Journal of Cellular Biochemistry 98:1471–1481 (2006)

Ginsenoside Rg1 Inhibits Tumor Necrosis Factor-α (TNF-α)-Induced Human Arterial Smooth Muscle Cells (HASMCs) Proliferation

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Abstract In China, the ginseng root began to be used in medicine over 2000 years ago. Ginsenosides are the most important component isolated from ginseng. The aim of this study was to determine the effects of ginsenoside Rg1 on the proliferation and molecular mechanism in cultured human arterial vascular smooth muscle cell (HASMC) induced by tumor necrosis factor-α (TNF-α). It was shown that ginsenoside Rg1 significantly inhibited TNF-α-induced HASMC proliferation in a dose-dependent manner. Treatment with ginsenoside Rg1, which blocked the cell cycle in the G1-phase, induced a downregulation of cyclin D1 and an upregulation in the expression of p53, $p21^{WAF/CIP1}$, and $p27^{KIP1}$. MEK inhibitors PD98059, U0126, and phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, but not p38-inhibitor SB203580 or JNK-inhibitor SP600125 significantly aggravated ginsenoside Rg1-inhibited HASMC proliferation. Ginsenoside Rg1 markedly inactivated the extracellular signal-regulated kinases (ERK1/2) and protein kinase B (PKB), indicating that the inhibition of ginsenoside Rg1 on HASMC proliferation was associated with ERK and PI3K/PKB pathways. The inactivation of ERK and PI3K/PKB pathways and modulation of cell-cycle proteins by ginsenoside Rg1 may be of importance in inhibition of HASMCs proliferation. J. Cell. Biochem. 98: 1471–1481, 2006.

Key words: TNF-α; ginsenoside Rg1; HASMC; G1 cell-cycle arrest; ERK; PI3K/PKB

The proliferation of vascular smooth muscle cells (VSMCs) is a key event in the development of advanced lesions associated with atherosclerosis [Ross, 1995]. The abnormal growth of VSMC also plays an important role in vascular diseases, including atherosclerosis, restenosis after angioplasty [Ross, 1995]. VSMC is the principal cell type in both atherosclerotic and restenotic lesions [Ross and Glomset, 1973]. In a normal artery, the VSMC are in a non-proliferative quiescent state and show a well-differentiated contractile phenotype. After the vascular injury, there is a loss of differentiated phenotype and a shift to a synthetic phenotype, which is also accompanied by entry into the cell cycle and proliferation [Owens et al., 2004]. Vascular lesions form during a number of pathological processes, which involve the accumulation of inflammatory cells and the release of cytokine. The cytokine tumor necrosis factor-α (TNF-α) is secreted by VSMC in the neointima after balloon-injury as well as by macrophages in atherosclerotic lesions [Barath et al., 1990; Ohta et al., 2005]. It has previously been shown that TNF-α induces an increase in DNA synthesis and activation of extracellular signal-regulated kinases (ERK1/2) in VSMC [Goetze et al., 1999, 2001]. A recent study reported that TNF-α may also play an important role in cell-cycle regulation in VSMC [Moon et al., 2003].

The proliferation of VSMC induced by TNF-α is involved in the mitogen-activated protein kinases (MAPKs) pathway and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) pathway. MAPKs pathway, including ERK1/2, c-Jun N-terminal protein kinase (JNK), and p38

Grant sponsor: The National Natural Sciences Foundation of China; Grant number: 30271617; Grant sponsor: State 863 High Technology Research and Development Project of China; Grant number: 2003AA2Z2042; Grant sponsor: China Postdoctoral Science Foundation; Grant number: 2005037391.

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Received 20 October 2005; Accepted 9 December 2005

DOI 10.1002/jcb.20799

MAP kinase [Chang and Karin, 2001], as well as PI3K/PKB pathway, regulates cellular processes of inflammation, proliferation, differentiation, apoptosis, and survival [Widmann et al., 1999]. In arterial media, VSMC are normally quiescent, proliferation at low indices (<0.05%), and remain in the G0/G1-phase of the cell cycle. After injury to the vessel, SMC migrate into the intimal layer of the arterial wall, where they leave their quiescent state and re-enter the cell cycle [Dzau et al., 2002]. $P21^{WAF/CIP1}$ (hereafter termed p21) and $p27^{KIP1}$ (hereafter termed p27) belong to the cip/kip family of cyclin-dependent kinase (CDK) inhibitors (CKIs), and have extensively characterized as negative regulators of G1-phase cell-cycle progression [Yew, 2001]. P21 and p27 are found in complexes comprised of CDKs and their cyclin-activating partners, and inhibit their catalytic activities [Braun-Dullaeus et al., 1998]. Antimitogenic signals activate p53, which induces expression of p21 and consequently inhibits the activity of the G1 cyclin–CDK complexes, resulting in G1-phase arrest [Levine, 1997].

Ginsenoside Rg1 (Fig. 1) is one of the active ingredients of a Chinese herbal medicine (ginseng). Ginseng is a key component in traditional Chinese medicine and is also one of the most extensively used botanical products in the West [Gillis, 1997]. Panax ginseng and its main active component, ginsenosides, exhibit a variety of cardiovascular actions [Chen, 1996], including an antihypertensive effect [Chen, 1996], protection against ischemical reperfusion injury [Zhan et al., 1994], a negative chronotropic effect [Wu and Chen, 1988], a negative inotropic effect [Wu and Chen, 1988], and a vasorelaxing effect [Chen et al., 1984]. In this study, there is considerable interest in defining the effects of ginsenoside Rg1 on MAPK, PI3K pathway, and cellular events from the G1-to S-phases, including p21, p27, p53, and cyclin D1 protein and gene expression in cultured human arterial smooth muscle cells (HASMCs) induced by TNF-α.

