RESEARCH ARTICLE

β-Sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells

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β-Sitosterol, normally present in vegetable-containing diets, comprises an important component of cholesterol controlling functional foods. It has been associated with cardiovascular protection, exerting its effect mainly through increasing the antioxidant defense system and effectively lowering the serum cholesterol levels in humans. However, its anti-inflammatory effect on endothelium is unknown. Attachment of leukocytes to the vascular endothelium and the subsequent migration of cells into the vessel wall are early events in atherogenesis, this process requiring the expression of endothelial adhesion molecules. We examined the effect of β-sitosterol (0.1–200 μM) on (i) the expression of vascular adhesion molecule 1 and intracellular adhesion molecule 1 by cell ELISA and (ii) the attachment of monocytes (U937 cells) in tumor necrosis factor-α (TNF-α)-stimulated human aortic endothelial cells (HAECs) by adhesion assay. The effect on nuclear factor-kB phosphorylation was also examined *via* a cell-based ELISA kit. Results showed that β-sitosterol inhibits significantly vascular adhesion molecule 1 and intracellular adhesion molecule 1 expression in TNF-α-stimulated HAEC as well as the binding of U937 cells to TNF-α-stimulated HAEC and attenuates the phosphorylation of nuclear factor-kB p65. This study extends existing data regarding the cardioprotective effect of β-sitosterol and provides new insights into understanding the molecular mechanism underlying the beneficial effect of β-sitosterol on endothelial function.

Keywords:
Adhesion molecules / Atherosclerosis / β-Sitosterol / Human aortic endothelial cell / Nuclear factor-kB

1 Introduction

Plant sterols (phytosterols) are naturally occurring plant constituents that are structurally similar to cholesterol. These compounds are not synthesized in mammals and are therefore derived solely from the diet, the most common being β-sitosterol, campesterol and stigmasterol. It is well established that dietary plant sterols lower plasma cholesterol concentrations by inhibiting intestinal cholesterol absorption [1]. Consumption of 1.8–2.0 g/day of plant sterols has been shown to lower both total and LDL cholesterol concentrations by 10–15% in a variety of different population groups [2, 3]. Because it has been demonstrated that plant sterols have additive effects in lowering LDL cholesterol when combined with statins [4], the National Cholesterol Education Program Adult Treatment Panel III has recognized the use of phytosterols as a key element of dietary therapy [5]. This has led to the development of functional foods containing high contents of these plant molecules or their esters as cholesterol controlling foods. In addition, anti-atherogenic effects of dietary plant sterols have been associated with inhibition of

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Abbreviations: BCECF-AM, 2′,7′-*bis*-(2-cabroxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester; **CAM,** cell adhesion molecule; **FBS,** fetal bovine serum; **HAEC,** human aortic endothelial cells; **ICAM-1,** intracellular adhesion molecule 1; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NFkB,** nuclear factor-kB; **PR,** phenol red; **RT,** room temperature; **TNF-α,** tumor necrosis factor-α; **VCAM-1,** vascular adhesion molecule 1

proinflammatory cytokine production in ApoE-KO mice [6]. It is remarkable that β-sitosterol, a natural compound normally present in vegetable-containing diets, shows beneficial effects directly on endothelial and monocytic cells [7]. Furthermore, *in vivo* studies support the topical anti-inflammatory effect of β-sitosterol [8, 9], while *in vitro* studies suggest the involvement of β-sitosterol in the arachidonic acid cascade [10].

Atherosclerosis is considered a chronic inflammatory process with increased oxidative stress in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are the pivotal early events in atherogenesis [11]. The interaction between monocytes and vascular endothelial cells is mediated by adhesion molecules including vascular adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1) and E-selectin on the surface of the vascular endothelium. The increased expression of adhesion molecules by endothelial cells in human atherosclerotic lesions, activated by inflammatory cytokines, may lead to further recruitment of leukocytes to atherosclerotic sites thereby resulting in the formation of atherosclerotic lesion, oxidative modification of LDL and endothelial dysfunction [12].

