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ARTICLE

β-Sitosterol Inhibits Cell Cycle Progression of Rat Aortic Smooth Muscle Cells through Increases of p21^{cip1} Protein

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Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays a central role in the pathogenesis of atherosclerosis. β-Sitosterol, an important phytosterol found in plant food, is known to exert anti-atherosclerosis activity. However, the molecular mechanisms underlying β-sitosterol-induced antiproliferation of VSMCs were still not clear. This study demonstrated that β-sitosterol (1−20 μM) concentration-dependently inhibited proliferation of rat aortic smooth muscle cells (RASMCs) without cytotoxic effect. Flow cytometric analysis revealed that β-sitosterol arrested cell cycle progression through down-regulation of cyclin E and cyclin-dependent kinase (CDK)2 and up-regulation of p21^{cip1}. In the β-sitosterol-treated RASMCs, the formation of the CDK2-p21^{cip1} complex was increased and the assayable CDK2 activity was decreased. Knockdown of the expression of p21^{cip1} gene prevented β-sitosterol-induced cell cycle arrest in RASMCs. In conclusion, β-sitosterol inhibited VSMC proliferation by increasing the levels of p21^{cip1} protein, which in turn inhibited the CDK2 activity, and finally interrupted the progress of the cell cycle.

KEYWORDS: β-Sitosterol; rat aortic smooth muscle cells; cell cycle arrest; p21^{cip1}; CDK2

INTRODUCTION

Proliferation of atherosclerosis remains a leading cause of morbidity and mortality worldwide. Vascular smooth muscle cells (VSMCs) have long been considered to be a key participant in the remodeling of vascular wall following vascular injury in diseases such as atherosclerosis and vascular restenosis after invasive intervention (*1*). Recently, the precise role of cell proliferation in atherogenesis has received more attention (*2*).

Proliferation of mammalian cells is governed by cell cycle, which is a complex and stepwise process (*3*). The activity of cyclin-dependent kinases (CDKs) is controlled by cyclin regulatory subunits. These form a complex with their catalytic subunit of CDKs and are regulated at a specific phase of the cell cycle (*4*−*6*). In many cell types, transition through the G1 phase of the cell cycle and entry into the S phase require the binding and activation of cyclin/CDK complexes, mainly cyclin D1/CDK4 and cyclin E/CDK2 (*3*). Thus, functional activation of CDK-cyclin is required for cell cycle progression (*1*, *7*). The kinase activity of these CDK−cyclin complexes is inhibited by two classes of cyclin-dependent kinase inhibitors (CKIs) (*8*). Members of the INK4 family (p16^{INK4a} and p15^{INK4b}) inhibit only CDK4 and CDK6 (*9*), whereas members of the cip family (p21^{cip1} and p27^{kip1}) inhibit all CDKs (*10*).

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Phytosterols are structurally similar to cholesterol with some modifications. These modifications include the addition of a double bond and/or methyl or ethyl group (*11*). Phytosterols are enriched in plant foods such as peanuts, nuts, soybean seeds, sesame seeds, and unrefined plant oils and grains as well as their products such as wheat bran, wheat germ, and cornflakes (*12*). The most common dietary phytosterols are β-sitosterol, campesterol, and stigmasterol. Previous studies have demonstrated that phytosterols and their derivates could reduce the absorption of dietary cholesterol, offer protection from cardiovascular diseases, and inhibit the growth of cancer cells (*13*, *14*). Animal studies have shown that a diet containing 2% mixed phytosterols inhibits cancer cell growth, reduces tumor size, and decreases the incidence of colon, breast, and prostate cancers induced by specific carcinogens (*15*−*18*). The results of in vitro studies have also shown that phytosterols, β-sitosterol in particular, inhibit proliferation and induce apoptosis in colon, breast, prostate, liver, and murine fibrosarcoma cancer cells at low concentrations (2.4−32 μM) (*19*−*23*). However, there is little information about the growth effect of β-sitosterol in VSMCs (*24*). The aims of this study were to investigate the effect of β-sitosterol on DNA synthesis and cell proliferation in cultured rat aortic smooth muscle cells (RASMCs) in vitro and to study the possible underlying mechanisms.