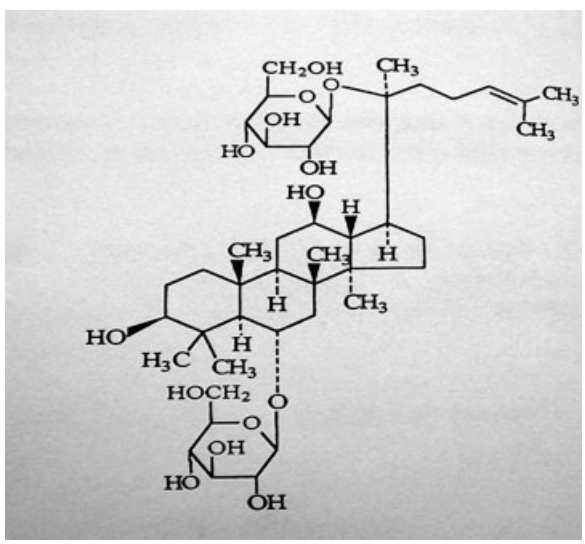


Fig. 1. Chemical structure of ginsenoside Rg1.

MATERIALS AND METHODS

Materials

Ginsenoside Rg1 (purity >99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ginsenoside Rg1 was dissolved in serum-free culture media. Recombinant human TNF-α was obtained from Sigma. The Reverse Transcription System, the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS), the PCR system were purchased from Promega (Madison, WI). Monoclonal antibody to β-actin, cyclin D1, p53, p27, and phospho ERK1/2 and polyclonal p21 antibody were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA), polyclonal antibody to phospho PKB (Thr 308), and total PKB were from Genex. Wortmannin, PD98059, U0126, SP600125, and SB203580 were purchased from Calbiochem (San Diego, CA).

Cell Cultures

HASMCs, medium 231, and SMC growth supplement were purchased from Cascade Biologics. HASMCs were grown in 231 medium with growth supplement at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells were used at passages 3 through 8. Subconfluent cells were serum-starved for 24 h and used for the experiments. For all experiments, HASMCs were grown to 80–90% confluence and made quiescent by starvation for at least 24 h. Ginsenoside Rg1, wortmannin, PD98059, U0126, SP600125, or SB203580 was given 30 min before treatment with TNF-α.

Cell Proliferation Assays

Cell proliferation was measured with a Cell Titer 96 (Promega) colorimetric assay, utilizing an MTS tetrazolium compound, as per the

manufacturer's instructions. Assays were performed by adding [3-(4,5-dimethylthiazol-2-yl]-5-(3-carbo-xymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium] (MTS) directly to the culture wells, incubating them for 2 h, and then recording the absorbance at 490 nm with a 96-well plate spectrophotometric reader (Dynatech MR600). Results were expressed as the percent changes from basal conditions using 4–6 culture wells for each experimental condition.

Cell-Cycle Analysis

Cells were harvested and fixed in 70% ethanol and stored at −20°C. They were then washed twice with ice-cold phospho-buffered saline (PBS) and incubated with 20 ng/ml RNase and 200 μg/ml DNA intercalating dye propidium iodide at room temperature for 30 min in the dark, cells were analyzed by a fluorescent activated cell sorter (FACS) can (Becton Dickinson, San Jose, CA, USA) using Cell Quest software (Becton Dickinson).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from HASMC cells using the Trizol Reagent (Invitrogen) and quantified by UV absorption at 260 and 280 nm. Two micrograms of total RNA was reverse-transcribed at the following conditions: 50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate, 1 mM dNTP mix, 15 U of avian RNase H-minus reverse transcriptase, 40 U of RNasin, 2.5 μM oligo d(T) primers in a final volume of 20 μl. The reaction was run at 65°C for 5 min and 50°C for 40 min, and then the enzyme was heat-inactivated at 85°C for 5 min. Reaction product (2 μl) was used for PCR reaction. PCR conditions were: 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 1 U *Taq* DNA polymerase, and 0.4 μM (final concentration) primers in 20 μl of total volume. The following primers with the predicted size were used for ampication: p21 (123 bp): 5′-CTG CCC AAG CTC TAC CTT CC-3′; 5′-CAG GTC CAC ATG GTC TTC CT-3′; Cyclin D (402 bp): 5′-ACC TGG ATG CTG GAG GTC TG-3′; 5′-GAA CTT CAC ATC TGT GGC ACA-3′; GAPDH (306 bp): 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′; 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′; p53 (297 bp): 5′-TCT GTC CCT TCC CAG AAA ACC-3′; 5′-TTG GGC AGT GCT CGC TTA GTG CTC C-3′; p27 (261 bp): 5′-TGG AGA AGC ACT GCA GAG AC-3′; 5′-GCG TGT CCT CAG AGT TAG CC-3′.

The PCR profile included an initial denaturation at 94°C for 5 min, followed by cycles of 94°C for 30 s, 53–58°C (depending on the primer set) for 30 s, and 72°C for 30 s, and a final extension of 5 min at 72°C. Amplification products were visualized on 1.5% agarose gel and visualized by ethidium bromide staining. The optimum number of cycles was determined experimentally for each gene product, and to verify uniform amplification.