In this study, we examined the hypothesis that β-sitosterol could inhibit the expression of adhesion molecules (VCAM-1 and ICAM-1) and the attachment of monocytes in human aortic endothelial cells (HAECs). The impact of treatment with β-sitosterol on tumor necrosis factor-α (TNF-α)-related signaling in HAEC, such as activation of nuclear factor-kB (NFkB) phosphorylation, was also examined.

2 Materials and methods

2.1 Culture of HAEC

HAEC were provided as cryopreserved cells by Clonetics (Cambrex, USA) and were grown in culture flasks at 37°C in a humidified 95% air-5% CO_2 atmosphere in endothelial cell basal medium (Clonetics, Cambrex) supplemented with fetal bovine serum (FBS, 2%), human epidermal growth factor (10 ng/mL), hydrocortisone (1.0 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL) and bovine brain extract (3 mg/mL). The growth medium was changed every other day until confluence. Cells under passage 8 were used for this study.

2.2 [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cultures of HAEC were grown in endothelial cell growth medium (Clonetics, Cambrex) supplemented with 2% FBS (Gibco BRL, Invitrogen, USA), in T-75 cm^2 flasks at 37°C, 95% humidity and 5% CO_2 atmosphere. Subcultures were carried out every 3–4 days using a trypsin 0.025% and EDTA 0.01% solution (Gibco BRL, Invitrogen). Cell viability was estimated by a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13], which determines the metabolically active mitochondria of cells. Briefly, cells were plated in their growth medium at a density of 6000 cells/well in 96 flat-bottomed well plates. Twenty-four hours after plating, β-sitosterol from soybean (purity ≥ 97%), (S9889, Sigma-Aldrich, Germany) was added at final concentrations ranging from 0.1 to 100 μM in DMEM phenol red (PR)-free medium (Gibco BRL Invitrogen). Cells in their growth medium – without test substances – were used as control samples (vehicle). After 48 h incubation, the medium was replaced with MTT (Sigma-Aldrich) dissolved at a final concentration of 1 mg/mL in serum-free, PR-free medium, for a further 4 h incubation. Then, the MTT-formazan product was solubilized thoroughly in isopropanol and the optical density was measured at a test wavelength of 550 nm and a reference wavelength of 690 nm.

2.3 Cell ELISA

To examine whether β-sitosterol could modify the expression of VCAM-1 and ICAM-1, cell ELISA was conducted. Briefly, to measure the cell-surface expression of adhesion molecules, HAEC monolayer in flat-bottomed 96-well plates (at 80% confluence) were pretreated with β-sitosterol (0.1, 1, 10, 50 and 100 μM) for 18 h, then stimulated for 6 h at 37°C with 1 ng/mL TNF-α (Sigma-Aldrich), after which the cells were fixed with 0.1% glutaraldehyde in PBS for 30 min at 4°C. Plates were blocked at 37°C for 1 h with 5% skimmed milk powder in PBS, this followed by an incubation at 4°C overnight with a primary monoclonal goat antibody against human ICAM-1 or VCAM-1, at final concentration 2 μg/mL in 5% skimmed milk PBS. Next, the plates were washed three times with 0.1% Tween-20 in PBS and incubated with a horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody at a dilution of 1:5000 at room temperature (RT) for 1 h. Subsequently, the plates were washed three times with 0.1% Tween-20 in PBS and finally the expression of cell adhesion molecules (CAMs) was quantified by the addition of the peroxides substrate *o*-phenylendiamine hydrochloride. As a positive control, we used vitamin E (α-tocopherol, 20 μM), an antioxidant known to exert its effect through modulation of cytokines, adhesion molecules, mobilization of NFkB transcription factor and interaction of immune cells with endothelial cells [14, 15]. The absorption of each well was measured at 450 nm using a microplate ELISA reader.

2.4 Fluorescent labeling of monocytes

U937 cells (human monocytic cell line which exhibits many characteristics of monocytes) (ECACC, UK) were fluores-

cently labeled with 2′,7′-*bis*-(2-cabroxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Invitrogen, USA) for the quantitative cell adhesion assay. Non-fluorescent BCECF-AM is lipophilic and when it is cleaved intercellularly it becomes a highly charged fluorescent BCECF, which is retained by viable cells. The BCECF-AM was obtained as a 1 g/L stock solution in anhydrous DMSO and was stored at -80°C. After labeling the U937 cells (10^6 cells/mL) with 10 μM BCECF-AM in RPMI-1640 PR(-) (Gibco BRL, Invitrogen), FBS(-) and L-glutamine(-) medium for 1 h at 37°C and 5% CO_2, the cells were washed two times with PBS to remove the excess dye. Finally, the cells were resuspended in RPMI-1640 PR(+), 2% FBS medium at a density of 2×10^4 cells/well, according to the manufacturer's instructions.

