition Sciences and

<sup>b</sup>Department of Pharmacology and Toxicology, State University of New York at Buffalo, Buffalo, NY 14214, USA

### Abstract

$\beta$ -Sitosterol is the most abundant phytosterol. Phytosterols are enriched in legumes, oil seeds and unrefined plant oils as found in foods such as peanut butter, pistachios and sunflower seeds.  $\beta$ -Sitosterol inhibits the growth of several specific types of tumor cells in vitro and decreases the size and the extent of tumor metastases in vivo. The effects of  $\beta$ -sitosterol on the extrinsic apoptotic programmed cell death pathway in human breast MCF-7 and MDA-MB-231 adenocarcinoma cells were examined, along with the extent of its incorporation into cellular membranes and its effects on cell growth, expression of Fas receptor pathway proteins, and caspase-8 activity. The results show that  $\beta$ -sitosterol exposure promotes its enrichment in transformed cell membranes and significantly inhibits tumor cell growth. Concurrently, Fas levels and caspase-8 activity are significantly increased. These actions are specific, as expression of other proteins of the Fas receptor pathway, including Fas ligand, FADD, p-FADD and caspase-8, remain unchanged. These findings support the hypothesis that  $\beta$ -sitosterol is an effective apoptosis-promoting agent and that incorporation of more phytosterols in the diet may serve a preventive measure for breast cancer.

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**Keywords:** Phytosterols; Breast cancer; Extrinsic pathway; Apoptosis; Signal transduction

### Introduction

According to the World Health Organization, more than 1.2 million people worldwide will be diagnosed with breast cancer this year. However, the occurrence of breast cancer varies widely among women from different countries and cultures, with higher incidences in European and North American women as compared to women in less developed countries and countries relying more heavily on vegetarian diets (American Cancer Society, 2003). Dietary factors, specifically the proportion of animal versus plant fats, may play a role in the development of breast cancer (Messina and

Barnes, 1991; Cho et al., 2003). Plant foodstuffs contain specific phytochemicals which may offer protection from breast cancer. Controlled dietary studies with animals suggest that one class of phytochemicals, the phytosterols, may offer protection from breast cancer as well as protection from other cancers common to Western societies, including cancers of the colon and prostate (Awad et al., 2001, 2004; Awad and Fink, 2000). Phytosterols are cholesterol-like chemicals made exclusively by plants. An attractive hypothesis which may account for the cancer protective action of phytosterols is that phytosterols induce apoptosis or programmed cell death in highly proliferative tumor cells.

Apoptosis occurs via two main pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor-mediated pathway. The intrinsic pathway

\*Corresponding author. Tel.: +1 716 829 3680x231; fax: +1 716 829 3700.

E-mail address: [awad@buffalo.edu](mailto:awad@buffalo.edu) (A.B. Awad).

induces apoptosis in response to internal signals. In this pathway, internal damage to the cell is sensed by the mitochondria and causes the release of apoptotic protease-activating factor-1 (Apaf-1), cytochrome *c*, and other pro-apoptotic factors from the intermitochondrial membrane space (Bunz, 2001; Zörnig et al., 2001; Saelens et al., 2004). These factors ultimately activate the effector protease, caspase-3, driving programmed cell death (Dragovich et al., 1998; Fesik, 2005). The extrinsic pathway of apoptosis is triggered by external signals that activate cell surface death receptors, including Fas. Upon binding their cognate ligands, the intracellular domains of death receptors recruit adapter molecules such as Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD) (Daniel et al., 2001), which in turn recruit procaspase-8, an initiator caspase. Activated caspase-8 drives downstream effector caspases, including caspase-3, ultimately culminating in cell death. Resultant apoptotic bodies are removed by phagocytosis.

The objective of this study was to assess the effect of cellular supplementation with either  $\beta$ -sitosterol or cholesterol on the extrinsic caspase-8 pathway in the two breast cancer cell lines, MCF-7 and MDA-MB-231. It is hypothesized that  $\beta$ -sitosterol may potentiate Fas-death domain signaling, leading to caspase-8 activation and ultimately to apoptosis. To test this hypothesis, the breast cancer cell lines were treated with  $\beta$ -sitosterol or with cholesterol as a control and effects on cell growth, sterol incorporation in cell membranes, expression of Fas-death domain signaling proteins, and caspase-8 activity were determined.