MATERIALS AND METHODS

Materials. The cell culture materials and FBS were obtained from Gibco-BRL (Gaithersburg, MD). β-Sitosterol was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine and [γ-^{32}P]ATP were

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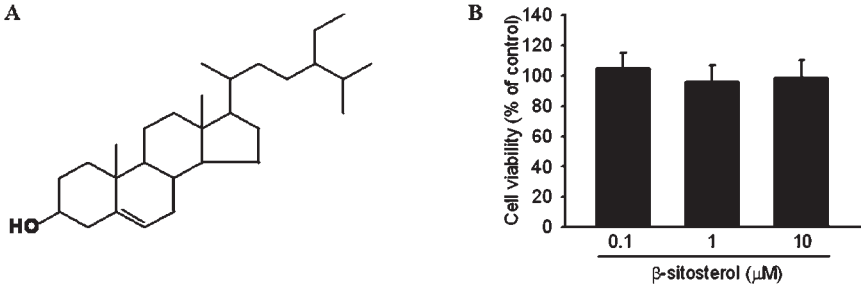

Figure 1. Effect of β-sitosterol on the viability of RASMCs: (**A**) chemical structure of β-sitosterol; (**B**) β-sitosterol at a range of concentrations (0.1−10 μM) did not affect cell viability. RASMCs were exposed to various concentrations (0.1−10 μM) of β-sitosterol for 24 h and then processed for the analysis of viability by MTT assay. Values represent the mean ± SEM (n = 3).

purchased from PerkinElmer Life Sciences (Boston, MA). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). Antibodies specific for $p21^{cip1}$, $p27^{kip1}$, cyclins D1, D3, A1, A2, and E, CDK 2, and CDK 4 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An antibody specific for glycerol-3-phosphate dehydrogenase (G3PDH) was purchased from Biogenesis (Kingston, NH). Unless otherwise specified, other chemicals used in this study were purchased from Sigma Chemical Co.

Cell Culture. RASMCs were harvested from the thoracic aortas of adult male Sprague−Dawley rats (200−250 g) by enzymatic dissociation. All procedures were approved by the Taipei Medical University Animal Care and Use Committee. The cells were grown in DMEM supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES (pH 7.4) in a humidified 37 °C incubator. After the cells had grown to confluence, they were disaggregated in trypsin solution, washed with DMEM containing 10% FBS, centrifuged at 125g for 5 min, resuspended, and then subcultured according to standard protocols. Cells from passages 5−9 were used. β-Sitosterol was prepared by dissolving in 100% ethanol, and the final concentration of ethanol in the medium was 0.5% (*25*). All of the control cells in this study were supplemented with medium containing the same concentration of ethanol (0.5%) as were the β-sitosterol-treated cultures.

Cell Viability Assay. RASMCs were grown to 80% confluence and treated with various concentrations of β-sitosterol for 24 h. Cell viability was determined by MTT assay.

[^{3}H]Thymidine Incorporation Assay. As previously described (*26*, *27*), RASMCs at a density of 1×10^4 cells/cm^3 were applied to 24-well plates in growth medium (DMEM plus 10% FBS). After the cells had grown to 70−80% confluence, they were rendered quiescent by incubation for 72 h in DMEM containing 0.04% FBS. β-Sitosterol at various concentrations or ethanol (control) was added to the cells, and the mixture was allowed to incubate for 24 h. During the last 3 h of the incubation with or without β-sitosterol, [^{3}H]thymidine was added at 1 μCi/mL (1 μCi = 37 kBq). The cells were incubated with 500 μL of 10% trichloroacetic acid (TCA) at 4 °C overnight, and incorporated [^{3}H]thymidine was then extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

Cell Proliferation Assay. The proliferation of RASMCs was measured by a direct cell counting. Briefly, cells were seeded at a concentration of 4×10^4 cells/well in a 12-well culture plate and grown in DMEM containing 10% FBS for 24 h. The cells were then rendered quiescent and challenged with 10% FBS. The cells were treated with various concentrations of β-sitosterol and counted at indicated times using a hemocytometer.