2.5 U937 cell adhesion assay

HAEC were cultured until confluence in 96-well plates and pretreated with β-sitosterol (0.1, 1, 10, 50 and 100 μM) for 18 h. Then cells were stimulated for 6 h at 37°C with TNF-α (2 ng/mL). BCECF-labeled U937 cells (2×10^4 cells/well) were incubated with HAEC for 1 h at 37°C and 5% CO_2. After incubation, non-adherent cells were removed by washing gently each well two times with PBS. The attached cells were lysed with 50 mmol/L Tris buffer (pH 7.6) containing 0.1% sodium dodecyl sulphate. α-Tocopherol was used as a positive control (20 μM). The fluorescent intensity of each well was measured with a fluorescence multiwell plate reader set at excitation and emission wavelengths of 485 and 530 nm, respectively.

2.6 Blocking antibody studies

mAbs against VCAM-1 or ICAM-1 were incubated with confluent HAEC in 96-well plates at 5 μg/mL final concentration, during the final 30 min of the TNF-α stimulation, before U937 were added [16, 17]. Adhesion assay was then performed in sections 2.4, 2.5.

2.7 Measurement of NFkB p65 phosphorylation

To measure NFkB phosphorylation, confluent HAEC were starved in serum free culture medium for 18 h and then pretreated without or with β-sitosterol (0.1, 1, 10, 50, 100 and 200 μM) for 18 h. Cells were then stimulated for 5 min at 37°C with TNF-α (2 ng/mL). To arrive at these conditions, we performed a time course experiment by stimulating HAEC with TNF-α (2 ng/mL) for 5, 15 and 30 min. Finally, NFkB p65 phosphorylation was measured with CASE™ kit (FE-005, SABiosciences, USA). The CASE™ kit can be used to monitor activation of a signal transduction pathway by measuring phosphorylation of a key mediator of a pathway of interest without the need to prepare cell lysates and to perform a Western blot. In our study, NFkB (p50/p65) is a key transcription factor which is implicated in the regulation of a variety of genes participating in inflammatory responses, including genes encoding VCAM-1 and ICAM-1. TNF-α induces the activation-phosphorylation and translocation of NFkB to the nucleus, promoting the VCAM-1 and ICAM-1 expression. Thus, we first detected the time course of TNF-α induced phosphorylation on Ser-536 of NFkB p65, and second we tested the impact on NFkB p65 phosphorylation β-sitosterol at the indicated concentrations. Briefly, the cells were fixed with 4% fixing buffer for 20 min, RT. Next, plates were blocked for 1 h, RT, with protein-based blocking buffer. After blocking, incubation with the primary antibody for 1 h, RT, followed. The plates were then washed three times with 1 × washing buffer and incubated with the secondary antibody for 1 h, RT. Subsequently, the plates were washed three times with 1 × washing buffer and finally colorimetric detection of antibodies was carried out by measuring the absorption of each well at 450 nm. Determination of relative cell number was followed by reading the absorption of each well at 595 nm. The absorption was measured by using a microplate ELISA reader.

2.8 Statistical analysis

Data are reported as mean ± SD of three independent experiments (each experiment was conducted in triplicate or quadruplicate). Data in figures are expressed as percentage of control, which was calculated as follows: (value for cells treated with test compound/value for control cells) × 100. Statistical analysis was performed using Student's *t*-test, two-tailed distribution, assuming two-sample unequal variance.