Western Immunoblotting

Cells were lysed in 50 mmol/L Tris-HCl–150 mmol/L NaCl, pH 7.5, buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mmol/L NaF, 2 mmol/L Na_3VO_4, 10 mmol/L phenylmethylsulfonylfluoride, 500 μmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride, 150 nmol/L aprotinin, and 1 μmol/L leupeptin. Protein concentrations were measured with the Bio-Rad Protein Assay. Equivalent amounts of protein were electrophoresed in 8–12% sodium dodecyl sulfate denaturing polyacrylamide slab gels. After transfer to nitrocellulose membranes, bands were visualized by reaction with primary antibody. In brief, membranes were blocked in 5% bovine serum albumin and probed with primary antibody for 1 h at room temperature. After secondary incubation in horseradish peroxidase-conjugated goat anti-mouse or goat-antirabbit IgG antibody (1:2,500) (Santa Cruz), the immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL) from Pierce. Bands corresponding to different proteins were quantified by scanning of photographs and then digitalized and analyzed with the Adobe Photoshop CS.

Statistical Analysis

Data were presented as mean ± SEM from at least three independent experiments. Statistical differences were determined using a Student's *t*-test ($P < 0.05$).

RESULTS

Ginsenoside Rg1 Inhibits HASMC Proliferation

In initial experiments, we investigated the effect of ginsenoside Rg1 on HASMC proliferation in response to TNF-α (10 ng/ml). TNF-α stimulated the proliferation of HASMC, as measured by CellTiter 96 Cell Proliferation

Assay, and the proliferation was significantly inhibited by ginsenoside Rg1 in a dose-dependent manner. Treatment with ginsenoside Rg1 resulted in a reduction in numbers of cells at levels of 0.1, 1, and 10 μM, respectively (Fig. 2A). While ginsenoside Rg1 alone had no effect on proliferation of HASMCs (Fig. 2B).

Ginsenoside Rg1 Induces HASMC G1 Cell-Cycle Arrest

To determine the phase of the cell cycle at which ginsenoside Rg1 exerts its growth-inhibitory effect, TNF-α-treated HASMCs were treated with different concentrations of ginsenoside Rg1 for 24 h and analyzed by flow cytometry (Fig. 3). G1 arrest was apparent after treatment with 0.1 μM ginsenoside Rg1 (72%) and increased to 84% in the presence of 10 μM ginsenoside Rg1. Ginsenoside Rg1 alone had no effect.

P27, p21, p53 Protein, and mRNA Levels Are Elevated and Cyclin D1 Protein and mRNA Levels Are Reduced Following Ginsenoside Rg1 Treatment

As p27, p21, p53, and cyclin D1 levels were a potential mediator of G1/S arrest, we evaluated its expression in the treated and untreated HASMCs. As shown in Figure 4A, p27, p21, p53 mRNA levels are elevated after 12 h of treatment with ginsenoside Rg1 in a dose-dependent manner; by contrast, cyclin D1 mRNA levels are reduced under similar experimental conditions.

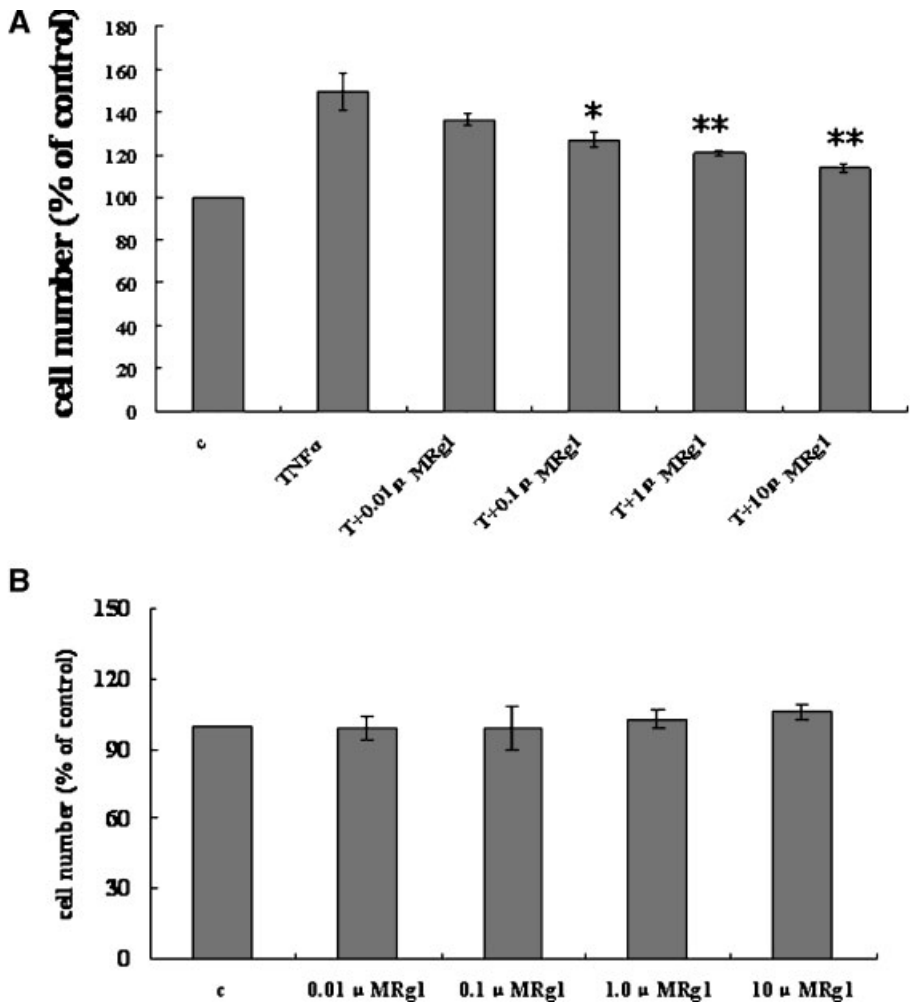
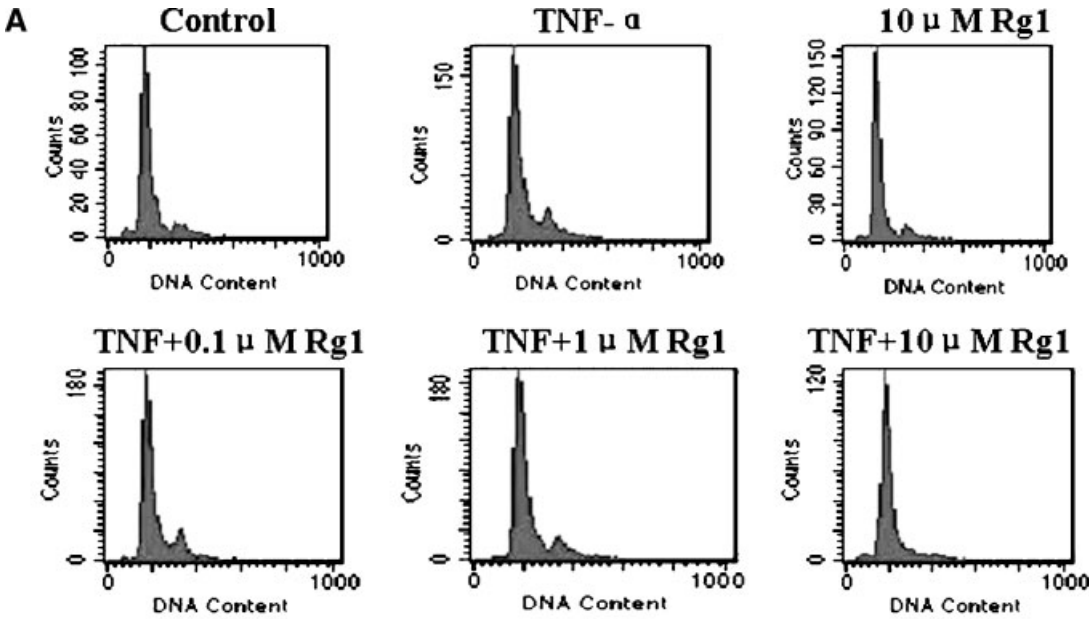


