JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Ginsenoside Rb1 Inhibits Proliferation and Inflammatory Responses in Rat Aortic Smooth Muscle Cells

Qun-Yi Li,[†,∥] Li Chen,[§,#,∥] Wen-Huan Fu,[†] Zhong-Dong Li,[†] Bin Wang,[†] Xiao-Jin Shi,[†] and Ming-Kang Zhong*[,†]

[†]Clinical Pharmacy Laboratory, Huashan Hospital, Fudan University, 12 Wu Lu Mu Qi M Road, Shanghai 200040, China

[§]Department of Preventive Medicine, Tongji University School of Medicine, Shanghai 200092, China

[#]Department of Pharmacy, Xuhui District Central Hospital, Shanghai 200031, China

ABSTRACT: Ginsenoside Rb1, a known phytoestrogen, is a major pharmacologically active component in ginseng. The present study was designed to investigate the effect of ginsenoside Rb1 on fetal bovine serum (FBS)-induced proliferation and tumor necrosis factor-α (TNF-α)-evoked inflammatory responses in cultured rat aortic vascular smooth muscle cells (VSMCs). The data showed that Rb1 potently inhibited VSMC proliferation and cell growth induced by 5% FBS. These inhibitory effects were associated with G_1 cell cycle arrest and down-regulation of cell cycle proteins. Treatment with Rb1 reduced FBS-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Furthermore, TNF-α-evoked inflammatory responses were inhibited by Rb1. Reporter gene assay indicated that Rb1 could transactivate ERβ especially. Moreover, Rb1-mediated inhibition of VSMCs proliferation was greatly blocked by transfection of ERβ siRNA. These results suggest that Rb1 inhibits FBS-induced proliferation and TNF-α-evoked inflammatory responses in VSMCs. The findings presented here highlight the possible therapeutic use of Rb1 in cardiovascular disease.

KEYWORDS: ginsenoside Rb1, phytoestrogen, estrogen receptor, vascular smooth muscle cells, proliferation, inflammation

INTRODUCTION

It is well accepted that the onset age of cardiovascular disease is on average delayed in women, despite the disease being an equally prevalent cause of death among men and women.[1] Coronary heart disease (CHD) in premenopausal women is extremely uncommon, suggesting a beneficial role of female sex steroid hormones.[2] To date, several lines of evidence have indicated the multiple cardioprotective effects of estrogens on the cardiovascular system.[3] More recently, the Women's Health Initiative (WHI) hormone trials found no CHD benefit among women aged 50 to 79 years with no prior CHD diagnosis and hormone replacement therapy (HRT) increased risk of stroke.[4,5] However, WHI has also provided evidence that CHD risks are reasonably low for short-term use by younger postmenopausal women, suggesting a need to better understand the relationship of circulating estrogen levels and aging of the cardiovascular system.[6]

Considering the unacceptable adverse effects caused by synthetic hormones, clinical use of HRT as a cardioprotective strategy is largely limited. As a result, many women turn to phytoestrogens as an alternative to hormone replacement therapy. There is emerging evidence that suggests the beneficial effects of estrogen-like agents on cardiovascular diseases.[7]

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays a critical role in intimal formation in the early stage of atherosclerosis and restenosis.[8] Several studies have indicated that reduction of VSMC proliferation using estrogens would give rise to vasoprotective effects.[9] Estrogen exhibits a variety of actions on the vascular wall that could be implicated in its atheroprotective properties. These include, but are not limited to, the inhibition of VSMC proliferation.[10,11]

Ginseng has become one of the most commonly consumed alternative herbal medicines in the West. Ginseng has been shown to exert beneficial effects in the cardiovascular, endocrine, immune, and central nervous systems.[12] Ginsenosides, triterpene saponins containing a rigid steroidal skeleton with sugar moieties, are believed to be responsible for the pharmacological actions of ginseng. Ginsenoside Rb1, a known phytoestrogen, is one of the major pharmacologically active components in ginseng.

Recently, an increasing amount of evidence has indicated that Rb1 possesses cardioprotective effects both in vitro and in vivo. In in vitro studies, ginsenoside Rb1 was reported to protect human endothelial cells against oxidized low-density lipoprotein (oxLDL),[13] homocysteine,[14] and tumor necrosis factor-α (TNF-α).[15] In vivo studies demonstrated the protective effects of Rb1 against homocysteine-induced intimal hyperplasia in C57BL/6J mice[16] and ApoE knockout mice.[17] These findings indicate the therapeutic potential of Rb1 as a novel strategy to treat cardio-related diseases; however, the role for Rb1 in modulating VSMC proliferation and the possible mechanism is still unknown.

In the present study, we aimed to investigate whether Rb1 inhibits VSMC proliferation and inflammatory responses. The results presented here clearly show that Rb1 potently inhibits VSMC proliferation, and this regulation is, at least in part, ERβ-dependent.

MATERIALS AND METHODS

Materials and Reagents. Rb1 is a reference compound (purity ≥ 97%) supplied by Tauto Biotech (Shanghai, China). β-Estradiol 17-acetate (E_2) and BrdU were obtained from Sigma (St. Louis, MO).

Received: January 29, 2011
Revised: April 26, 2011
Accepted: April 27, 2011
Published: April 27, 2011

TNF-α was the product of Invitrogen Corp. (Carlsbad, CA). Polyclonal anti-β-actin, monoclonal antiphospho-ERK1/2, and polyclonal antibody to ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA for silencing ERβ was from Dharmacon (Lafayette, CO).