3 Results

3.1 β-Sitosterol inhibits CAMs expression in HAEC

The effect of different concentrations of TNF-α (1 or 2 ng/mL) on VCAM-1 and ICAM-1 expression was initially determined after 6-, 12- or 24-h incubation. Incubation of confluent HAEC with TNF-α (1 ng/mL) caused a maximal surface expression of VCAM-1 and ICAM-1 after 6 h of incubation (data not shown). In subsequent experiments, we used TNF-α (1 ng/mL) for 6 h to induce stimulation of cells. TNF-α increased the basal expression (control) of CAMs VCAM-1 and ICAM-1 of confluent HAEC. α-Tocopherol decreased significantly the TNF-α-induced endothelial expression of both VCAM-1 and ICAM-1 ($p<0.001$), as expected [18]. The effects of β-sitosterol on the expression of VCAM-1 and ICAM-1 by HAEC are shown in Figs. 1A and B. β-Sitosterol (50, 100 μM) caused a significant dose dependent decrease in VCAM-1 and ICAM-1 expression

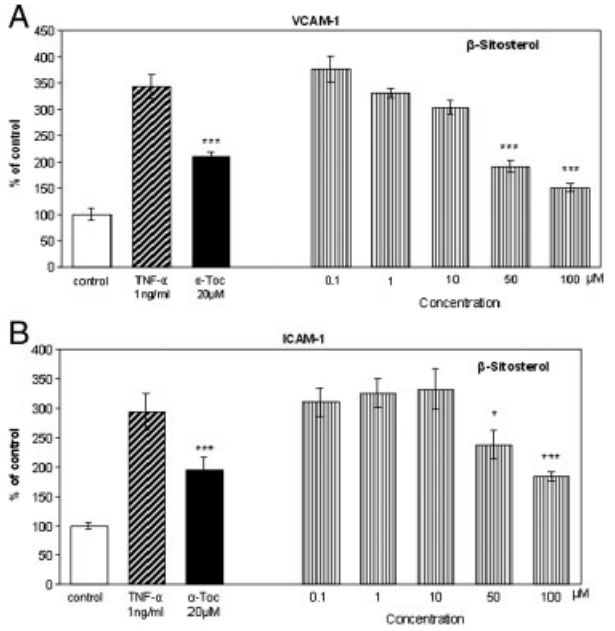

Figure 1. (A) β-Sitosterol inhibits TNF-α-induced VCAM-1 protein expression in HAEC. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with different concentrations of β-sitosterol (0.1–100 μM) for 18 h, followed by stimulation with TNF-α (1 ng/mL) for up to 24 h. Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared to TNF-α-treated cells ($^{**}p<0.01$, $^{***}p<0.001$). (B) β-Sitosterol inhibits TNF-α-induced ICAM-1 protein expression in HAEC. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with different concentrations of β-sitosterol (0.1–100 μM) for 18 h, followed by stimulation with TNF-α (1 ng/mL) for up to 24 h. Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared with TNF-α-treated cells ($^{**}p<0.01$, $^{***}p<0.001$).

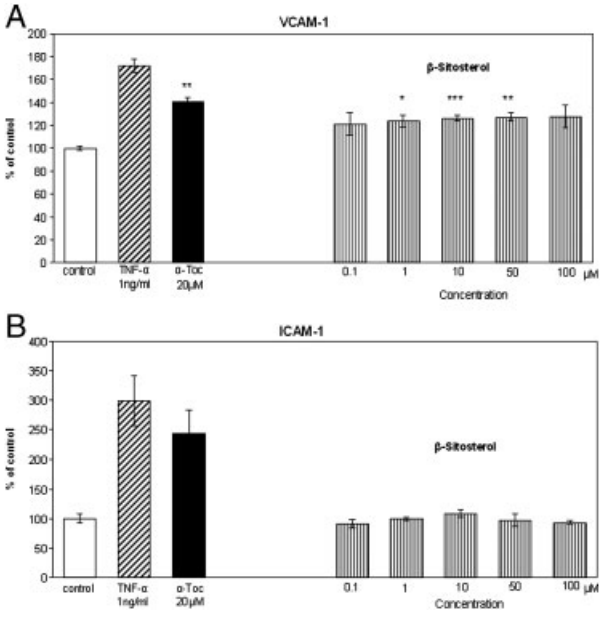

Figure 2. (A) β-sitosterol has no significant effect on VCAM-1 protein expression in HAEC when tested alone. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with different concentrations of β-sitosterol (0.1–100 μM) for 18 h (without stimulation with TNF-α). Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared with control ($^{**}p<0.01$, $^{***}p<0.001$). (B) β-Sitosterol has no significant effect on ICAM-1 protein expression in HAEC when tested alone. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with different concentrations of β-sitosterol (0.1-100 μM) for 18 h (without stimulation with TNF-α). Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared to control ($^{**}p<0.01$, $^{***}p<0.001$).