Immunoblotting Analysis. Protein lysates were prepared as previously described (*28*). Western blot analysis was performed using primary antibodies against $p21^{cip1}$, $p27^{kip1}$, cyclins A1, A2, D1, D3, and E, CDKs 2 and 4, or G3PDH.

Immunoprecipitation. As previously described (*27*), CDK2 or CDK4 was immunoprecipitated from 200 μg of protein by using anti-CDK2 or anti-CDK4 antibody (2 μg) and protein A agarose beads (20 μL). The precipitates were washed five times with lysis buffer and once with PBS. The pellet was then resuspended in sample buffer (50 mm Tris, pH 6.8; 100 mM bromophenol blue; 10% glycerol) and incubated at 90 °C for 10 min before electrophoresis to release the proteins from the beads.

Kinase Assay. As previously described (*29*), CDK2 or CDK4 immunoprecipitates from β-sitosterol- or vehicle-treated RASMCs were washed three times with lysis buffer and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 1 mM DTT). Phosphorylation of histone H1 (for CDK2) and glutathione-*S*-transferase fusion protein (for CDK4) was measured by incubating the beads with 40 μL of hot kinase solution [0.25 μL (2.5 μg) of histone H1, 0.5 μL of (γ-^{32}P) ATP, 0.5 μL of 0.1 mM ATP, and 38.75 μL of kinase buffer] at 37 °C for 30 min. The reaction was stopped by boiling the sample in SDS sample buffer for 5 min, and the products were analyzed by 10% SDS-PAGE. The gel was dried and visualized by autoradiography.

Flow Cytometric Analysis. As previously described (*18*), RASMCs were grown in DMEM supplemented with 10% FBS. After the cells had grown to subconfluence, they were rendered quiescent and then challenged with 10% FBS. After release using trypsin−EDTA, they were harvested at indicated times, washed twice with PBS/0.1% dextrose, and fixed in 70% ethanol at 4 °C. Nuclear DNA was stained with a reagent containing propidium iodine (50 mg mL^{-1}) and DNase-free RNase (2 U mL^{-1}) and measured using a fluorescence-activated cell sorter (FACS). The proportion of nuclei in each phase of the cell cycle was determined using established WinMDI 2.9 DNA analysis software.

Small Interfering RNA Transfection. Silencer predesigned small interference RNA (siRNA) for rat $p21^{cip1}$ (sc-108036) and a nonrelated control siRNA-A (sc-37007) were obtained from Santa Cruz Biotechnology Inc. RASMCs were seeded in 6-well culture plates. After the cells had grown to 70% confluency, the cells were transfected with the siRNA duplexes using GenMute siRNA transfection reagent (SignaGen Laboratories) according to the manufacturer's instructions. Cellular levels of the protein specific for the siRNA transfection were checked by immunoblotting analysis, and all experiments were performed at 6 h after transfection.