## Materials and methods

### Materials

Breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). RPMI-1640 media, trypsin-EDTA, antibiotic–antimycotic and fetal bovine serum were obtained from Gibco™ Invitrogen Corporation (Grand Island, NY, USA). Cholesterol and  $\beta$ -sitosterol were purchased from Sigma Chemical Company (St. Louis, MO, USA). Protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany). Ready gels (12% Tris–HCl, 50  $\mu$ l, 10 wells), prestained SDS-PAGE standards and Bio-Rad DC protein assay kit were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Ac-IETD-AFC (caspase-8 substrate) and Ac-IETD-CHO (caspase-8 inhibitor) were obtained from BIOMOL® Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Cyclodextrin (2-hydroxypropyl beta-cyclodextrin; CD) was obtained from Cerestar USA, Inc. (Hammond, IN,

USA). Antibodies, chemiluminescence luminol reagent and Cruz Marker™ molecular weight standards were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### Cell culture

MCF-7 and MDA-MB-231 cells, representing estrogen-dependent and estrogen-independent stages of human breast carcinoma, were cultured at 37 °C in 5% CO<sub>2</sub>/95% air as monolayers using RPMI 1640 growth medium supplemented with 2 g/l sodium bicarbonate, 5% fetal bovine serum and 1% antibiotic–antimycotic.

### Preparation of sterol supplemented media and measurement of cell growth

RPMI 1640 media supplemented with cholesterol or  $\beta$ -sitosterol as CD complexes were prepared as described (4) and added to growth medium to achieve a ratio of 8–16  $\mu$ M sterols: 5 mM CD. Control groups were treated with 5 mM CD vehicle. For growth studies, cells were seeded at 5000 cells/cm<sup>2</sup> into 24-well plates and incubated for 24 h. On day 1, media were replaced with that containing  $\beta$ -sitosterol or cholesterol in graded concentrations or CD vehicle. Media were similarly changed on days 3 and 5. Cells were trypsinized and counted on days 2, 4 and 6 by Coulter Counter using the electrical sensing zone method. Growth curves were generated from the Coulter Counter data.

### Measurement of sterol content of cell membranes by GLC

Cells were seeded into 6-well plates, treated with sterols or vehicle for 2 d, and then harvested by scraping. Cells were suspended, washed 3 times and then frozen –80 °C in 350  $\mu$ l 10 mM Tris–20 mM mannitol buffer (pH 7.4) until further processing. Samples were thawed and briefly sonicated on ice. An aliquot of each sample was used for protein analysis. The samples were then saponified at 80 °C in 95% ethanolic KOH in the presence of 5 $\alpha$ -cholestane as an internal GLC standard. After the addition of 2 ml of water and 2 ml of hexane the upper organic phase containing the non-saponifiable lipids (sterols) was removed and dried under nitrogen. The samples were reconstituted with carbon disulfide and injected into a gas–liquid chromatograph (Shimadzu, model GC-17A) fitted with a 30 m DB-5M5, ID 0.25 mm column (JRW Scientific, Folsom, CA, USA). The column temperature was set at 265 °C and that of injection port and detector at 290 °C. Sterol contents were normalized to the internal standard and expressed per mg protein.

## Determination of caspase-8 activity

Cells were seeded in T-75 flasks at a density of 10,000 cells/cm<sup>2</sup> and allowed to attach for 24 h at which time the media were replaced with sterol-supplemented or control media. After 3 d treatment, cells were scraped, washed in PBS (pH 7.4), and lysed by suspension on ice of 10<sup>7</sup> cells/200  $\mu$ l in buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.15 CHAPS, 0.1% Triton X-100 and 5 mM DTT. After 30 min, the lysates were aspirated several times through a syringe fitted with 26-gauge needle and then centrifuged at 10,000g for 30 min at 4 °C. The protein concentrations of the supernatants (cell lysates) were analyzed by the Bio-Rad DC protein assay modified for thiol containing samples.

To quantify caspase-8 activity, cell lysates (100  $\mu$ g protein) were assayed with the specific caspase-8 substrate, Ac-IETD-AFC, in the presence and absence of the specific caspase-8 inhibitor, Ac-IETD-CHO. Samples were incubated with 50  $\mu$ M Ac-IETD-CHO for 30 min at 37 °C. This inhibitor blank was used to correct each sample for its own respective caspase-8 activity. Ac-IETD-AFC was then added to 50  $\mu$ M and the samples incubated at 37 °C. Relative fluorescence was monitored over time (1, 2, 3, 4, 19, and 20 h) at an excitation wavelength of 360 nm and an emission wavelength of 530 nm using a fluorescence multi-well plate reader (CytoFluor<sup>TM</sup> II, PerSeptive Biosystems). Fluorescence values obtained for samples treated with the specific caspase-8 inhibitor were subtracted from those that did not receive inhibitor.