($p<0.001$), compared to TNF-α-treated HAEC. Furthermore, when we performed the same procedure, but without the stimulation of the cells with TNF-α, β-sitosterol caused a small increase in VCAM-1 expression (1, 10 and 50 μM) and had no effect on ICAM-1 expression (in all concentrations tested), compared with control (Figs. 2A and B).

3.2 Cell viability

The assessment of cell viability revealed that neither the morphology nor the reduction of MTT salt in HAEC cells was affected by any of the tested compounds (β-sitosterol, α-tocopherol or TNF-α) in any concentration range or experimental conditions used. The lowering effect of β-sitosterol on the expression of adhesion molecules without affecting the viability rate of cells supports their anti-inflammatory activity.

3.3 β-Sitosterol inhibits binding of U937 cells to TNF-α-stimulated HAEC

The effects of the test compounds on the binding of U937 cells to TNF-α-stimulated HAEC were determined and results are shown in Fig. 3. Control confluent HAEC showed minimal binding to U937 cells and results are expressed as percentage of control. Adhesion was significantly increased when the HAEC were treated with TNF-α (2 ng/mL). Pretreatment with α-tocopherol (20 μM) or β-sitosterol (1, 10,

50 and 100 μM) significantly ($p<0.01$) reduced the adhesion of U937 cells to TNF-α-stimulated HAEC.

3.4 Effect of antibodies against adhesion molecules on U937 adhesion to HAEC

By using mAbs against human VCAM-1 or ICAM-1, we evaluated their relative involvement in TNF-α induced monocyte adhesion to HAEC. TNF-α-stimulated HAEC (2 ng/mL), pretreated with β-sitosterol (100 μM) or not, were incubated with anti-ICAM-1 or anti-VCAM-1 antibodies at 5 μg/mL for 30 min prior to the adhesion assay. As shown in Fig. 4, anti-VCAM-1 mAb alone inhibited significantly ($p<0.05$) the number of adherent monocytes. Co-incubation of anti-VCAM-1 antibody with 100 μM of β-sitosterol enhanced the inhibition of U937 monocyte adhesion in TNF-α-stimulated HAEC ($p<0.01$). Interestingly, anti-ICAM-1 mAb treatment of HAEC or co-incubation of anti-ICAM-1 with 100 μM of β-sitosterol markedly increased U937 cell adhesion to HAEC (Fig. 4).

3.5 β-Sitosterol attenuates phosphorylation of NFkB p65 in TNF-α-stimulated HAEC

The time course experiment we performed to monitor the NFkB phosphorylation, by stimulating HAEC with TNF-α (2 ng/mL) for 5, 15 and 30 min, showed that stimulation of the cells for 5 min was more significant (Fig. 5). Results showed that pretreatment with β-sitosterol decrease significantly ($p<0.001$) the phosphorylation of NFkB p65 in TNF-α-stimulated HAEC compared with control HAEC (Fig. 6).

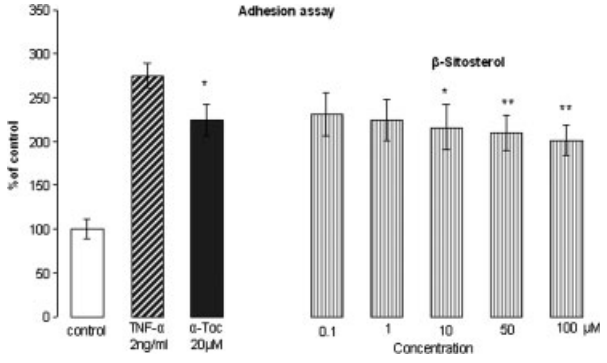

Figure 3. β-Sitosterol and α-tocopherol (α-Toc) inhibit TNF-α-induced monocyte adhesion to HAEC. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with different concentrations of β-sitosterol (0.1-100 μM) for 18 h, followed by stimulation with TNF-α (2 ng/mL) for up to 24 h. Monocyte adhesion was determined by the adhesion assay. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared with TNF-α-treated cells ($^{**}p<0.01$, $^{***}p<0.001$).