Cell Culture. Primary VSMCs were obtained from the thoracic aorta of 3-month-old female Sprague−Dawley rats weighing 150−180 g using the tissue explant method, as described previously.[18] The experimental protocol was approved by the local ethics committee. More than 98% of the cells were positive for smooth muscle-specific α-actin and exhibited the typical hill-and-valley morphology of VSMCs. Cells between passages 2 and 5 were used in these studies to ensure the genetic stability of the culture. Cells grown to 80−95% confluence were made quiescent by starvation (0.1% FBS) for 24 h. Rb1 was administered 2 h before treatment with FBS.

BrdU Incorporation Assay. DNA synthesis in VSMCs was examined using the BrdU incorporation assay according to the method described previously.[19] Quiescent VSMCs were pretreated with or without Rb1 for 2 h prior to stimulation with 5% FBS for 48 h. Cell transfection was performed using siLentfect regent (Bio-Rad, Carlsbad, CA) according to the manufacturer's instructions. In total, 10 nM ERβ small interfering RNA (siRNA) was added per well. After 24 h, the cells were incubated with or without Rb1 for 2 h prior to stimulation with 5% FBS for another 24 h.

Subsequently, 10 μM BrdU was added to the cells and incubated for another 24 h. To immunostain for BrdU, the cells were washed with PBS, fixed in 4% polyformaldehyde, and then permeabilized with 0.1% Triton X-100. After DNA denaturation with 4 M HCl, nonspecific binding sites were blocked with 5% nonfat milk. The cells were then stained with antibody for BrdU followed by incubation with the Alexa Flour 568 goat anti-mouse IgG (Invitrogen) secondary antibody. The cell nuclei were stained with Hoechst 33342 and evaluated by fluorescence microscopy with the appropriate fluorescent filters. Results are presented as mitotic index and defined as the percentage of BrdU-positive nuclei per number of cells.

Cell Number Counting. To assess VSMC growth, the number of cells in each sample was determined by removing the cells from the plates using trysinization and then counting them using a hemocytometer, as previously described.[20]

Flow Cytometry Analysis of Cell Cycle. Quiescent VSMCs were pretreated with or without Rb1 for 2 h, followed by 5% FBS treatment for 24 h. Cells were then trypsinized, collected, and washed twice with cold PBS. Cells pellets were fixed in 70% ethanol and stored at 4 °C. Next, the fixed cells were treated with RNase A (10 μg/mL). DNA was stained with propidium iodide (50 μg/mL) for 30 min at 37 °C, and 1×10^4 cells were analyzed by flow cytometry. The rates of G_0/G_1, S, and G_2/M phases were determined using the computer program ModiFit LT.

Western Blot Analysis. VSMCs were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1% Triton. Equal amounts of protein from each sample were subjected to SDS-PAGE and blotted on PVDF membrane, which was incubated for 2 h at room temperature with blocking buffer (5% nonfat milk, 0.1% Tween 20, in TBS, pH 7.6) and then probed with primary antibodies overnight at 4 °C. After incubation with the appropriate secondary antibodies, the immunoreactive band was detected by an ECL Western blotting detection system (GE Healthcare) and subsequently photographed by an LAS-3000 luminescent image system (Fujifilm, Tokyo, Japan).

Quantitative Real-Time PCR. Total RNA from VSMCs was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed (RT) using Superscript II First-strand Synthesis SuperMix (Invitrogen). Primers and real-time PCR conditions for cell cycle proteins[21] and inflammatory mediators[22] were described previously. For RT-PCR, SYBR Premix Ex Taq (Takara Bio Inc., Dalian, China) was used. Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. Reactions with no RT sample and no template were included as negative controls. Relative quantitative evaluation of target gene levels was performed by the comparative CT (cycle threshold) method[23] and performed in triplicate.

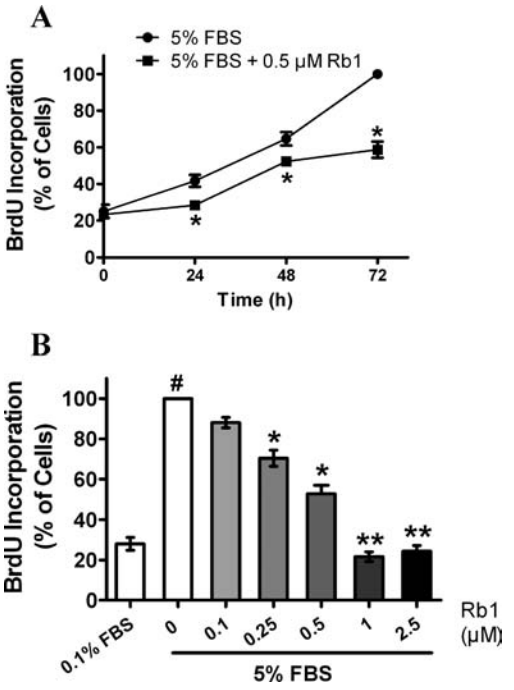

Figure 1. Effects of Rb1 on FBS-induced DNA synthesis in VSMCs. (A) DNA synthesis, determined by measuring the incorporation of BrdU, was assessed in VSMCs isolated from female rats treated with 0.5 μM Rb1. Relative proliferation (%) is displayed using untreated control cells as a standard ($n = 3$). (B) DNA synthesis was measured in VSMCs treated for 72 h with 0.1−2.5 μM. Data (mean ± SEM) are representative of three independent experiments. #, $P < 0.01$ versus control; ∗, $P < 0.05$, and ∗∗, $P < 0.01$, versus FBS induction.

