

Full-length article

Ginsenoside Rg1 inhibits proliferation of vascular smooth muscle cells stimulated by tumor necrosis factor- α ¹Zeng-chun MA, Yue GAO², Yu-guang WANG, Hong-ling TAN, Cheng-rong XIAO, Sheng-qi WANG*Institute of Radiation Medicine Sciences, Academy of Military Medical Sciences, Beijing 100850, China***Key words**cell cycle; ginsenoside Rg1; proteomics; tumor necrosis factor- α ; vascular smooth muscle cells¹ Project supported by National Natural Sciences Foundation of China (The basic research of traditional Chinese medicine on genomics and proteomics, No 30271617)² Correspondence to Yue GAO.
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Abstract

Aim: To investigate the proliferation of vascular smooth muscle cells (VSMC) affected by ginsenoside Rg1 and further explore the molecular mechanism of ginsenoside Rg1 using proteomics. **Methods:** The proliferation of VSMC was measured by MTS assay kit and flow cytometry. Proteomic alterations were analyzed using two-dimensional electrophoresis and peptide mass fingerprinting. Differential proteins found in proteomics were confirmed by RT-PCR. **Results:** The proliferation of VSMC was enhanced significantly after tumor necrosis factor- α (TNF- α) treatment, and ginsenoside Rg1 treatment inhibited proliferation in a dose-dependent manner. Proteomic analysis showed 24 protein spots were changed, including 17 spots that were increased and 7 spots that were decreased. Ginsenoside Rg1 could restore the expression levels of these proteins, at least partly, to basic levels of untreated cells. The expression of G-protein coupled receptor kinase, protein kinase C (PKC)- ζ , N-ras protein were decreased, while cycle related protein p21 was increased by ginsenoside Rg1 in TNF- α treated VSMC. **Conclusion:** PKC- ζ and p21 pathway might be the mechanism for inhibitory effects of ginsenoside Rg1 on proliferation of VSMC.

Introduction

Several natural products have been used for vascular diseases and some traditional herbal prescriptions have been employed for treatment of atherosclerosis. Ginsenosides are the most important active constituents identified in ginseng^[1], which can protect against atherosclerosis^[2]. Ginsenoside Rg1, a steroidal saponin of high abundance in ginseng, can relax the rat aorta and enhance NO production^[2-3]. Gene chip analyses find ginsenoside Rg1 can affect the expression levels of genes involved in vascular constriction, cell adherence, coagulation, cell growth and signal transduction in TNF- α stimulated endothelial cells^[4]. Atherosclerosis is the principal vascular lesion which is initiated by vascular endothelial cell damage followed by an intimal hyperplasia of VSMC. VSMC change their phenotype from a contractile to a synthetic state and actively proliferate in the intima of atherosclerotic arteries, secreting excess amounts of extracellular matrix components including collagen and proteoglycans^[5]. Thus inhibition of VSMC hyperproliferation is

one of the key pharmacological strategies for prevention of atherosclerosis. TNF- α , a cytokine that is secreted by VSMC in the atherosclerotic neointima, can promote cell growth and movement, both of which are critical for the initiation and progression of vascular lesions^[6]. An investigation of ginsenoside Rg1-induced alterations in protein expression in TNF- α -activated VSMC will aid in understanding the molecular mechanism of ginsenoside Rg1.

Proteomics describe all the proteins encoded from a specific genome^[7]. Proteomic analysis involves the qualitative alterations in proteins along with the quantitative changes in protein expression levels that occur in response to a given set of conditions^[8]. The rapid increase in proteomic-based drug studies stems from the potential benefits associated with the elucidation of drug mechanisms.

As little is known regarding the vascular molecular mechanism of ginsenoside Rg1 on VSMC and because proteomic technique has more advantages in molecular identification, we attempted to use proteomic analysis to explain the molecular mechanism of ginsenoside Rg1 on the prolifera-

tion of cultural VSMC.

Materials and methods

Reagents and materials Electrophoresis reagents including acrylamide, methylenebisacrylamide, *N,N,N',N'*-tetramethylethyl-diamide (TEMED), hydroxymethyl aminomethane (Tris), glycine, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), 3-[3-(cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), Immobiline DryStrips, Immobilized pH gradient (IPG) buffer, IPG coverfluid and low molecular marker were purchased from Amersham Biosciences (Sweden). Iodoacetamide and trifluoroacetic acid (TFA) were purchased from Acros (Geel, Belgium). Trypsin was obtained from Boehringer Mannheim (GE, Germany).

TNF- α , 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) was obtained from Sigma (St Louis, Mo, USA), ginsenoside Rg1 was obtained from Beijing Institute of Biological Products (Beijing, China), Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Los Angeles, USA). Male Sprague-Dawley rats were obtained from the Experimental Animal Center of the Academy of Military Medical Sciences (Beijing, China).

Cell culture Primary VSMC cultures were isolated after removal of the adventitia and scraping of the endothelium. Arterial strips were then incubated in DMEM with 20% fetal bovine serum (FBS), penicillin 100 U/mL, streptomycin 100 mg/L at 37 °C in 5% CO₂ humidified atmosphere. VSMC were propagated in DMEM with 10% FBS in subsequent passages. Mean incubation time was 5 d. Cell viability was confirmed by Trypan blue exclusion after each passage and assay^[9]. All assays were conducted with subconfluent cells from passages 3–6.