Fig. 2. Effect of on HASMC proliferation induced by TNF-α. Arrested HASMC were stimulated with 0.01, 0.1, 1, 10 μM ginsenoside Rg1 (Rg1) for 30 min before treatment with (**A**) or without (**B**) 10 ng/ml TNF-α during 24 h. Control group was not treated with TNF-α and ginsenoside Rg1. HASMC proliferation was assayed by CellTiter 96 Cell Proliferation Assay. Results are expressed as percents of control. Each bar represents the mean ± SEM for four experiments. C indicates control. *significantly different from TNF-α alone, $P < 0.05$; **$P < 0.01$.

A



B

	G0-G1	G2-M	S
Control	91.37 ± 3.66	0.68 ± 0.43	7.95 ± 3.51
TNF-α	63.17 ± 4.33	1.99 ± 0.13	34.84 ± 4.27
10 μ MRg1	88.3 ± 1.99	2.27 ± 0.23	9.43 ± 1.81
TNF+0.1 μ M Rg1	72.49 ± 0.65	1.45 ± 0.36	26.08 ± 1.01
TNF+1 μ M Rg1	78.93 ± 1.22	1.97 ± 0.18	1.91 ± 1.25
TNF+10 μ M Rg1	84.25 ± 0.91	2.49 ± 0.35	13.27 ± 0.85

Fig. 3. Ginsenoside Rg1 induces G1 cell-cycle arrest in VSMC. **A**: Representative results of FACS measurements to determine the cell-cycle stages of HASMC. HASMC were incubated in serum-free medium for 24 h to induce quiescence, followed by the addition of TNF-α (10 ng/ml) or TNF-α after treatment with 0.1, 1, 10 μM ginsenoside Rg1 for 30 min, and cultured for an additional 24 h, followed by FACS analysis as described under Materials and Methods. **B**: Data indicate the percentage of cells in each phase of the cell cycle. The figure is representative of at least three experiments with similar results.

An immunoblot analysis revealed that ginsenoside Rg1 treatment of the TNF-α-induced HASMC resulted in the significantly dose-dependent induction of p27, p21, p53 protein levels compared with the basal levels (Fig. 4B). Under similar experimental conditions, the levels of expression of cyclin D1 levels were reduced in a dose-dependent manner. While ginsenoside Rg1 alone did not affect on the levels of cell-cycle-related proteins.

ERK1/2, PI3K, but not p38 MAPK and JNK Inhibitors Potentiated Ginsenoside Rg1-Inhibted HASMC Proliferation

To better understand the molecular mechanisms involved in ginsenoside Rg1-inhibted HASMC proliferation, we investigated the possible involvement of MAPKs and PI3K signaling pathways. To determine whether ERK1/2, p38, JNK, or PI3K/PKB signaling pathway is involved in ginsenoside Rg1-inhibted HASMC proliferation, cells were treated with MEK1 inhibitors PD98059 and U0126, p38 inhibitor SB203580, JNK inhibitor SP600125, PI3K inhibitor wortmannin for 30 min before and for the duration of the stimulation, and proliferation assays were performed with the use of the CellTiter 96 Cell Proliferation Assay. PD98059, U0126, and wortmannin potentiated, in a dose-dependent manner, ginsenoside Rg1-stimulated inhibition of HASMCs proliferation (Fig. 5). Under similar experimental conditions, the p38 inhibitor SB203580 (10 μM) and the JNK inhibitor SP600125 (10 μM) did not modify the effects of ginsenoside Rg1 on TNF-α-treated HASMCs.