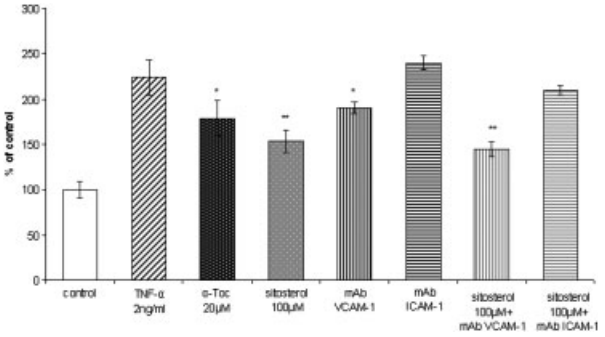

Figure 4. Blocking antibody studies. HAEC were incubated as described in Section 2, in the absence of TNF-α or compounds (control), with α-tocopherol (α-Toc) (20 μM), or with β-sitosterol (100 μM) for 18 h, followed by stimulation with TNF-α (2 ng/mL) for up to 24 h. During the last 30 min of TNF-α stimulation and just before conducting the U937 adhesion assay, mAb to human VCAM-1 or ICAM-1 were added (5 μg/mL). Monocyte adhesion was determined by the adhesion assay. Data are expressed as percentage of control and shown as means ± SD of two independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared to TNF-α-treated cells ($^{**}p<0.01$, $^{***}p<0.001$).

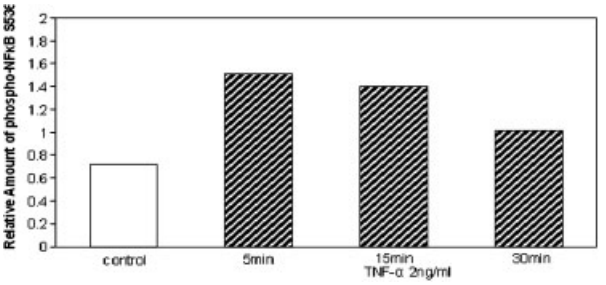

Figure 5. Monitoring of NFkB phosphorylation over a time course. HAEC were grown in 96-well plates and starved before treatment. Cells were then treated with 2 ng/mL of NFkB activator TNF-α for 5, 15 and 30 min. Phosphorylation levels at Serine-536 of NFkB p65 were measured using the CASE^{TM} kit for NFkB S536 (Cat. No. FE-005).

4 Discussion

Accumulating evidence indicates that phytosterols exert cardiovascular protective effects mainly *via* their cholesterol lowering ability, modulation of endothelial function and antioxidant capacity [19, 20]. More important, the cardiovascular protective effect of β-sitosterol, a key component of cholesterol controlling functional foods, has been related to its antioxidant and hypocholesterolemic capacity [1].

Data, however, on the role of β-sitosterol in the inflammatory process of atherosclerosis are sparse. Since the binding and recruitment of circulating monocytes to vascular endothelial cells are early steps in the development of inflammation and atherosclerosis, mediated through CAMs that are expressed on the surface of endothelial cells, we evaluated the potential of β-sitosterol to influence the

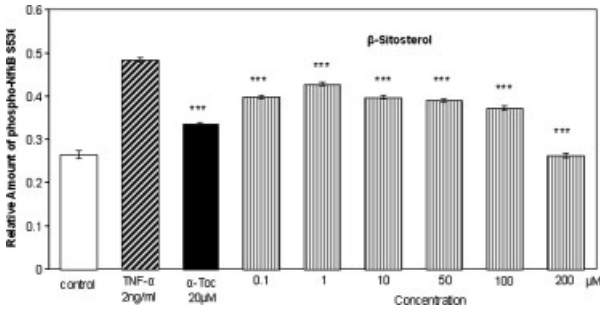

Figure 6. β-Sitosterol inhibits TNFα-induced phosphorylation of NFkB. HAEC were grown in 96-well plates and starved before treatment with test compound (0.1–200 μM). Cells were then treated with 2 ng/mL of NFkB activator TNF-α (2 ng/mL) for 5 min. Phosphorylation levels at Serine-536 of NFkB p65 were measured using the CASE^{TM} kit for NFkB S536. Data are expressed as relative amount of phospho-NFkB and shown as means ± SD of three independent experiments (each conducted in triplicates). A $^{*}p<0.05$ value was considered statistically significant when compared to TNF-α-treated cells ($^{**}p<0.01$, $^{***}p<0.001$).