## Western immunoblot analysis

Western immunoblot analyses for Fas, FasL, FADD, p-FADD, caspase 8 and  $\beta$ -actin were performed on cell lysates (50  $\mu$ g protein) resolved by SDS-PAGE (12% polyacrylamide gels) and electroblotted onto PVDF membranes. After blocking with 5% nonfat dried milk and 0.05% Tween20 Tris-buffered saline (TBS-T), blots were incubated for 1 h with primary antibody, washed 3 times with TBS-T and incubated with the secondary antibody. Blots were treated with luminol reagent for 1 min and exposed to X-ray film. Intensities were analyzed by densitometry (Bio-Rad Imaging Densitometer, Model GS-700) and quantified using Quantity One (Version 4.2.3) software. Blots were stripped and re-probed for  $\beta$ -actin reactivity. Experimental values were normalized to  $\beta$ -actin reactivity.

## Statistical analyses

All experiments were performed in triplicate. Values are expressed as means and standard errors of the

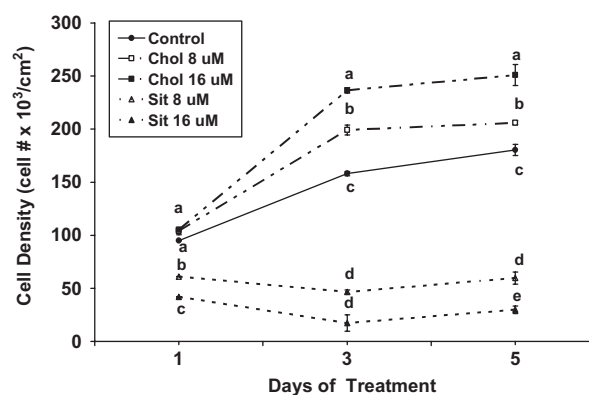
means. Data were analyzed using one-way analysis of variance (ANOVA). Differences between mean values of the treatment groups were tested for significance ( $p < 0.05$ ) using the Student's Newman–Keuls post hoc test. The statistical software package used was ProStat (Poly Software Int., Pearl River, NY, USA).

## Results

### $\beta$ -Sitosterol supplementation inhibits MCF-7 and MDA-MB-231 cell growth

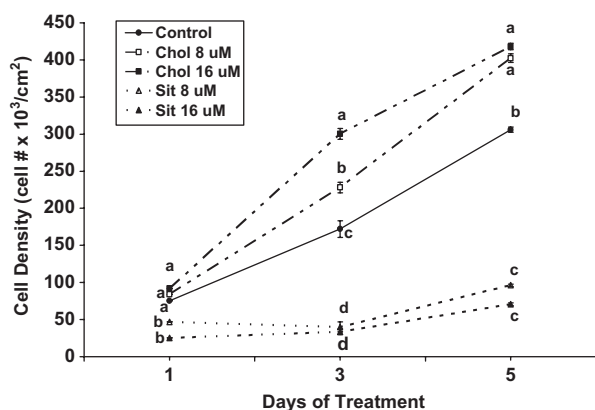
The effects of supplementation with cholesterol or  $\beta$ -sitosterol on cell growth were studied with the breast cancer cell lines MCF-7 and MDA-MB-231. For MCF-7 cells, cholesterol supplementation (8 and 16  $\mu$ M) resulted in significantly increased cell growth when compared to vehicle control after 3 and 5 d supplementation (Fig. 1). There was a dose-dependency of the effect, with 8 and 16  $\mu$ M cholesterol significantly increasing cell growth by 29% and 60%, respectively. In contrast, supplementation with 8 or 16  $\mu$ M  $\beta$ -sitosterol for 1, 3, and 5 d resulted in significantly decreased cell growth when compared to vehicle control. At 5 d, the effect of 16  $\mu$ M  $\beta$ -sitosterol (81% decrease) was greater than that of 8  $\mu$ M  $\beta$ -sitosterol (63% decrease) after 5 d supplementation.