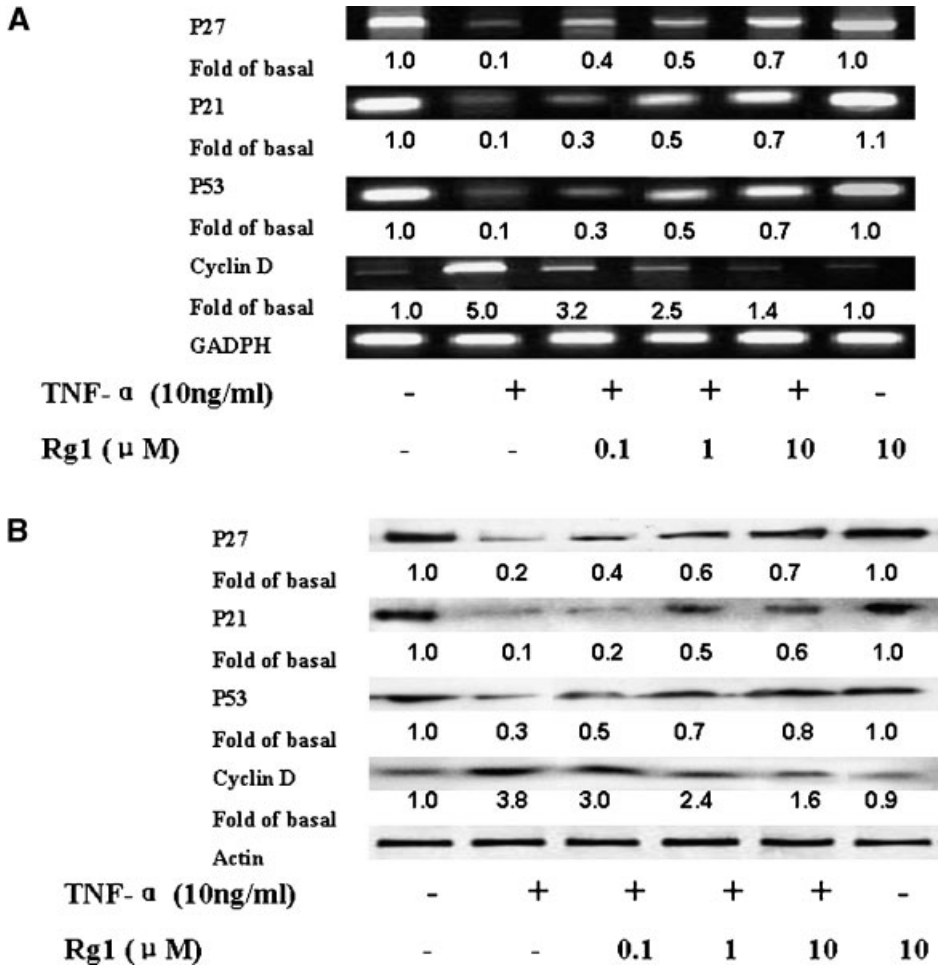


Fig. 4. Effect of ginsenoside Rg1 on G1 cell-cycle regulator cyclin D1, p27, p21, p53 mRNA, and protein levels. **A**: HASMC were stimulated with 0.1, 1, 10 μM ginsenoside Rg1 for 30 min before treatment with TNF-α (10 ng/ml) or 10 μM ginsenoside Rg1 for 12 h. Total RNA was harvested and RT-PCR was performed and products were resolved by gel electrophoresis. RT-PCR products were normalized to GAPDH products. The intensity of PCR product bands shown were quantitated by scanning densitometry. Data are representative of at least three independent experiments. **B**: HASMC were stimulated with 0.1, 1, 10 μM ginsenoside Rg1 for 30 min before treatment with TNF-α (10 ng/ml) or 10 μM ginsenoside Rg1 for 24 h. Cell lysates containing equal amounts of protein were subjected to Western blot analyses with antibodies specific for cyclin D1, p27, p21, and p53. The results of representative experiments were normalized to β-actin expression. The intensity of Western blotting bands shown was quantitated by scanning densitometry. Data are representative of at least three independent experiments.

Ginsenoside Rg1 Inhibits TNF-α-Induced HASMC Proliferation Through ERK1/2 and PI3K/PKB Pathway

The previously experimental findings prompted us to further define whether ginsenoside Rg1 inhibits two major signaling cascades such as ERK1/2 and PI3K pathways. To address this issue, the cell lysates were probed for ERK1/2 and PKB, a downstream substrate of PI3K, protein levels of their phosphorylation forms. Western blot analysis showed that increased ERK1/2 and PKB phosphorylation in HASMCs treated with 10 ng/ml was detected after 5 min, with maximal increase occurring after 30 min of treatment and decreasing after 1 h (Fig. 6A). ERK1/2 and PKB phosphorylation levels were inhibited by ginsenoside Rg1 in a dose-dependent manner following treated with TNF-α for 30 min, while ginsenoside Rg1 alone had no effect (Fig. 6B).