Statistical Analysis. Values represent the means ± standard error of the mean (SEM). Three to six samples were analyzed in each experiment. Comparisons were subjected to one-way ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

RESULTS

Effects of β-Sitosterol on RASMCs Proliferation and DNA Synthesis. Initially, we examined the effect of β-sitosterol (**Figure 1A**) on the viability of RASMCs. As shown in **Figure 1B**, the concentrations of β-sitosterol (0.1−10 μM) used in this experiment and at other laboratories did not affect the viability of RASMCs. To further evaluate the effect of β-sitosterol on cell proliferation and DNA synthesis, RASMCs were rendered quiescent and their cell cycle activities synchronized by incubation of the cells in medium containing 0.04% FBS for 72 h. They were then returned to medium with 10% FBS and treated with various concentrations of β-sitosterol at various time points. **Figure 2A** shows that treatment of RASMCs with β-sitosterol for 3 or 6 days resulted in a significant decrease in cell numbers in a concentration-dependent manner. The level of [^{3}H]thymidine incorporation into RASMCs was also measured as an index of cell proliferation. Similarly, treatment of RASMCs with β-sitosterol concentration-dependently inhibited the [^{3}H]thymidine incorporation into the cells (**Figure 2B**).

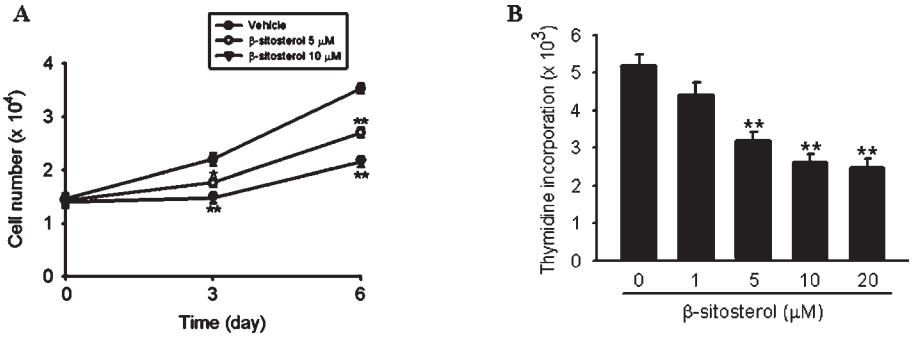

Figure 2. Effects of β-sitosterol on the proliferation and DNA synthesis of RASMCs. Cells were released from quiescence by incubation in culture medium supplemented with 10% FBS and ethanol vehicle (0.5%) or β-sitosterol (1−20 μM) for the indicated times (3−6 days for proliferation assay; 24 h for [^{3}H]thymidine incorporation assay). The cell number (**A**) and [^{3}H]thymidine level incorporated (**B**) were determined as described under Materials and Methods. Values represent the mean ± SEM (n = 3). ∗, P < 0.05; ∗∗, P < 0.01, as compared with the vehicle-treated control group.