Statistical Analysis. Results are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). The intergroup comparisons (post hoc analysis) among the data with equal variances were made by the LSD method, whereas Tamhane's T2 method was used for the data with unequal variances. A P value of <0.05 was considered to be significant.

RESULTS

Rb1 Inhibited FBS-Induced VSMCs Proliferation. In this study, we first investigated the effects of Rb1 on the proliferation of rat VSMCs. Rates of DNA synthesis as measured by BrdU incorporation assay have been used as an indication of VSMC proliferation. As shown in Figure 1A, a low dose of Rb1 (0.5 μM) signtificantly attenuated VSMC proliferation by approximately 30% versus control (5 FBS% alone) at 24 h. Furthermore, this effect was sustained through 72 h of treatment.

Next, we determined the dose−response for Rb1-mediated effects on VSMC proliferation. Figure 1B shows that Rb1 displayed a dose-dependent, 12−76% inhibitory effect in VSMC

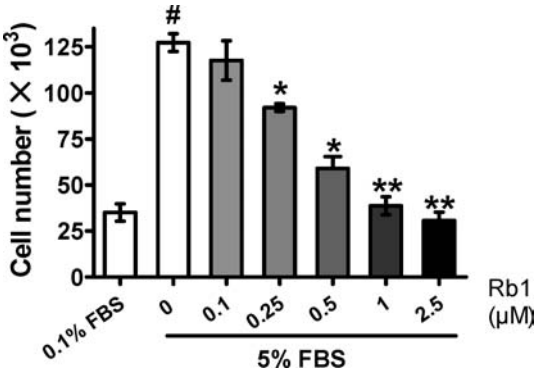

Figure 2. Effects of Rb1 on FBS-induced VSMC growth. VSMCs were treated with vehicle or Rb1 for 72 h, and cell number was quantified to indicate cell growth. Data (mean ± SEM) are representative of three independent experiments. #, $P < 0.01$ versus control; ∗, $P < 0.05$, and ∗∗, $P < 0.01$, versus FBS induction.

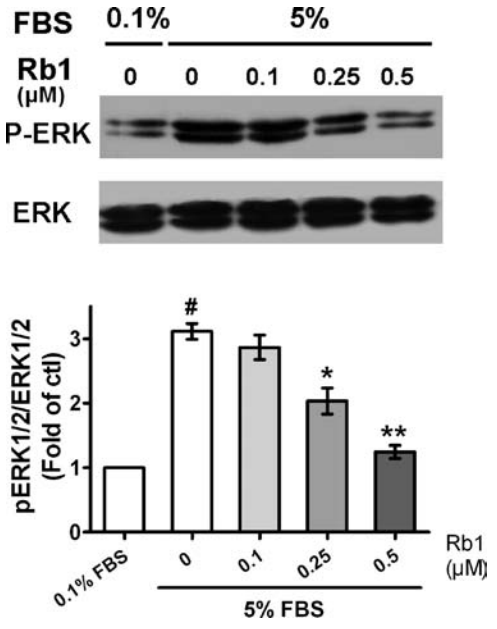

Figure 3. Effects of Rb1 on FBS-induced ERK1/2 activation in VSMCs. VSMCs were treated with Rb1 for 2 h prior to FBS (5%) stimulation for 15 min. Whole cell lysates were prepared and subsequently used for detection of p-ERK1/2 and ERK1/2 by Western blot ($n = 3$). #, $P < 0.01$ versus control; ∗, $P < 0.05$, and ∗∗, $P < 0.01$, versus FBS induction.

DNA synthesis at 72 h after treatment. The IC_{50} value of Rb1-mediated inhibition on VSMC proliferation was 0.57 ± 0.13 μM, with doses as low as 0.1 μM decreasing proliferation (Figure 1B).

To ascertain whether effects of Rb1 on DNA synthesis were translated into inhibition on cell growth, the cell number of VSMCs was measured 72 h after 0.5 μM Rb1 was added to cells. In line with the observation of Rb1-mediated inhibition of DNA synthesis, Rb1 potently reduced the cell number of VSMCs in a dose-dependent manner after 72 h of treatment (Figure 2).

Rb1 Suppressed FBS-Induced ERK1/2 Phosphorylation in VSMCs. It has been suggested that ERK1/2 activation plays a critical role in the proliferation of VSMCs.[24] Inhibition of this signaling protein proved to be a useful method for preventing intimal hyperplasia.[24] Therefore, we investigated the effect of

A

	G0/G1	S	G2/M
Quiescence (0.1% FBS)	75.0	5.7	19.3
5% FBS	55.3	15.4	26.3
5% FBS + Rb1 (1 μM)	69.4	6.5	24.1

B

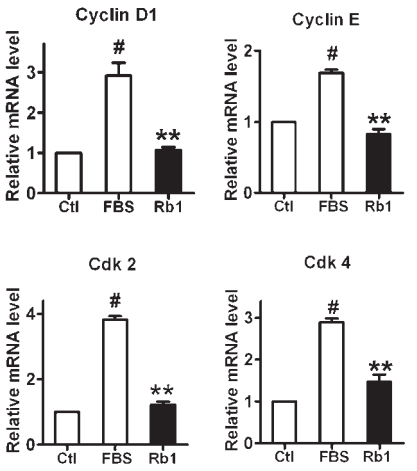

Figure 4. Effects of Rb1 on cell cycle distribution and mRNA levels of cell cycle regulators. (A) Effect of Rb1 on cell cycle distribution. VSMCs were incubated with 0.1% FBS for 24 h to induce quiescence, followed by addition 5% FBS with or without 1 μM Rb1 for 24 h of incubation and FACS analysis ($n = 2$). (B) VSMCs were stimulated with 5% FBS in the presence or absence of 1 μM Rb1, and real-time PCR was performed to determine the expression of cyclin D1, cyclin E, CDK 2, and CDK 4. Data (mean ± SEM) are representative of three independent experiments. #, $P < 0.01$ versus control; ∗∗,$P < 0.05$, and ∗∗, $P < 0.01$, versus FBS induction.