Effect of Wortmannin, PD98059, or U0126 on p27, p21, p53 Protein and mRNA Levels Are Elevated and Cyclin D1 Protein and mRNA Levels Are Reduced Following Ginsenoside Rg1 Treatment

The following experiments were aimed at understanding whether PI3K or ERK

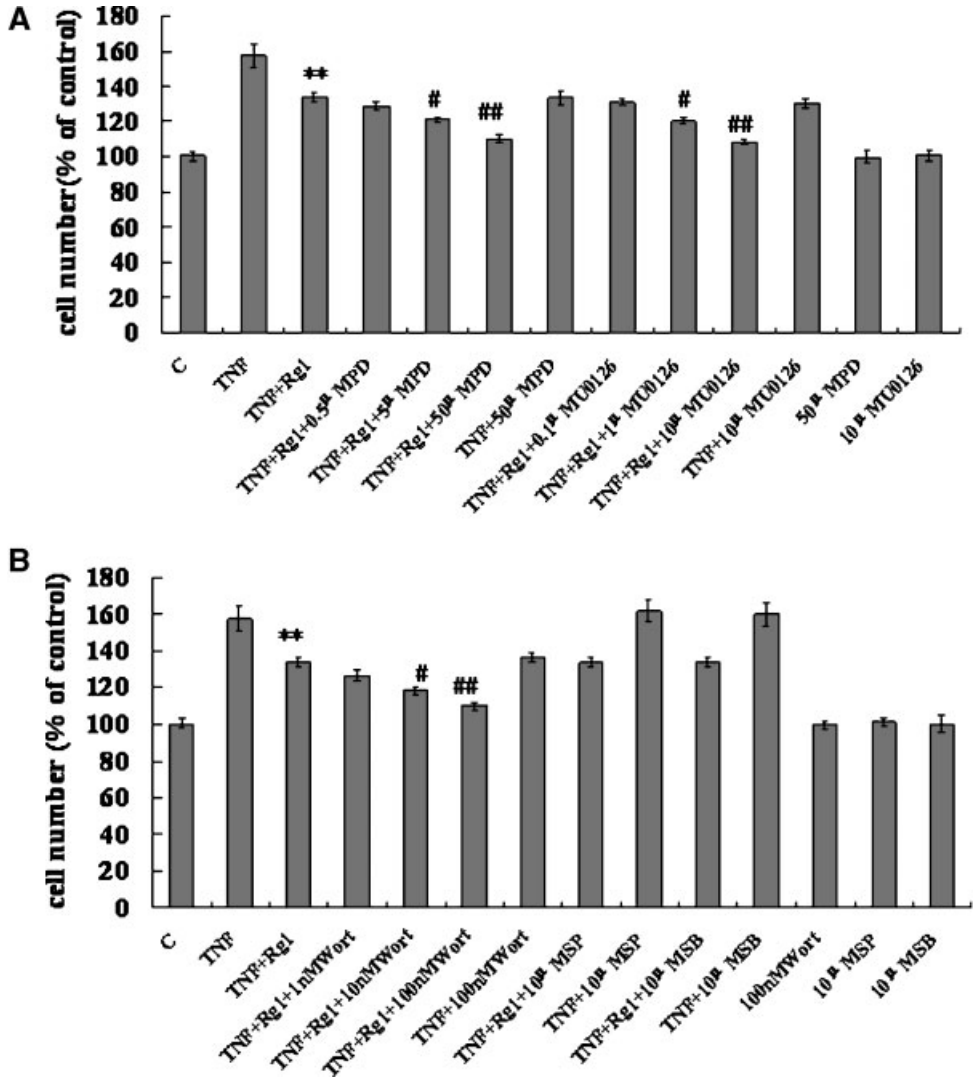


Fig. 5. Involvement of ERK, p38 MAPK, JNK, and PI3K on the effect of ginsenoside Rg1 on proliferation of HASMC proliferation. Arrested HASMC were stimulated with 10 μM ginsenoside Rg1 (Rg1) for 30 min before treatment with 10 ng/ml TNF-α for 24 h, in the absence or presence of (**A**) 0.5, 5, 50 μM PD98059, 0.1, 1, 10 μM U0126; (**B**) 1, 10, 100 nM wotmannin, 10 μM SP600125 and 10 μM SB203580, introduction 30 min before TNF-α. Cell proliferation was measured using CellTiter 96 Cell Proliferation Assay. Results are expressed as percents of control. Each bar represents the mean ± SEM for six experiments. *Significantly different from TNF-α alone, $P < 0.05$; **$P < 0.01$. #Significantly different from TNF-α plus ginsenoside Rg1, $P < 0.05$; ##, $P < 0.01$.

signaling pathway was involved in ginsenoside Rg1-induced changing in protein levels related to G1/S arrest. As shown in Figure 7, in the presence of wortmannin, PD98059 or U0126, the protein (Fig. 7B) and mRNA (Fig. 7A) levels of p27, p21, p53 were elevated compared with ginsenoside Rg1 and TNF-α or inhibitors and TNF-α or inhibitors alone; bycontrast, the protein (Fig. 7B) and mRNA (Fig. 7A) levels of cyclin D1 were decreased compared with ginsenoside Rg1 and TNF-α or inhibitors and TNF-α or inhibitors alone.

DISCUSSION

VSMC proliferation and migration induced by various growth factors can develop a variety of pathological processes including atherosclerosis, hypertension, and restenosis after balloon angioplasty [Ross, 1999]. Inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the treatment of diseases such as atherosclerosis and restenosis [Dzau et al., 2002]. Panax ginseng has long been used in Traditional Chinese Medicine. It has a broad spectrum of physiological activities