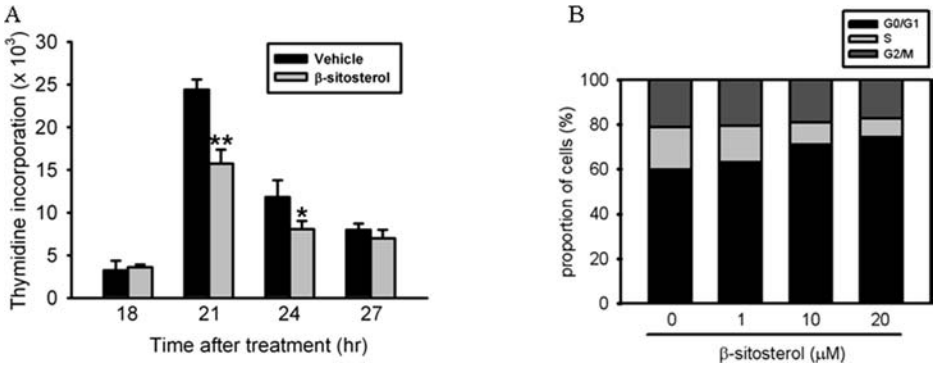

Figure 3. Time-dependent inhibition of cell cycle progress in RASMCs by β-sitosterol. To study the time-dependent effect of β-sitosterol on the cell cycle progress, [^{3}H]thymidine incorporation was conducted after cells were released from quiescence by incubation in culture media supplemented with 10% FBS and ethanol vehicle (0.5%) or 20 μM β-sitosterol for the indicated times (**A**). The mean amount of [^{3}H]thymidine level incorporated was calculated. Values represent the mean ± SEM (n = 3). ∗, P < 0.05; ∗∗, P < 0.01, as compared with the vehicle-treated control group. FACS analysis of DNA content was performed 21 h after release from quiescence by incubation in culture media supplemented with 10% FBS and ethanol vehicle (0.5%) or with 20 μM β-sitosterol (**B**). The percentage of cells at the G0/G1, S, or G2/M phase of the cell cycle was determined using established WinMDI 2.9 DNA analysis software. The results are from one representative experiment of three performances showing similar patterns.

Effects of β-Sitosterol on RAMSCs Entry into the S Phase. To further study the actions of β-sitosterol on the cell cycle, RASMCs were synchronized by switching them to medium with 0.04% FBS for 72 h to render them quiescent. **Figure 3A** shows a reduction of the [^{3}H]thymidine incorporation into RASMCs during the S phase of the cell cycle, and the maximum response time point was at 21 h after β-sitosterol treatment. **Figure 3B** shows the FACS analyses of DNA content at 21 h after release from quiescence by incubation in culture medium supplemented with 10% FBS and ethanol or β-sitosterol (1−20 μM) in ethanol. The data reveal that β-sitosterol induced a significant accumulation of cells at the G0/G1 phase of the cell cycle, suggesting that the observed growth inhibition effect of β-sitosterol was due to a retardation of DNA replication, thereby inhibiting further progress in the cell cycle.

Effects of β-Sitosterol on Cell Cycle Regulatory Protein. It has been generally believed that coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S phase. This CDK activation is in turn modulated by association with a number of regulatory subunits called cyclins and a group of CDK-inhibitory proteins designated CKIs. Cyclins have been identified as cyclins A1, A2, D1, D3, and E. As illustrated in **Figure 4A**, Western blot analysis demonstrated that β-sitosterol (1−20 μM) inhibited the protein levels of cyclin E, but not cyclins A1, A2, D1, and D3. We also examined the changes of CDK levels in the β-sitosterol-treated RASMCs. In response to β-sitosterol treatment, the protein levels

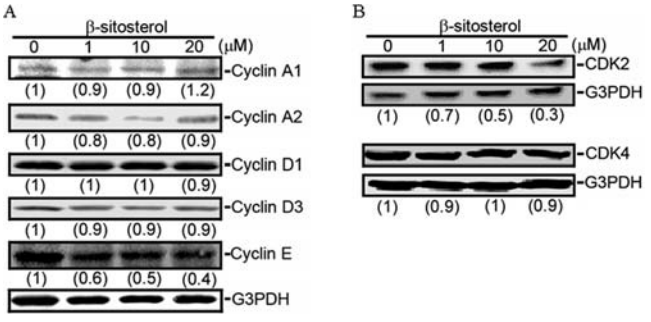

Figure 4. Effect of β-sitosterol on the protein levels of cyclins and CDKs in RASMCs. Cells were treated with various concentrations (1−20 μM) of β-sitosterol for 21 h, and the cell lysates for protein extraction were prepared and adjusted to Western blot analysis. The membrane was probed with proper dilutions of specific antibodies. The membrane was also probed with anti-G3PDH antibody to verify equivalent loading. The results are from one representative experiment of three performances.

of CDK2, but not CDK4, were decreased significantly (**Figure 4B**). Because not only cyclins and CDKs but also CKIs can control the CDK activity, we further examined the protein levels of p21^{cip1} and p27^{kip1}, two known CKIs, in the β-sitosterol-treated RASMCs. **Figure 5A** shows that the protein level of p21^{cip1}, but not p27^{kip1}, was concentration-dependently increased in the β-sitosterol-treated RASMCs. We then conducted an immunoprecipitation