Rb1 on ERK1/2 activation, and the results indicate that exposure of VSMCs to FBS for 15 min enhanced ERK1/2 phosphorylation considerably. Conversely, addition of Rb1 dose-dependently abrogated ERK1/2 activation in FBS-stimulated VSMCs: at a concentration of 0.5 μM, Rb1 completely blocked the phosphorylation of ERK1/2 (Figure 3).

Rb1 Arrested FBS-Stimulated VSMCs in G_1 Phase and Abrogated Cell Cycle Protein Transcription. Proliferative cells pass through several cell cycle checkpoints, mainly the G_1 to S and G_2 to M transitions. The former checkpoint is considered to be the most important step in DNA replication. Accordingly, flow cytometric assessment was performed to determine whether the Rb1-induced cell proliferation inhibition is due to an arrest in a specific point of the cell cycle. As shown in Figure 4A, the percentages of G_0/G_1 or S phase cells in the 5% FBS-stimulated group were 55.3 and 15.4%, respectively. Rb1 at concentration of 1 μM effectively increased the proportion of cells in the G_0/G_1 phase and simultaneously decreased the S phase cell population.

We next examined the mRNA levels of cell cycle-related proteins by use of real-time PCR to determine whether Rb1 induced changes in this pathway. It was shown that treatment

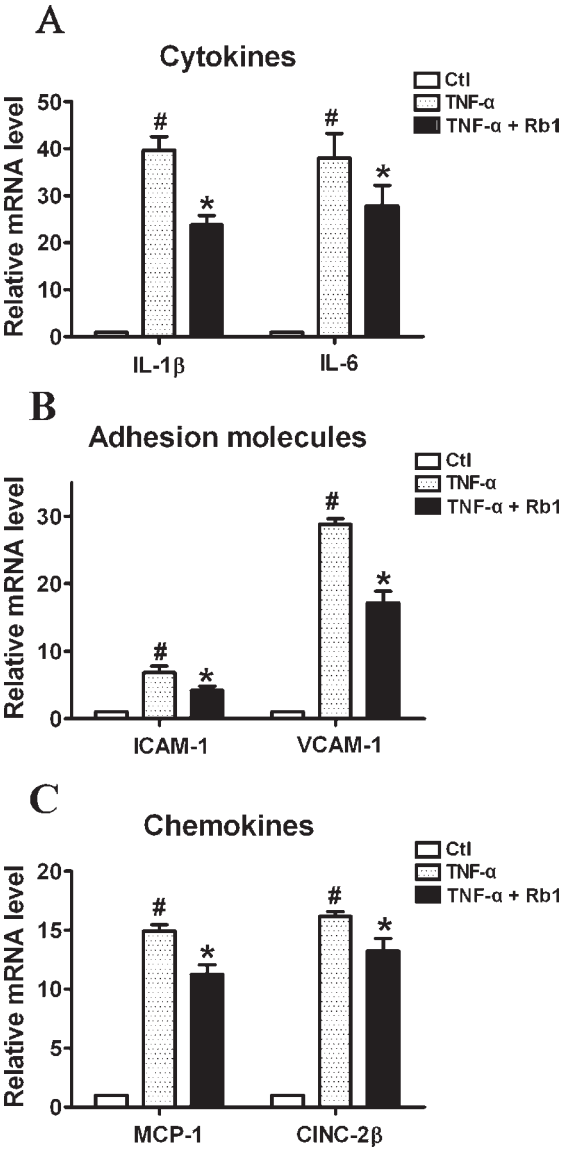

Figure 5. Effects of Rb1 on TNF-α-induced mRNA expression of cytokines (A), adhesion molecules (B), and chemokines (C) in VSMCs. Data (mean ± SEM) are from real-time quantitative RT-PCR assays and are normalized by β-actin ($n = 3$). #, $P < 0.01$ versus control; ∗, $P < 0.05$ versus 5 ng/mL TNF-α induction.