expression of VCAM-1 and ICAM-1 by HAEC. We used the Cell ELISA to measure VCAM-1 and ICAM-1, a well-recognized *in vitro* assay, to evaluate the anti-inflammatory effect of test compounds [21–23]. As a positive control, we used vitamin E (α-tocopherol), an antioxidant known to exert its effect through modulation of cytokines, adhesion molecules, mobilization of NFkB transcription factor and interaction of immune cells with endothelial cells [14, 15]. For further evaluation of the anti-inflammatory effect of β-sitosterol, we used the adhesion assay to measure the monocyte adhesion to TNF-α-stimulated HAEC [24]. To decide the proper dosages of β-sitosterol to be tested in our *in vitro* systems, we considered it important to take into account the following: (i) β-sitosterol, the major phytosterol of higher plants, is found in the tissues and plasma of healthy individuals at concentrations 800–1000 times lower than that of endogenous cholesterol [25] and (ii) a recent report demonstrates that plasma concentrations of β-sitosterol range from 2.8 to 16 μmol/L [1]. In view of the above, we decided to assess the biological effects of β-sitosterol at a wide concentration range from 0.1 to 200 μM. Vitamin E inhibited the TNF-α induced expression of both ICAM-1 and VCAM-1, as expected [14, 18]. The inhibition of TNF-α induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules by β-sitosterol, at a concentration range 0.1–100 μM, implicates the anti-inflammatory effect of β-sitosterol on endothelial cells. Meanwhile, β-sitosterol, when tested alone (in the absence of HAEC stimulation with TNF-α), had no significant action (compared with control). In our study, the small increase in VCAM-1 expression by β-sitosterol treatment without TNF-α stimulation suggests that the lowering effect of β-sitosterol on adhesion molecule expression is more pronounced under inflammatory conditions, whereas at basal conditions it may induce inflammatory processes. However, further studies are warranted to elucidate the clinical impact of this observation, *i.e.* whether β-sitosterol supplementation in a healthy population may be a risk factor for low-grade inflammation and atherogenesis.

More importantly, the adhesion assay showed that in the presence of β-sitosterol (0.1–100 μM), the binding of human monocytic cell line, U937, to TNF-α-stimulated HAEC was inhibited significantly. We further investigated the role of VCAM-1 and ICAM-1 expression in mediating the inhibitory effects of β-sitosterol on adhesion of U937 cells to HAEC. We examined the effects of blocking antibodies (anti-VCAM-1 and anti-ICAM-1) on adhesion of U937 to HAEC stimulated with TNF-α and found that the use of anti-VCAM-1 inhibited significantly the U937 cell adhesion, whereas anti-ICAM-1 was without effect. Such data suggest that VCAM-1 expression plays an important role in U937 adhesion to HAEC. The small decrease by β-sitosterol in enhanced ICAM-1 expression induced by TNF-α (as shown in cell ELISA) thus seems to be of no significance in U937 adhesion to HAEC. Interestingly, a combination of β-sitosterol and anti-VCAM-1 antibody increased the magnitude of inhibition of U937 cell adhesion to a greater degree than the individual anti-VCAM-1 antibody. The degree of inhibition was similar to that observed by β-sitosterol alone. This may be due to the expression of other available adhesion molecules on the endothelial surface being occupied by U937 cells, which molecules, in the presence of β-sitosterol, become unavailable thus resulting in attenuation of U937 binding. It is important to note that the combination of anti-ICAM-1 antibody with β-sitosterol attenuated the inhibitory effect of β-sitosterol alone in U937 cell adhesion to HAEC. Previous reports have shown that ICAM-1 adhesion molecules, which are expressed in a wide variety of cell types including U937 [26], may use ICAM-1 antibody (bound to ICAM-1 molecules expressed on HAEC) as a bridge to facilitate further binding of several U937 cells [16]. In view of the above, we speculate that similar interactions may have taken place in our study, thus masking the inhibitory effect of β-sitosterol alone in U937 cell adhesion to HAEC. Taken together, our study demonstrates that β-sitosterol inhibits TNF-α induced U937 cell adhesion to HAEC by lowering the expression of various adhesion molecules, of which the suppression of VCAM-1 adhesion molecule plays a key role in U937 adhesion to HAEC.