The effect of sterol supplementation on the cell growth of MDA-MB-231 cells was also examined and



**Fig. 1.** Effect of sterol supplementation on growth of MCF-7 cells. Cells were grown in RPMI-1640 growth medium supplemented with different concentrations of sterols: 8 or 16  $\mu$ M cholesterol; 8 or 16  $\mu$ M  $\beta$ -sitosterol. Control group received only 5 mM of the cyclodextrin vehicle. Cell growth, as assessed by counter coulter, was studied up to 5 d sterol supplementation. Values (mean  $\pm$  SEM,  $n = 3$ ) with different letters (a–e) in one time point are significantly different ( $p < 0.05$ ) [Control: 5 mM cyclodextrin (CD) vehicle; Chol 8  $\mu$ M: 8  $\mu$ M cholesterol in CD; Chol 16  $\mu$ M: 16  $\mu$ M cholesterol in CD; Sit 8  $\mu$ M: 8  $\mu$ M  $\beta$ -sitosterol in CD; Sit 16  $\mu$ M: 16  $\mu$ M  $\beta$ -sitosterol in CD].

observed to be similar to the effect on MCF-7 cells (Fig. 2). Cholesterol supplementation at 8 and 16  $\mu\text{M}$  for 5 d resulted in significantly increased cell growth compared to vehicle control. After 3 d, the effect of 16  $\mu\text{M}$  cholesterol was greater than that of 8  $\mu\text{M}$  cholesterol; however, there was no difference in cell growth between the two cholesterol concentrations after 5 d sterol supplementation. Like the results seen with MCF-7 cells, supplementation of MDA-MB-231 cells



**Fig. 2.** Effect of sterol supplementation on growth of MDA-MB-231 cells. Cells were grown in RPMI-1640 growth medium supplemented with different concentrations of sterols: 8 or 16  $\mu\text{M}$  cholesterol; 8 or 16  $\mu\text{M}$   $\beta$ -sitosterol. Control group received only 5 mM of cyclodextrin vehicle. Cell growth, as assessed by counter coulter, was studied up to 5 d sterol supplementation. Values (mean  $\pm$  SEM,  $n = 3$ ) with different letters (a–d) are significantly different ( $p < 0.05$ ) [Control: 5 mM cyclodextrin (CD) vehicle; Chol 8  $\mu\text{M}$ : 8  $\mu\text{M}$  cholesterol in CD; Chol 16  $\mu\text{M}$ : 16  $\mu\text{M}$  cholesterol in CD; Sit 8  $\mu\text{M}$ : 8  $\mu\text{M}$   $\beta$ -sitosterol in CD; Sit 16  $\mu\text{M}$ : 16  $\mu\text{M}$   $\beta$ -sitosterol in CD].

with either 8 or 16  $\mu\text{M}$   $\beta$ -sitosterol significantly decreased cell growth compared to vehicle control after 1, 3 and 5 d sterol supplementation. However, there was no significant difference between the two  $\beta$ -sitosterol concentrations on any of the days studied.

### Sterol supplementation alters composition of MCF-7 and MDA-MB-231 cell membranes

Supplementation of MCF-7 cells with 16  $\mu\text{M}$  cholesterol resulted in significantly increased cholesterol content of the crude membrane preparations compared to CD vehicle control or  $\beta$ -sitosterol groups (Table 1). Likewise, supplementation of MCF-7 cells with 16  $\mu\text{M}$   $\beta$ -sitosterol significantly increased the  $\beta$ -sitosterol content of crude cell membranes. In the control and cholesterol supplementation groups, no detectable levels of  $\beta$ -sitosterol were observed. However, total sterol content of membranes from  $\beta$ -sitosterol-supplemented MCF-7 cells was significantly greater and about twice that of the CD control and cholesterol groups, reflecting the enrichment of membranes with  $\beta$ -sitosterol.

With MDA-MB-231 cells, cholesterol supplementation resulted in significant elevation of total membrane sterol content compared to the CD vehicle control or  $\beta$ -sitosterol groups (Table 2). Likewise, when treated with  $\beta$ -sitosterol, MDA-MB-231 cell membranes contained significantly greater amounts of  $\beta$ -sitosterol when compared to control or cholesterol-treated groups.  $\beta$ -Sitosterol was not detected in either the control or cholesterol treatment group.  $\beta$ -Sitosterol constituted 61% of the total sterol content of crude cell membranes of MDA-MB-231 cells after supplementation with  $\beta$ -