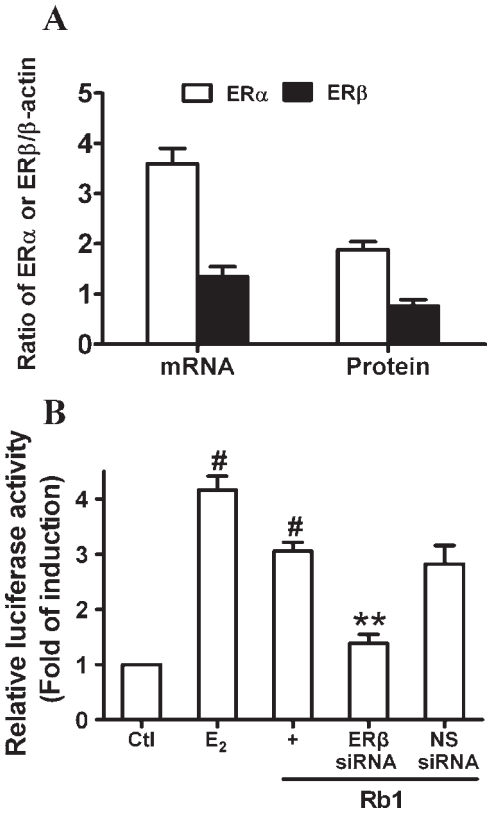

Figure 6. Rb1 activates ERE-mediated transcription in VSMCs: (A) expression of both ER types in VSMCs determined by real-time PCR and Western blot analysis ($n = 3$); (B) cells cotransfected with pERE-luc and β-gal expression plasmids treated with 1 μM Rb1 with or without 10 nM ERβ siRNA. E_2 (100 nM) was used as a positive control. Relative luciferase units (RLU) normalized to transfection efficiency were calculated as the ratio of luciferase activity to β-galactosidase. Data (mean ± SEM) are representative of three independent experiments. #, $P < 0.01$ versus control; ∗∗, $P < 0.01$ versus Rb1 treatment.

with Rb1 not only produced a significant down-regulation in cyclin D1 and E gene transcription but also markedly decreased the expression of CDK 2 and CDK 4 genes (Figure 4B).

Rb1 Down-Regulated mRNA Levels of Inflammatory Mediators Induced by TNF-α in VSMCs. Inflammation plays an important role in the pathogenesis of many forms of vascular disease, including atherosclerosis and the response to acute vascular injury.[25] It has been shown that VSMCs would produce inflammatory mediators such as adhesion molecules and chemokines when stimulated with cytokines.[25] Therefore, we evaluated the effects of Rb1 on TNF-α-induced excretion of inflammatory factors in VSMCs. All mediators were expressed at low levels in unstimulated vehicle-treated VSMCs (Figure 5). Pretreatment of Rb1 (1 μM) significantly inhibited expression of IL-1β, IL-6, ICAM-1, VCAM-1, CINC-2β, and MCP-1 in cells treated with TNF-α (5 ng/mL, Figure 5).

Rb1 Transactivated ERβ in VSMCs. It has been suggested that Rb1 binds speficially to ERβ ($K_d = 70$ nM) in a competitive binding assay and suppresses the formation of endothelial tube-like structures in HUVEC via ERβ, but not ERα.[26] Thus, we want to examine the possible role of ER in Rb1-induced antiproliferation and anti-inflammation activity in VSMCs. First, we evaluated the expression of ERα and ERβ by both real-time PCR and Western blot analysis in VSMCs. Figure 6A illustrates that protein and mRNA of both ER subtypes were present in the VSMCs derived from female Sprague−Dawley rats.

Thereafter, an ERE-luciferase reporter gene assay was performed to study whether Rb1 acts as a phytoestrogen in VSMCs. As shown in Figure 6B, Rb1 (1 μM) was able to activate ER transcription from a luciferase reporter gene (pERE-luc) under the control of a promoter containing two copies of the ERE in VSMCs, showing a 3.1-fold induction over control and the efficacy was comparable to that of E_2. Furthermore, the ERE-driven luciferase activity induced by Rb1 was greatly blocked by the introduction of ERβ siRNA, implying that Rb1 may exert its phytoestrogen action via ERβ.

ERβ Was Involved in the Inhibitory Effects of Rb1 on VSMC Proliferation. Previous work has suggested the involvement of ERβ in the inhibitory effects of estrogen on VSMCs.[27,28] Our results also showed that the selective ERβ agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN, 1 μM) closely resembled E_2

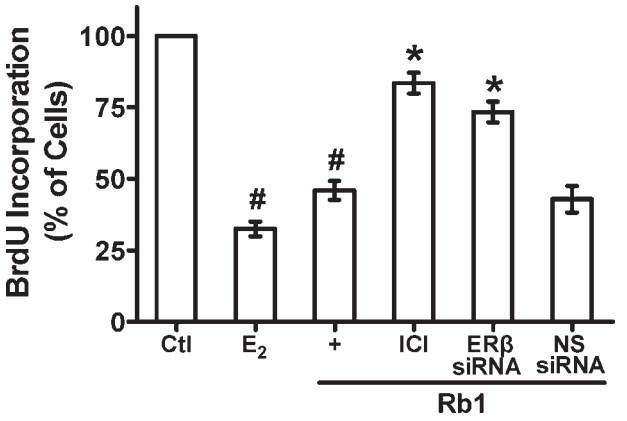

Figure 7. Inhibition on VSMC DNA synthesis by Rb1 was reversed by ERβ siRNA. Cells were treated with or without 1 μM Rb1, in the absence or presence of ERβ siRNA (10 nM), nonspecifc (NS) siRNA, or ICI182,780 (ICI; 10 μM). E_2 (100 nM) was used as a positive control. Data (mean $\pm$ SEM) are representative of three independent experiments. #, $P < 0.01$ versus control; **, $P < 0.01$ versus Rb1 treatment.

in significantly reducing DNA synthesis in VSMCs (data not shown).

The antiproliferation effect of Rb1 was almost completely blocked by ER antagonist ICI182,780, confirming its estrogenic nature in VSMCs (Figure 7). Moreover, ERβ siRNA was chosen to characterize the role of ERβ in Rb1-mediated effects. As shown in Figure 7, diminished ERβ expression caused by siRNA remarkably attenuated the inhibitory effect of Rb1 on VSMC proliferation, when compared to cells transfected with nonspecific siRNA. Taken together, the data suggest that ERβ is, at least in part, involved in Rb1-mediated inhibition on VSMC proliferation.