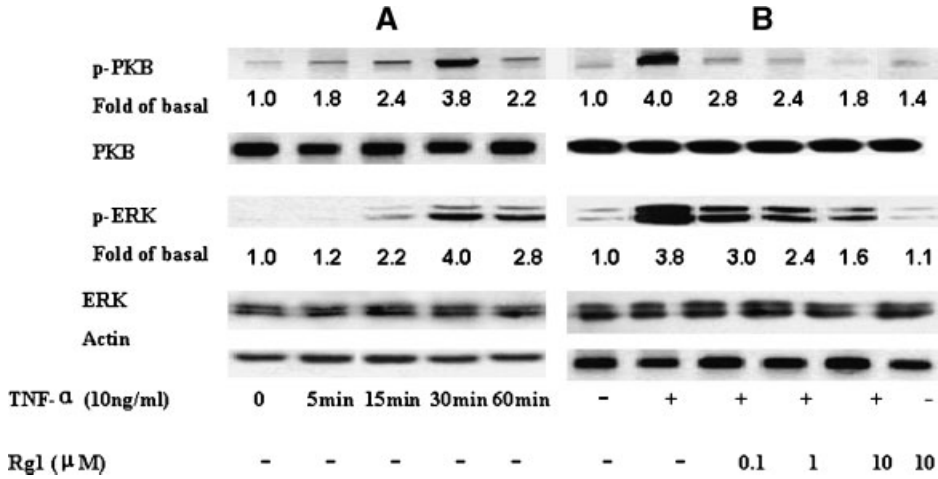


Fig. 6. Dose-dependent effect of ginsenoside Rg1 on ERK and PKB activation. **A**: Time course of PKB and ERK activation in HASMC stimulated with TNF-α. HASMC treated with 10 ng/ml TNF-α for 5, 15, 30, 60 min. **B**: Arrested HASMC were stimulated with 0.01, 0.1, 1, 10 μM ginsenoside Rg1 (Rg1) for 30 min before treatment with 10 ng/ml TNF-α or 10 μM ginsenoside Rg1 for 30 min. Cell lysates containing equal amounts of protein were analyzed by Western blot for phophorylated PKB (p-PKB), total PKB, phophorylated ERK (p-ERK), and total ERK. The results of representative experiments were normalized to β-actin expression. The intensity of Western blotting bands shown was quantitated by scanning densitometry. Data are representative of at least three independent experiments.

[Attele et al., 1999; Sengupta et al., 2004]. Ginsenosides are the main active components of Panax ginseng [Jin et al., 1999]. Among these, ginsenoside Rg1 is the most active saponin even though it is only found in trace amounts [Nah et al., 1995]. In our study, our data suggest that ginsenoside Rg1 significantly inhibited VSMC proliferation induced by TNF-α. These results indicate that ginsenoside Rg1 may be a potential pharmaceutic to prevent VSMC proliferation.

The number of VSMCs was determined by a colorimetric enzyme assay in TNF-α-stimulated VSMC after ginsenoside Rg1 treatment, indicated that an inhibition of VSMC proliferation induced by ginsenoside Rg1 occurred. Our data also showed that ginsenoside Rg1 led to G1 cell-cycle arrest, as compared with the TNF-α-stimulated VSMC. G1 to S cell-cycle progression is controlled by several CDK complexes, including cyclin D1/CDK4 and cyclin E/CDK2, the activities of which are dependent on the balance of cyclins and CKIs, such as p27 and p21 [Sriram and Patterson, 2001; Massagué, 2004]. The expression or activity of cyclin D1 affects the cell progression from G1-to S-phase [Sherr, 1994; Lavoie et al., 1996; Massagué, 2004]. Cyclin D1 binds and activates Cdk4 (6), and then phosphorylates its target protein, pRb; subsequently, the release of transcription factors E2F by phosphorylation of pRb promotes cell proliferation [Massagué, 2004]. The high level of p21 and p27 can inhibit the cyclin D1 expression, resulting in decline of pRb phosphorylation [Massagué, 2004]. A number of studies have shown that the regulation of G1 cell-cycle arrest can be attributed to a number of cellular proteins, including p53 [Massagué, 2004]. To investigate the issue of whether ginsenoside Rg1-induced cell growth inhibition is due to the downregulation of cyclin D1 or the upregulation of CKIs, we then analyzed the expression of these cell-cycle regulators in VSMC after ginsenoside Rg1 treatment. Our experiment indicated that treatment of VSMC with ginsenoside Rg1 resulted in the significant downmodulation of cyclin D1 protein and mRNA levels. Our data also demonstrated a significant upregulation in p53, p21, and p27, as determined by immunoblot analysis and RT-PCR, during the G1-phase arrest of VSMC by ginsenoside Rg1.

ERK has been thought to play a pivotal role in controlling VSMC proliferation [Mii et al., 1996; Gennaro et al., 2003]. Moreover, specific inhibition of MAPK phosphorylation markedly inhibits VSMC growth in vitro. The activation of ERK1/2 can stimulate the downstream transcription gene expression, such as cyclin D1, resulting in protein synthesis and cell proliferation [Bonni et al., 1999]. Our data suggest that the proliferation of HASMCs induced by TNF-α treatment was associated with an induction of ERK activation. Ginsenoside Rg1 significantly