**Table 1.** Effect of 2 d-sterol supplementation on sterol content of MCF-7 cell membranes\*

Supplementation	Cholesterol ( $\mu\text{g}/\text{mg}$ protein)	$\beta$ -Sitosterol ( $\mu\text{g}/\text{mg}$ protein)	Total sterol ( $\mu\text{g}/\text{mg}$ protein)	$\beta$ -Sitosterol (% total sterol)
CD vehicle	43 $\pm$ 2 <sup>a</sup>	0.0 $\pm$ 0 <sup>a</sup>	43 $\pm$ 2 <sup>a</sup>	0 <sup>a</sup>
Cholesterol	49 $\pm$ 2 <sup>b</sup>	0.0 $\pm$ 0 <sup>a</sup>	49 $\pm$ 2 <sup>a</sup>	0 <sup>a</sup>
$\beta$ -Sitosterol	40 $\pm$ 1 <sup>a</sup>	50 $\pm$ 5 <sup>b</sup>	90 $\pm$ 5 <sup>b</sup>	56 <sup>b</sup>

\*MCF-7 cells were treated for 2 d with 16  $\mu\text{M}$  sterol or vehicle and sterol contents of membranes determined by gas–liquid chromatography as described. Data are means  $\pm$  SEM ( $n = 3$ ) and letters (a, b) of values in each column are significantly different ( $p < 0.05$ ).

**Table 2.** Effect of 2 d-sterol supplementation on sterol content of MDA-MB-231 cell membranes\*

Supplementation	Cholesterol ( $\mu\text{g}/\text{mg}$ protein)	$\beta$ -Sitosterol ( $\mu\text{g}/\text{mg}$ protein)	Total sterol ( $\mu\text{g}/\text{mg}$ protein)	$\beta$ -Sitosterol (% total sterol)
CD vehicle	49 $\pm$ 7 <sup>a</sup>	0.0 $\pm$ 0 <sup>a</sup>	49 $\pm$ 7 <sup>a</sup>	0 <sup>a</sup>
Cholesterol	73 $\pm$ 4 <sup>b</sup>	0.0 $\pm$ 0 <sup>a</sup>	73 $\pm$ 4 <sup>b</sup>	0 <sup>a</sup>
$\beta$ -Sitosterol	33 $\pm$ 2 <sup>a</sup>	51 $\pm$ 6 <sup>b</sup>	84 $\pm$ 8 <sup>b</sup>	61 <sup>b</sup>

\*MDA-MB-231 cells were treated for 2 d with 16  $\mu\text{M}$  sterol or vehicle and sterol contents of membranes determined by gas–liquid chromatography as described. Data are means  $\pm$  SEM ( $n = 3$ ) and letters (a, b) of values in each column are significantly different ( $p < 0.05$ ).

sitosterol. The total membrane sterol levels of the cholesterol- and  $\beta$ -sitosterol-supplemented groups were similar to each other and both significantly greater than the CD control group.

### $\beta$ -Sitosterol increases caspase-8 activity in MCF-7 and MDA-MB-231 cells

Caspase-8 activities in lysates from 3 d sterol-supplemented MCF-7 and MDA-MB-231 cells were measured kinetically over 1–20 h of incubation with the caspase-8 substrate, Ac-IETD-AFC. Activities increased linearly and in parallel for the 20 h incubation period in all the treatment groups and results for only the 20 h time points are reported. In  $\beta$ -sitosterol-supplemented MCF-7 cells, caspase-8 activity was significantly increased

compared to the CD vehicle control group (1.9-fold increase) or the cholesterol treatment group (2.9-fold increase) (Table 3). There was no significant difference in caspase-8 activity between the CD vehicle control and cholesterol groups at any time point. Similarly, in  $\beta$ -sitosterol-supplemented MDA-MB-231 cells, caspase-8 activity was significantly increased when compared to the CD vehicle control or cholesterol treatment groups (Table 3). There was no significant difference in caspase-8 activity between the vehicle control and the cholesterol treated groups.