Summarizing, since monocyte recruitment into the vascular wall, after their adhesion to endothelial cells, is a crucial step in the pathogenesis of atherosclerosis, our data strongly indicate that β-sitosterol has a noticeable anti-atherogenic potential. In agreement with the present findings, animal studies have concluded that PS (mixture of: β-sitosterol, campesterol and stigmasterol) exerts protective effects on atherosclerotic lesion development, plaque formation, foam cell formation and vascular endothelium damage [27]. More recently, Bustos *et al.* showed that β-sitosterol

inhibited expression of ICAM-1 and decreased migration and adhesion of THP-1 cells to oxLDL-stimulated HUVEC [7]. Furthermore, the excessive production of reactive oxygen species contributes to the pathogenesis of cardiovascular disease. To this effect, the cardioprotection of β-sitosterol, mechanistically, has been shown to be mediated *via* its ability to strengthen the intracellular antioxidant defense. In particular, β-sitosterol has been shown to protect against oxidative stress *via* modulation of antioxidant enzymes [28]. More importantly, a reduction by β-sitosterol in arachidonic acid release and prostanglandin E2 production, induced by oxidized low-density lipoproteins, has been observed [29]. Such data implicate possible mechanism(s) involved in the regulatory effects of β-sitosterol in adhesion molecule expression, cell-cell interaction and inflammation [30]. However, De Jong *et al.* [31] demonstrated that β-sitosterol did not modify soluble adhesion molecules, neither did it affect markers of antioxidant status or oxidative stress, despite a significant reduction in LDL cholesterol in patients treated with statins. Such observations suggest that under *in vivo* conditions, the combination of therapeutic regimes may result in synergistic or antagonistic effects and thus variable biological activity. The clinical impact of such effects should be taken into consideration.

To further elucidate the mechanism of action of β-sitosterol, we investigated their effects on the phosphorylation of NFkB p65, which is a key transcription factor regulating a variety of genes participating in inflammatory responses, including genes encoding VCAM-1 and ICAM-1 [32]. NFkB is a cytoplasmatic component which is an inactive complex with its inhibitor IkB. Its activation is the critical process for the downstream activation and gene expression in endothelial cells. TNF-α can induce the phosphorylation of IkB, which results in dissociation of IkB and finally activation-phosphorylation and translocation of NFkB to the nucleus, promoting the expression of downstream genes, such as adhesion molecules VCAM-1 and ICAM-1.

In our study, we found that TNF-α treatment induced higher levels of NFkB phosphorylation in HAEC, an indication that NFkB was activated. Treatment with β-sitosterol (0.1–200 μM) significantly inhibited the phosphorylation of NFkB, suggesting that its anti-inflammatory activity *in vitro* is mediated, at least in part, *via* the inactivation of NFkB. In the same line with our findings, Moreno showed that β-sitosterol decreased the activation of NFkB transcription factor in PMA-stimulated macrophage cells [10]. Although it is widely recognized that induction of endothelial adhesion molecules by inflammatory cytokines depends greatly on NFkB activation and its DNA-binding ability, however, the promoters of adhesion molecules, including ICAM-1 and VCAM-1 promoters, contain binding sites for other transcription factors such as AP-1 or SP-1 [32–36]. In view of the above, the anti-inflammatory effect of β-sitosterol on HAEC may be mediated by activation of multiple transcription factors and certainly warrants further investigation.

In conclusion, our study extends existing data regarding the cardioprotective effect of β-sitosterol and provides new insights into understanding the molecular mechanism underlying the beneficial effect of β-sitosterol on endothelial function and cardioprotection.

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