DISCUSSION

Ginsenosides, the major pharmacologically active ingredients in ginseng, are considered to be responsible for most of ginseng's actions. Various studies have described the beneficial effects of ginsenosides on cardiovascular disease either in vivo or in vitro.[17,29,30] At present, little is known about the antimitogenic and anti-inflammatory roles of Rb1 in VSMCs. Here, we show for the first time that Rb1 possesses estrogenic properties and mimics E_2 to suppress VSMC proliferation and inflammatory responses in vitro. In addition, we also demonstrate that the effect exerted by Rb1 on cell cycle involves the participation of ERβ. Thus, our data provide a potential molecular target through which Rb1 manifests as an antimitogenic agent.

To simulate the multiple factors environment in vivo, a single growth factor was replaced with 5% FBS to induce cell proliferation in this study. FBS is known to contain a range of growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor, serotonin, and thrombin.[31] Indeed, an increase in VSMC proliferation evoked by 5% FBS added to the culture medium was blocked by Rb1 in a concentration-dependent manner (Figures 1 and 2).

After mitogenic stimulation, ERK1/2 is phosphorylated by MEK kinase and enters the nucleus, where it regulates the expression of cell cycle regulators and subsequent DNA synthesis in VSMCs.[32] Rb1 significantly decreased FBS-induced phosphorylation of ERK1/2, thereby suggesting that Rb1 is a negative effector for ERK1/2 activation leading to suppression of the expression of the cell cycle regulators and eventual growth arrest. The cell cycle is a highly regulated process that involves a complex cascade of events. Modulation of expression and function of cell cycle regulatory proteins provides an important approach to the control of cell growth. According to flow cytometry analysis, Rb1 profoundly decreased FBS-induced transition to the S phase (Figure 4A). It is known that the move from G_1 to S phase is closely linked to the activation of cell cycle regulatory proteins such as CDKs.[33] In particular, the cyclin D1/CDK4 and cyclin E/CDK2 complexes are essential for entering the S phase.[33] As shown in Figure 4B, Rb1 displayed a potent inhibitory effect on the expression of a number of cell cycle regulatory proteins (cyclin D1, cyclin E, CDK2, and CDK4). It appears that Rb1 exerts its action through down-regulation of positive regulators in the cell cycle.

It has been suggested that serum levels of TNF-α would be elevated in postmenopausal women.[34] TNF-α participates in innate immunity and adaptive immune responses via TNF-α receptors, especially TNFR1, on almost all kinds of cells.[34] TNF-α is up-regulated in VSMCs after balloon injury, restenotic lesions, and intimal VSMCs, as well as plaques of atherosclerotic arteries.[35] TNF-α induces rapid depolymerization of F-actin fibers and the disappearance of vinculin from focal adhesions in VSMC, and migration of cultured rat aortic VSMC was linked to VSMC proliferation both in vitro and in vivo.[36] Moreover, TNF-α-mediated proinflammatory effector production, suggesting a pivotal role in these processes. Considering the important role of TNF-α as a regulator of cardiovascular pathophysiology, it is thus of great value for Rb1 to attenuate TNF-α-mediated production of cytokines and adhesion molecules, as well as chemokines, in VSMCs (Figure 5).

The estrogenic effects of Rb1 have been hypothesized, and Leung et al. confirmed that Rb1 potently activated ERβ signaling in HUVEC, with a direct binding to ERβ, but not ERα.[26] In rat VSMCs, Rb1 was shown to activate ERE-mediated gene transcription (Figure 6). Further investigation using ERβ siRNA showed that the transactivation effect of Rb1 in VSMCs was mediated by ERβ (Figure 6).

These are two types of ER presented in VSMCs, and the mechanism by which of estrogens exert inhibitory actions on VSMCs has been intensely investigated. Although previous studies using knockout mice suggested that both subtypes of ER are responsible for the antimitogenic effect observed, an increasing amount of evidence showed that ERβ would be more potent than ERα in inhibiting VSMC proliferation.[37,38] Due to the preferential activation of ERβ signaling, we speculate that this pathway might be involved in the beneficial effects of Rb1 on the cardiovascular system. Of course, further study should be well-designed to test the hypothesis.

In summary, the present study provides new experimental evidence supporting the postulated property of ginsenoside Rb1 as a functional phytoestrogen capable of inhibiting FBS-induced proliferation and TNF-α-evoked inflammatory responses in rat smooth muscle cells. Furthermore, the effects exerted by Rb1 are cell cycle related and ERβ specific. The findings presented here also highlight the possible therapeutic use of Rb1 in cardiovascular disease, in which excess proliferation and inflammation play important pathological roles.

AUTHOR INFORMATION

Corresponding Author

*Phone: + 86 21 5288 8380. Fax: + 86 21 6248 6927. E-mail: mkzhong@medmail.com.cn.

Author Contributions

‖These authors contributed to this work equally.

Funding Sources

The study was supported in part by grants from the Shanghai Bureau of Health (2009Y006 and 2009Y069) and the Program for Young Excellent Talents in Tongji University (2009KJ113).

ACKNOWLEDGMENT

We thank Drs. Yin Zhou, Liang Huang, and Shi Yang for their excellent technical assistance and helpful discussion.

REFERENCES

(1) Ling, S.; Komesaroff, P.; Sudhir, K. Cellular mechanisms underlying the cardiovascular actions of oestrogens. *Clin. Sci. (London)* **2006**, *111*, 107–118.