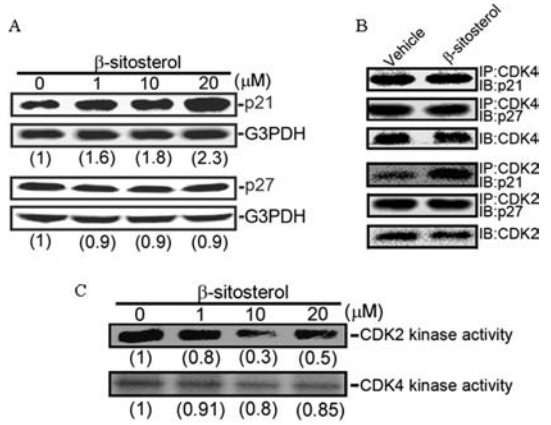

Figure 5. Effect of β-sitosterol on levels of CKI protein, CKI−CDK association, and CDK kinase activity. (**A**) Concentration-dependent increase of p21 but not p27 protein level after β-sitosterol (1−20 μM) treatment for 21 h in RASMCs. The membrane was probed with anti-G3PDH antibody to verify equivalent loading. Values shown in parentheses represent the quantified results after adjustment with their own G3PHD. (**B**) Treatment of RASMCs with 20 μM β-sitosterol induced increase of the formation of CDK2−p21 complex. The formations of CDK2-p27, CDK4-p21, and CDK4-p27 complex were not changed significantly by β-sitosterol treatment. CDK2 was immunoprecipitated by anti-CDK2 antibody, and CDK2−p21 complex was detected by anti-p21 antibody, whereas CDK2−p27 complex was detected by anti-p27 antibody. CDK4 was immunoprecipitated by anti-CDK4 antibody, and CDK4−p21 complex was detected by anti-p21 antibody, whereas CDK4−p27 complex was detected by anti-p27 antibody. (**C**) CDK2 kinase activity was decreased dose dependently by β-sitosterol treatment, whereas CDK4 kinase activity was not significantly changed. The activities of CDK2 and CDK4 were determined as described under Materials and Methods. The results are from one representative experiment of three performances showing similar patterns. Values shown in parentheses represent the quantified results.

assay to examine the effect of β-sitosterol on the formation of CDK−CKI complex. In β-sitosterol-treated cells, the formation of the CDK2−$p21^{cip1}$ complex, but not CDK2−$p27^{kip1}$, CDK4−$p21^{cip1}$, and CDK4−$p27^{kip1}$ complex, was increased (**Figure 5B**), and the assayable CDK2, but not CDK, activity was decreased (**Figure 5C**). These findings suggest that β-sitosterol induced an inhibition of the CDK2 activity, which in turn led to an impairment of RASMCs in the transition from the G1 to the S phase.

$p21^{cip1}$ Is the Key Regulator for β-Sitosterol-Induced G0/G1 Arrest. As illustrated in **Figure 5A**,**B**, the levels of $p21^{cip1}$ protein and formation of CDK2−$p21^{cip1}$ complex were increased significantly in β-sitosterol-treated RASMCs, suggesting that up-regulation of $p21^{cip1}$ might be responsible for the β-sitosterol-induced G0/G1 arrest in these cells. To further demonstrate that in the β-sitosterol-treated RASMCs, increased $p21^{cip1}$ expressions correlated with G0/G1 arrest, the experiment illustrated in **Figure 6** was conducted. **Figure 6A** shows that transfection of RAMSCs with $p21^{cip1}$ siRNA, but not nonspecific siRNA, prevented the β-sitosterol-induced increase of the $p21^{cip1}$ protein level. The blockage of $p21^{cip1}$ induction by siRNA transfection significantly inhibited the β-sitosterol-induced decreases in the cell cycle progression (**Figure 6B**). These findings verify that β-sitosterol induced cell cycle arrest of RASMCs at the G0/G1 phase through the up-regulation of $p21^{cip1}$.