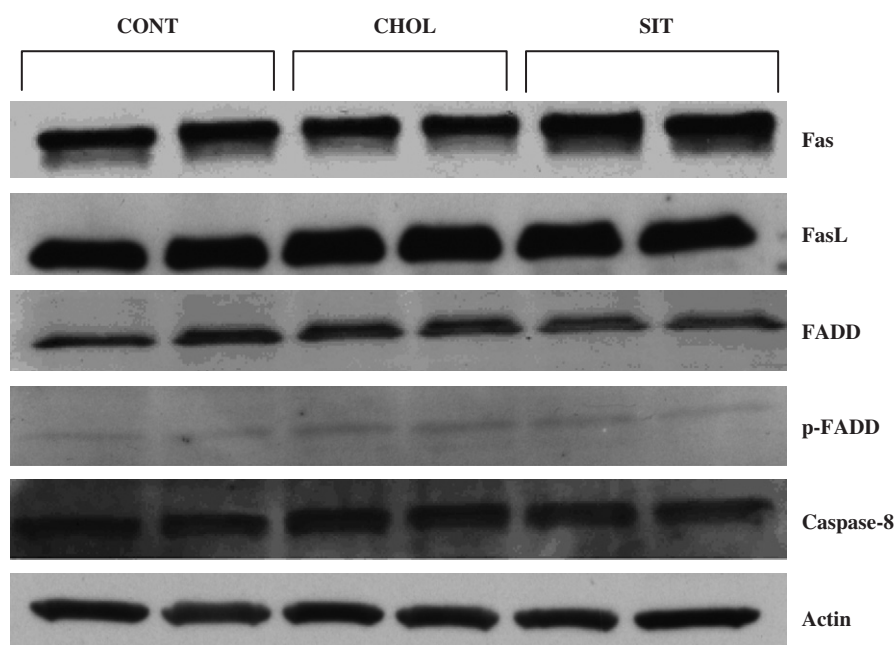
### Fas expression is selectively induced by $\beta$ -sitosterol

The expression levels of the Fas-related proteins Fas, FasL, FADD, phosphorylated FADD (p-FADD), and caspase 8 were assessed by quantitative immunoblot. After 6 h of sterol supplementation of MCF-7 cells, there were no detectable differences in the amounts of Fas, FasL, FADD, p-FADD and caspase 8 between the two sterol treatment groups (data not shown). However, after 24 h  $\beta$ -sitosterol supplementation of MCF-7 cells, there was a 30% increase in Fas protein level compared to MCF-7 cells supplemented with cholesterol or CD vehicle control (Fig. 3). No differences in any other Fas-related proteins were observed at either time among the three supplementation groups. Similar data were obtained from sterol-supplemented MDA-MB-231 cells; however, the  $\beta$ -sitosterol dependent increase in Fas protein levels, though significant, was only 10% above control after 24 h (Fig. 4).

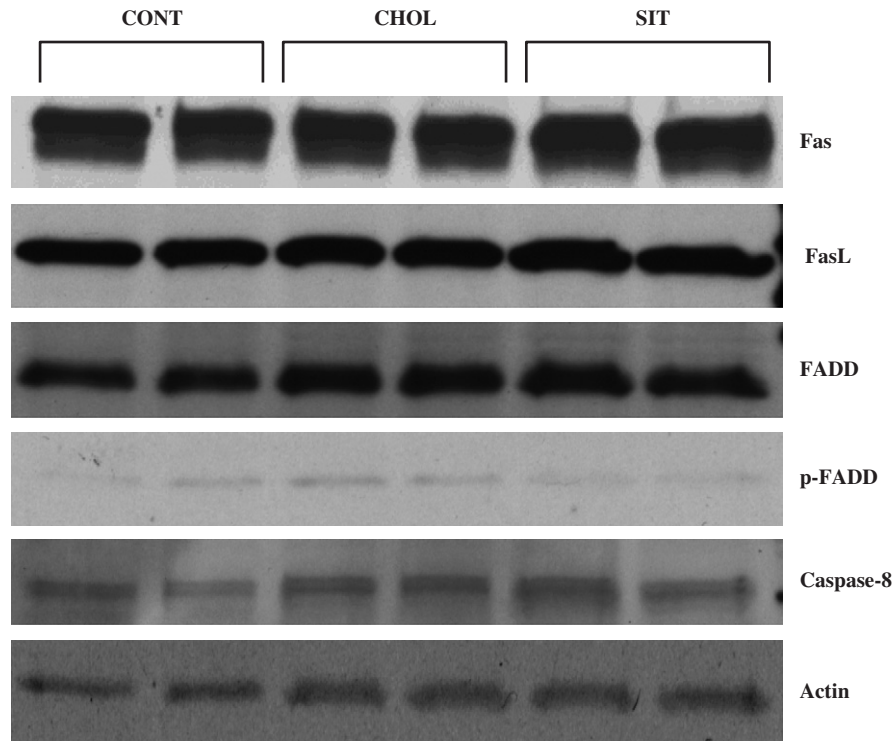
**Table 3.** Effect of 3 d-sterol supplementation on caspase-8 activity of MCF-7 and MDA-MB-231 cell lysates\*