(2) Isles, C. G.; Hole, D. J.; Hawthorne, V. M.; Lever, A. F. Relation between coronary risk and coronary mortality in women of the Renfrew and Paisley survey: comparison with men. *Lancet* **1992**, *339*, 702–706.

(3) Kuller, L. H. Cardiovascular disease is preventable among women. *Expert Rev. Cardiovasc. Ther.* **2010**, *8*, 175–187.

(4) Rossouw, J. E.; Anderson, G. L.; Prentice, R. L.; LaCroix, A. Z.; Kooperberg, C.; Stefanick, M. L.; Jackson, R. D.; Beresford, S. A.; Howard, B. V.; Johnson, K. C.; Kotchen, J. M.; Ockene, J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA, J. Am. Med. Assoc.* **2002**, *288*, 321–333.

(5) Anderson, G. L.; Limacher, M.; Assaf, A. R.; Bassford, T.; Beresford, S. A.; Black, H.; Bonds, D.; Brunner, R.; Brzyski, R.; Caan, B.; Chlebowski, R.; Curb, D.; Gass, M.; Hays, J.; Heiss, G.; Hendrix, S.; Howard, B. V.; Hsia, J.; Hubbell, A.; Jackson, R.; Johnson, K. C.; Judd, H.; Kotchen, J. M.; Kuller, L.; LaCroix, A. Z.; Lane, D.; Langer, R. D.; Lasser, N.; Lewis, C. E.; Manson, J.; Margolis, K.; Ockene, J.; O'Sullivan, M. J.; Phillips, L.; Prentice, R. L.; Ritenbaugh, C.; Robbins, J.; Rossouw, J. E.; Sarto, G.; Stefanick, M. L.; Van Horn, L.; Wactawski-Wende, J.; Wallace, R.; Wassertheil-Smoller, S. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA, J. Am. Med. Assoc.* **2004**, *291*, 1701–1712.

(6) Hsia, J.; Langer, R. D.; Manson, J. E.; Kuller, L.; Johnson, K. C.; Hendrix, S. L.; Pettinger, M.; Heckbert, S. R.; Greep, N.; Crawford, S.; Eaton, C. B.; Kostis, J. B.; Caralis, P.; Prentice, R. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch. Intern. Med.* **2006**, *166*, 357–365.

(7) Simpkins, J. W.; Yang, S. H.; Liu, R.; Perez, E.; Cai, Z. Y.; Covey, D. F.; Green, P. S. Estrogen-like compounds for ischemic neuroprotection. *Stroke* **2004**, *35*, 2648–2651.

(8) Rivard, A.; Andres, V. Vascular smooth muscle cell proliferation in the pathogenesis of atherosclerotic cardiovascular diseases. *Histol. Histopathol.* **2000**, *15*, 557–571.

(9) Andres, V.; Castro, C. Antiproliferative strategies for the treatment of vascular proliferative disease. *Curr. Vasc. Pharmacol.* **2003**, *1*, 85–98.

(10) Dubey, R. K.; Gillespie, D. G.; Mi, Z.; Rosselli, M.; Keller, P. J.; Jackson, E. K. Estradiol inhibits smooth muscle cell growth in part by activating the cAMP-adenosine pathway. *Hypertension* **2000**, *35*, 262–266.

(11) Akishita, M.; Ouchi, Y.; Miyoshi, H.; Kozaki, K.; Inoue, S.; Ishikawa, M.; Eto, M.; Toba, K.; Orimo, H. Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* **1997**, *130*, 1–10.

(12) Jia, L.; Zhao, Y.; Liang, X. J. Current evaluation of the millennium phytomedicine − ginseng (II): collected chemical entities, modern pharmacology, and clinical applications emanated from traditional Chinese medicine. *Curr. Med. Chem.* **2009**, *16*, 2924–2942.

(13) He, F.; Guo, R.; Wu, S. L.; Sun, M.; Li, M. Protective effects of ginsenoside Rb1 on human umbilical vein endothelial cells in vitro. *J. Cardiovasc. Pharmacol.* **2007**, *50*, 314–320.

(14) Ohashi, R.; Yan, S.; Mu, H.; Chai, H.; Yao, Q.; Lin, P. H.; Chen, C. Effects of homocysteine and ginsenoside Rb1 on endothelial proliferation and superoxide anion production. *J. Surg. Res.* **2006**, *133*, 89–94.

(15) Chai, H.; Wang, Q.; Huang, L.; Xie, T.; Fu, Y. Ginsenoside Rb1 inhibits tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Biol. Pharm. Bull.* **2008**, *31*, 2050–2056.

(16) Chai, H.; Dong, Y.; Wang, X.; Zhou, W. Ginsenoside Rb1 attenuates homocysteine-augmented guidewire injury-induced intimal hyperplasia in mice. *J. Surg. Res.* **2009**, *157*, 193–198.

(17) Chai, H.; Schultz, G.; Aghaie, K.; Zhou, W. In vivo assessment of the effects of ginsenoside Rb1 on intimal hyperplasia in ApoE knockout mice. *J. Surg. Res.* **2010**, *162*, 26–32.

(18) Rodriguez, A.; Fortuno, A.; Gomez-Ambrosi, J.; Zalba, G.; Diez, J.; Fruhbeck, G. The inhibitory effect of leptin on angiotensin II-induced vasoconstriction in vascular smooth muscle cells is mediated via a nitric oxide-dependent mechanism. *Endocrinology* **2007**, *148*, 324–331.