DISCUSSION

Cardiovascular diseases are associated with a multitude of pathophysiologic conditions, such as inflammation, pulmonary hypertension, coronary artery restenosis following balloon angioplasty, and proliferation of VSMCs in response to vessel injury (*30*). Inhibiting the proliferation of VSMCs is an important strategy for the treatment of cardiovascular diseases. Numerous studies have suggested that the beneficial effects of β-sitosterol may be due to its inhibitory activity against abnormal cell proliferation and of its capability in inducing apoptosis in prostate (*23*),

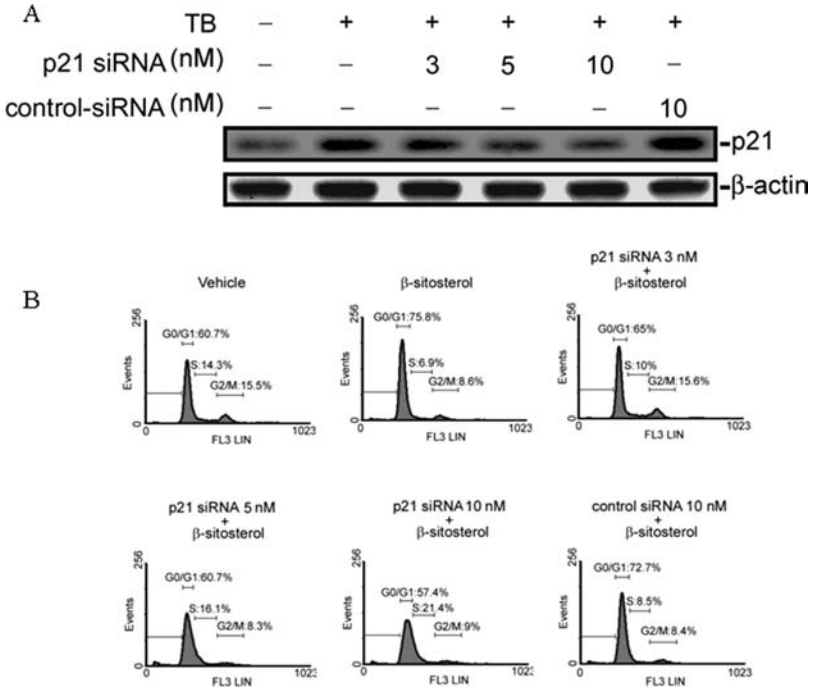

Figure 6. Critical role of $p21^{cip1}$ in sitosterol-induced cell cycle arrest in RASMCs. (**A**) RASMCs were transfected with either $p21^{cip1}$-specific siRNA (3−10 nM) or control siRNA (10 nM) using GenMute siRNA transfection reagent, and at 6 h after transfection, the expression level of $p21^{cip1}$ was determined by Western blot assay. (**B**) In addition, the transfected cells were synchronized for 48 h by using medium containing 0.04% FBS and treated with 10% FBS and ethanol vehicle (0.5%) or 20 μM β-sitosterol for an additional 21 h, and then the cells were processed for the analysis of cell cycle progression.

colon (*19*), breast (*20*), stomach (*31*), and leukemia (*32*) cancer cells. Phytosterols have been known to reduce serum cholesterol concentrations by competing with dietary and biliary cholesterol for intestinal absorption. Clinically, phytosterols and their esters are used to reduce the plasma levels of LDL-C and atherosclerotic risk. However, a direct effect of phytosterols on VSMC proliferation has not been studied. In the present study, we examined the antiproliferative activity of β-sitosterol, one kind of phytosterol, on primary cultured RASMCs. Our current study demonstrates for the first time the cellular mechanism underlying β-sitosterol-induced antiproliferation on RASMCs.