Supplementation	Caspase-8 activity of MCF-7 lysates	Caspase-8 activity of MDA-MB-231 lysates
CD vehicle	6000 $\pm$ 800 <sup>a</sup>	4600 $\pm$ 200 <sup>a</sup>
Cholesterol	5000 $\pm$ 200 <sup>a</sup>	4600 $\pm$ 200 <sup>a</sup>
$\beta$ -Sitosterol	9800 $\pm$ 400 <sup>b</sup>	8100 $\pm$ 800 <sup>b</sup>

\*Cells were treated for 3 d with 16  $\mu$ M sterol or vehicle and caspase-8 activities of cell lysates determined. Data are RFU/100  $\mu$ g lysate protein and represent means  $\pm$  SEM ( $n = 3$ ). Letters (a, b) of values in each column are significantly different ( $p < 0.05$ ).



**Fig. 3.** Effect of sterol supplementation on expression levels of Fas-related signaling proteins in MCF-7 cells. MCF-7 cells were treated with sterols for 24 h as described. Expression of Fas-related signaling pathway proteins was quantified by immunoblot.



**Fig. 4.** Effect of sterol supplementation on expression levels of Fas-related signaling proteins in MDA-MB-231 cells. MDA-MB-231 cells were treated with sterols for 24 h as described. Expression of Fas-related signaling proteins was quantified by immunoblot.

## Discussion

Preliminary studies showed that phytosterol supplementation of MDA-MB-231 human breast cancer cells increases the activities of caspases-3, 8, and 9 (Awad et al., 2003). The purpose of the present study was to expand this work by examining the effects of  $\beta$ -sitosterol on caspase activity in MCF-7 human breast cancer cells as well as to determine the effects of  $\beta$ -sitosterol on cell growth, membrane sterol content, and expression levels of Fas-related apoptotic proteins in both cell lines. The MCF-7 cell line is estrogen receptor-positive and considered a model of early stage breast cancer whereas the MDA-MB-231 cell line is estrogen receptor-negative, hormone-insensitive, and a model of late or advanced stage breast cancer. The effect of sterols on the cell growth and the expression of the extrinsic apoptotic pathway in these cells have not been studied.

$\beta$ -Sitosterol was observed to have growth inhibitory effects on both breast cancer cell lines and these effects occurred over similar time (3–5 d) and concentration ranges (8–16  $\mu$ M) in both cell types. The growth inhibitory effect of phytosterols is at concentrations relevant to vegetarian diets similar to that observed with other tumor cell lines, including the human prostate cancer LNCaP cell line (von Holtz et al., 1998) and the human colon cancer HT-29 cell line (Awad et al., 1998). These findings indicate that  $\beta$ -sitosterol inhibits the

growth of several specific cancer cell lines representing common cancers of Western society. The present findings are contrary to a previous report (Ju et al., 2004) in which treatment of MCF-7 cells with  $\beta$ -sitosterol at concentrations of 150  $\mu$ M increased cell growth. Several reasons could account for this discrepancy, perhaps most significant is that  $\beta$ -sitosterol is not readily soluble at concentrations in the hundred micromolar range.

In addition to the negative effect of  $\beta$ -sitosterol on cell growth,  $\beta$ -sitosterol treatment of tumor cells is associated with increased apoptosis (Awad et al., 2004). In the present study, potential cellular mechanisms underlying phytosterol-induced apoptosis were investigated. Specific effects on caspase-8, the initiator caspase in the extrinsic apoptotic pathway, were identified. After 3 d,  $\beta$ -sitosterol induced a 1.9-fold increase in caspase-8 activity in MCF-7 cells and a 2.9-fold increase in MDA-MB-231 cells. These effects were specific and not mimicked by cholesterol.

$\beta$ -Sitosterol is a plant sterol similar in structure to cholesterol. Supplementation of cells with  $\beta$ -sitosterol was hypothesized to lead to its incorporation into cellular membranes. This action might affect signal transduction pathways leading to cell death. Treatment of MCF-7 and MDA-MB-231 cells with  $\beta$ -sitosterol and cholesterol resulted in significant increases in the amounts of sterol incorporation in cellular membranes.