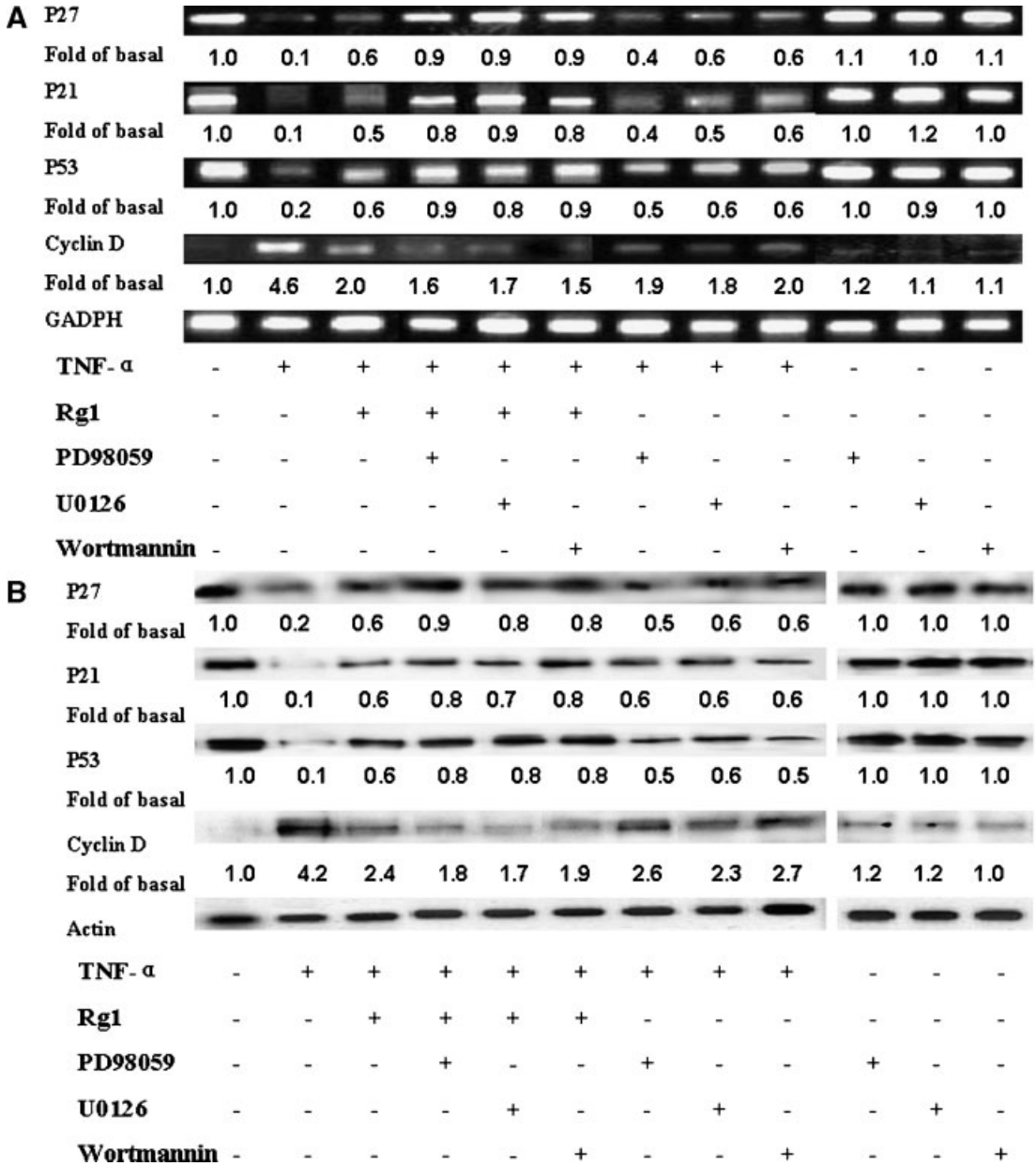


Fig. 7. Involvement of ERK and PI3K/PKB on the effect of ginsenoside Rg1 on G1 cell-cycle regulator cyclin D1, p27, p21, p53 mRNA, and protein levels. Arrested HASMC were stimulated with 10 μM ginsenoside Rg1 (Rg1) for 30 min before treatment with 10 ng/ml TNF-α or 10 ng/ml TNF-α alone for 12 h (**A**) or 24 h (**B**), in the absence or presence of 50 μM PD98059, 10 μM U0126, 100 nM wotmannin, introduction 30 min before TNF-α. A: Total RNA was harvested and RT-PCR was performed and products were resolved by gel electrophoresis. RT-PCR products were normalized to GAPDH products. The intensity of PCR product bands shown was quantitated by scanning densitometry. Data are representative of at least three independent experiments. B: Cell lysates containing equal amounts of protein were subjected to Western blot analyses with antibodies specific for cyclin D1, p27, p21, p53. The results of representative experiments were normalized to β-actin expression. The intensity of Western blotting bands shown was quantitated by scanning densitometry. Data are representative of at least three independent experiments.

attenuated TNF-α-induced ERK phosphorylation. Moreover, the inhibition of this signaling pathway, with the use of specific MEK inhibitors, significantly aggravated ginsenoside Rg1-inhibited HASMC proliferation. The PI3K/PKB pathway is an important mediator of cell growth and survival in response to growth factors and other signals [Cantley, 2002; Sears and Nevins, 2002; Vivanco and Sawyers, 2002]. PI3K activates the PKB serine/threonine kinase by

generating specific inositol phospholipids, which recruit PKB to the cell membrane and enable its activation. PKB mediates cell survival and growth by phosphorylating and inactivating proapoptotic proteins. PKB was found to influence cell-cycle progression of SMCs both in vitro and in vivo. In the present study, we also found that the PI3K/PKB pathway was involvedin ginsenoside Rg1-inhibited HASMC proliferation, and we demonstrated that the use of a specific inhibitor of this pathway, wortmannin, dramatically aggravated ginsenoside Rg1-inhibited HASMC proliferation.

In summary, we have demonstrated for the first time that ginsenoside Rg1 significantly inhibited proliferation on HASMC induced by TNF-α. The antiproliferative effect is involved in inhibition of ERK and PI3K/PKB activation, which may result in downregulation cyclin D1 levels and upregulation of p53, p21, and p27 levels. Our data provide a possible molecular mechanism mediating the inhibitive effect of ginsenoside Rg1 on HASMC proliferation. The results also give evidence that ginsenoside Rg1 may be an effective agent for cardiovascular disease.

ACKNOWLEDGMENTS

The work was supported by grants from State 863 High Technology Research and Development Project of China and the National Natural Sciences Foundation of China and China Postdoctoral Science Foundation.

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