It is commonly accepted that an active substance inhibits cell proliferation by either a cytostatic or a direct cytotoxic action. β-Sitosterol significantly decreased the cell number and [^{3}H]thymidine uptake into RASMCs (**Figure 2**), but did not affect their viability at the same conditions (**Figure 1B**), suggesting that β-sitosterol appeared to exert an antiproliferative effect on RASMCs through cytostatic rather than other actions. We also conducted flow cytometric analysis to further confirm that β-sitosterol arrested the RASMCs at the G0/G1 phase of the cell cycle (**Figure 3B**).

Observation of intracellular events associated with the progression of cell cycle activity has suggested that coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S phase (*33*, *34*). This CDK activation is in turn modulated by association with a series of regulatory subunits called cyclins and with a group of CDK-inhibitory proteins designated CKIs (*35*). Cyclins have been identified as cyclins A, D1, D3, and E, whereas the most common CDKs are designated CDK2 and CDK4. CKIs such as $p21^{cip1}$ and $p27^{kip1}$ are important regulators of cyclin−CDK complexes. They can inhibit the kinase activities of several cyclin−CDK complexes, such as cyclin D−CDK4/6 and cyclin E−CDK2, and also arrest cell proliferation at the G0/G1 and G1/S boundary (*1*, *7*−*10*). Our current findings demonstrate that β-sitosterol decreased the levels of cyclin E and CDK2 protein, but not cyclins A1, A2, D1, and D3, and CDK4 protein. Moreover, treatment of RASMCs with β-sitosterol resulted in an increase in the protein level of $p21^{cip1}$, but not $p27^{kip1}$. In the β-sitosterol-treated RASMCs, the formation of the CDK2−$p21^{cip1}$ complex was increased and the assayable CDK2 kinase activity was decreased. In contrast, the formations of the CDK2−$p27^{kip1}$, CDK4−$p21^{cip1}$, and CDK4−$p27^{kip1}$ complex and the assayable CDK4 kinase activity were not changed significantly. All of these findings were similar to the results of previous studies showing that some drugs exert their antiproliferative activity by up-regulating the $p21^{cip1}$ and/or $p27^{kip1}$ expression in vascular smooth muscle cells (*36*, *37*). However, in other kinds of cells, β-sitosterol exerted an antiproliferative effect via a different mechanism from VSMCs. For example, β-sitosterol induces G2/M arrest, endoreduplication, and up-regulation of $p21^{cip1}$ and CDK2 in leukemic cells (*32*) or activation of pro-caspase-3 and suppression of Bcl-2 in stomach cancer cells (*31*).

Previously, it has been demonstrated that $p21^{cip1}$ is a very important regulator of RASMCs proliferation in vitro and in vivo (*37*). The important role of $p21^{cip1}$ in the β-sitosterol-induced G0/G1 cell cycle arrest was further confirmed by demonstration that knockdown of $p21^{cip1}$ in the RASMCs with a $p21^{cip1}$-specific siRNA reduced β-sitosterol-induced inhibition in the cell cycle progression. Accordingly, we concluded that β-sitosterol inhibited RASMCs proliferation through increasing the levels of $p21^{cip1}$ protein, which in turn inhibited the CDK2 kinase activity and, finally, impaired the transition of the cells from the G1 phase to the S phase.

β-Sitosterol is a major variety of phytosterol in plant food. Thus, people can ingest phytosterols from their everyday diet, and the daily intake amount is 160−400 mg/day (*38*). Therefore, the physiological concentrations in human blood can be kept relatively constant by eating a diet enriched in phytosterols. The effective concentration of β-sitosterol used in this studies is even lower than the physiological human blood ranges (4−70 μM) (*39*). Thus, the findings from the present studies and previous in vivo studies suggest the potential applications of β-sitosterol in the treatment of atherosclerosis.

ABBREVIATIONS USED

CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; G3PDH, glycerol-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; RASMCs, rat aortic smooth muscle cells; siRNA, small interference RNA; VSMCs, vascular smooth muscle cell.

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