(19) Sasu, S.; LaVerda, D.; Qureshi, N.; Golenbock, D. T.; Beasley, D. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ. Res.* **2001**, *89*, 244–250.

(20) Ekshyyan, V. P.; Hebert, V. Y.; Khandelwal, A.; Dugas, T. R. Resveratrol inhibits rat aortic vascular smooth muscle cell proliferation via estrogen receptor dependent nitric oxide production. *J. Cardiovasc. Pharmacol.* **2007**, *50*, 83–93.

(21) Pizarro, J. G.; Yeste-Velasco, M.; Esparza, J. L.; Verdaguer, E.; Pallas, M.; Camins, A.; Folch, J. The antiproliferative activity of melatonin in B65 rat dopaminergic neuroblastoma cells is related to the downregulation of cell cycle-related genes. *J. Pineal Res.* **2008**, *45*, 8–16.

(22) Miller, A. P.; Feng, W.; Xing, D.; Weathington, N. M.; Blalock, J. E.; Chen, Y. F.; Oparil, S. Estrogen modulates inflammatory mediator expression and neutrophil chemotaxis in injured arteries. *Circulation* **2004**, *110*, 1664–1669.

(23) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method. *Methods* **2001**, *25*, 402–408.

(24) Nelson, P. R.; Yamamura, S.; Mureebe, L.; Itoh, H.; Kent, K. C. Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase. *J. Vasc. Surg.* **1998**, *27*, 117–125.

(25) Welt, F. G.; Rogers, C. Inflammation and restenosis in the stent era. *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 1769–1776.

(26) Leung, K. W.; Cheung, L. W.; Pon, Y. L.; Wong, R. N.; Mak, N. K.; Fan, T. P.; Au, S. C.; Tombran-Tink, J.; Wong, A. S. Ginsenoside Rb1 inhibits tube-like structure formation of endothelial cells by regulating pigment epithelium-derived factor through the oestrogen β receptor. *Br. J. Pharmacol.* **2007**, *152*, 207–215.

(27) Geraldes, P.; Sirois, M. G.; Tanguay, J. F. Specific contribution of estrogen receptors on mitogen-activated protein kinase pathways and vascular cell activation. *Circ. Res.* **2003**, *93*, 399–405.

(28) Xing, D.; Feng, W.; Miller, A. P.; Weathington, N. M.; Chen, Y. F.; Novak, L.; Blalock, J. E.; Oparil, S. Estrogen modulates TNF-α-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-β activation. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292*, H2607–H2612.

(29) Zhu, D.; Wu, L.; Li, C. R.; Wang, X. W.; Ma, Y. J.; Zhong, Z. Y.; Zhao, H. B.; Cui, J.; Xun, S. F.; Huang, X. L.; Zhou, Z.; Wang, S. Q. Ginsenoside Rg1 protects rat cardiomyocyte from hypoxia/reoxygenation oxidative injury via antioxidant and intracellular calcium homeostasis. *J. Cell Biochem.* **2009**, *108*, 117–124.

(30) Lu, J. M.; Yao, Q.; Chen, C. Ginseng compounds: an update on their molecular mechanisms and medical applications. *Curr. Vasc. Pharmacol.* **2009**, *7*, 293–302.

(31) Peng, C. Y.; Pan, S. L.; Huang, Y. W.; Guh, J. H.; Chang, Y. L.; Teng, C. M. Baicalein attenuates intimal hyperplasia after rat carotid balloon injury through arresting cell-cycle progression and inhibiting ERK, Akt, and NF-κB activity in vascular smooth-muscle cells. *Naunyn−Schmiedeberg's Arch. Pharmacol.* **2008**, *6*, 579–588.

(32) Adam, R. M.; Borer, J. G.; Williams, J.; Eastham, J. A.; Loughlin, K. R.; Freeman, M. R. Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate. *Endocrinology* **1999**, *140*, 5866–5875.

(33) Wei, G. L.; Krasinski, K.; Kearney, M.; Isner, J. M.; Walsh, K.; Andres, V. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. *Circ. Res.* **1997**, *80*, 418–426.

(34) Sites, C. K.; Toth, M. J.; Cushman, M.; L'Hommedieu, G. D.; Tchernof, A.; Tracy, R. P.; Poehlman, E. T. Menopause-related differences in inflammation markers and their relationship to body fat distribution and insulin-stimulated glucose disposal. *Fertil. Steril.* **2002**, *77*, 128–135.

(35) Clausell, N.; de Lima, V. C.; Molossi, S.; Liu, P.; Turley, E.; Gotlieb, A. I.; Adelman, A. G.; Rabinovitch, M. Expression of tumour necrosis factor α and accumulation of fibronectin in coronary artery restenotic lesions retrieved by atherectomy. *Br. Heart J.* **1995**, *73*, 534–539.

(36) Ferreri, N. R. Estrogen-TNF interactions and vascular inflammation. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292*, H2566–H2569.

(37) Watanabe, T.; Akishita, M.; Nakaoka, T.; Kozaki, K.; Miyahara, Y.; He, H.; Ohike, Y.; Ogita, T.; Inoue, S.; Muramatsu, M.; Yamashita, N.; Ouchi, Y. Estrogen receptor β mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation. *Cardiovasc. Res.* **2003**, *59*, 734–744.

(38) Lindner, V.; Kim, S. K.; Karas, R. H.; Kuiper, G. G.; Gustafsson, J. A.; Mendelsohn, M. E. Increased expression of estrogen receptor-β mRNA in male blood vessels after vascular injury. *Circ. Res.* **1998**, *83*, 224–229.