$\beta$ -Sitosterol treatment of MCF-7 cells did not lower the membrane cholesterol content when compared with the control group but rather enriched the cellular membranes with  $\beta$ -sitosterol, which under the specific incubation conditions, resulted in  $\beta$ -sitosterol representing more than half of the total membrane sterols. The total sterol content of membranes from  $\beta$ -sitosterol treatment group was about two-fold that of the control or cholesterol treatment groups. The cholesterol-treatment control produced a 14% increase in cholesterol content of MCF-7 membranes when compared with the vehicle control. Similar results were obtained in MDA-MB-231 cells, wherein  $\beta$ -sitosterol constituted 61% of total sterols. Treatment of MDA-MB-231 cells with cholesterol however resulted in about 49% increase in cholesterol content of cell membranes. The total sterol contents in cholesterol and  $\beta$ -sitosterol treatment groups were similar and both were significantly greater than that of the control group in this cell line. This enrichment of membranes with sterols could influence the cell signaling mechanisms.

These observations led to the suggestion that the Fas receptor-mediated signal transduction pathway leading to activation of caspase-8 might be influenced by phytosterol-induced changes in cellular membranes. Western blot evaluation of a series of Fas-related apoptotic proteins in  $\beta$ -sitosterol-treated cells showed no effects on the expression levels of FasL, FADD, p-FADD and caspase-8. However, there was a 30% increase in Fas expression in MCF-7 cells after 24 h  $\beta$ -sitosterol treatment. This finding suggests a specific effect of  $\beta$ -sitosterol supplementation on Fas expression and that this increase might be responsible for the increased activity (1.9-fold) of the initiator caspase, caspase-8.

The increased expression of Fas, a transmembrane protein, upon treatment with  $\beta$ -sitosterol may be related to the enrichment of cellular membranes with  $\beta$ -sitosterol. The kinetics of these events deserves consideration. After 6 h treatment (data not shown) of MCF-7 cells with  $\beta$ -sitosterol there were no detectable differences in expression of Fas, FasL, FADD, p-FADD and caspase-8 across the treatment groups. However, at 24 h there was a  $\beta$ -sitosterol-stimulated increase in Fas levels and at 48 h (the only time measured) there was a 50–100% increase in membrane sterols (predominantly  $\beta$ -sitosterol). During the first 24 h treatment caspase-8 activity was increased. It is possible that the increase in membrane sterol levels drives apoptotic changes and that only late in the process do Fas levels change appreciably.

The effects of  $\beta$ -sitosterol on Fas levels in MDA-MB-231 cells were not as dramatic as those seen in MCF-7 cells: 24 h treatment resulted in only approximately a 10% increase as revealed by Western immunoblot. This increase was reproducible and significant and may also

be a consequence of enrichment of  $\beta$ -sitosterol into cellular membranes. Similar to MCF-7 cells, treatment of MDA-MB-231 cells with  $\beta$ -sitosterol for 24 h did not show any detectable changes in the levels of other proteins of the extrinsic apoptotic pathway such as FasL, FADD, p-FADD and caspase-8 when compared with the control and cholesterol treatment groups.

In summary, the present study demonstrates that membrane enrichment with the phytosterol,  $\beta$ -sitosterol, may affect the amounts and activity of components of the extrinsic apoptotic pathway in human breast adenocarcinoma cells. This is a significant observation and investigation of any cause and effect relationship between these observations is warranted. It is interesting to consider that the Fas receptor has been reported to be localized in cholesterol- and sphingomyelin-rich membrane rafts (Hueber et al., 2002). Perhaps any disruption or change in the cholesterol–sphingomyelin ratio or sterol content by the addition of phytosterols may influence the activities of lipid rafts and thus expression of and signaling through Fas receptors. Our previous observations indicate that  $\beta$ -sitosterol induces a reduction in membrane sphingomyelin and an increase the ceramide levels in some tumor cells (von Holtz et al., 1998; Awad et al., 1998). Ceramide could play a role in the activation of the extrinsic pathway as suggested by observations of death receptor clustering in ceramide-rich lipid rafts (Hueber, 2003; Scheel-Toellner et al., 2004). These findings suggest that the effect of  $\beta$ -sitosterol treatment to increase caspase-8 activity and apoptosis in these cells may be mediated, at least in part, by changes in membrane sterol content and effects on the Fas apoptotic pathway.

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