Proliferation assay VSMC were detached using trypsin and seeded at a density of 5000 cells/well in a 96-well plate and rendered quiescent. Then VSMC were incubated in DMEM with various concentrations of ginsenoside Rg1 and TNF- α for 48 h, and 20 μ L of MTS was added. The cultures were incubated for a further 1.5 h, after which the absorbance was read in a multiplate reader. MTS assay is a colorimetric method for determining the number of viable cells in proliferation. It is composed of a solution of tetrazolium, which is bio-reduced by metabolically active cells into a soluble formazan product in culture medium. Its absorbance can be measured at 490 nm. The quantity of formazan produced is directly proportional to the number of living cells.

Flow cytometry Aliquots of 5×10^5 VSMC were centri-

fuged at $200 \times g$ for 5 min, and cell pellets were fixed with 70% ethanol overnight. The cells were then washed twice with PBS and incubated with 1 g/L RNaseA for 30 min. The cells were then resuspended in 0.5 mL of solution containing 50 mg/L propidium iodide and stored in the dark for 20 min. Cells were analyzed on a FACScan Flow Cytometer (FACSCalibur; Becton Dickinson, CA, USA).

Protein extraction and determination of concentrations

Following growth arrest, VSMC were incubated in DMEM with various concentrations of ginsenoside Rg1 and TNF- α for 24 h. Cells were then trypsinized and washed with PBS buffer; 1×10^7 cells were added to 100 μ L lysis buffer (8 mol/L urea, 4 % CHAPS, 40 mmol/L Tris base). After a few cycles of quick freezing and subsequent thawing in liquid nitrogen, 4 μ L RNase (10 g/L) was added. After deposition for 20 min, the cells were centrifuged at $12\,000 \times g$ for 30 min. The supernatant was transferred and stored. Protein concentrations were determined using the Bradford assay.

Two-dimensional electrophoresis (2-DE) and image analysis

Isoelectric focusing (IEF) was carried out on gel strips as described by Ying *et al*^[10]. After IEF separation, the gel strips were immediately equilibrated for two steps in equilibrium buffer, and the second dimension separation was performed using ETTANDALT six (Amersham Biosciences). The gels were stained and scanned with an ImageScanner (Amersham Biosciences) in transmission mode. Spot detection and matching were performed using ImageMaster 2D Elite 4.01 (Amersham Biosciences). The gel of TNF- α treated cells was selected as a reference gel. Other gels were matched with the reference gel. Values of spot abundance were normalized and exported for statistical analysis.

In-gel digestion and MALDI-TOF-MS identification

In-gel digestion of proteins from 2D gels was carried out as described by Steiner *et al*^[11]. Spots were excised and destained with 25 mmol/L ammonium bicarbonate/50% acetonitrile, then dried in a vacuum concentrator (Savant, Holbrook, NY, USA). The dried gel pieces were rehydrated with 5 μ L of 20 mg/L trypsin solution, and digested at 37 °C for 18–20 h. Tryptic peptides were extracted using 5% TFA and dried in a vacuum concentrator. MS was performed on a Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Database searching was performed in the NCBI database and pI were acquired from image analysis as described by Lim *et al*^[12].

RNA isolation and reverse transcriptase polymerase

chain reaction (RT-PCR) Total RNA was extracted from cultured cells according to the supplied protocol. Total RNA was reverse transcribed at 42 °C for 1 h using reverse transcriptase. The cDNA was amplified using glyseralde-

hyde-3-phosphate dehydrogenase(GAPDH) cDNA as an internal control for the amplification of p21 and protein kinase C- ζ (PKC- ζ) expressed by the cells. Primers for the GAPDH were as follows: sense: 5'-ACCACAGTCCATGCC-ATCAC-3'; antisense: 5'-TCCACCACCCTGTTGCTGTA-3'. Primer sequences for p21 were as follows: sense 5'-CCGATA-CAGGTGATGATGATGG-3', antisense: 5'-GCCAGAAGTG-AAGCCAAGG-3'; Primer sequences for PKC- ζ were as follows: sense: 5'-GCCTCCAGTAGATGACAAGAAC-3', antisense: 5'-GAGTGTAAGCCAACCAGGAAG-3'. The sample was amplified for 28 cycles with the following parameters: 94 °C 30 s, 58 °C 20 s, 72 °C 30 s. The PCR products were subjected to 2% agarose gel electro-phoresis. Quantitative data normalized to GAPDH were obtained from a densitometer and analyzed with the included Quantity One 4.4.0 software (BIO-RAD, USA).

Statistical analysis Data was reported as mean \pm SD. One-way ANOVA or Student's *t*-test was used for statistical analysis of the original data, and significance was accepted at $P < 0.05$.

Results

Effects of ginsenoside Rg1 on proliferation of TNF- α stimulated VSMC Following growth arrest, VSMC were stimulated with TNF- α for 24 h. TNF- α induced VSMC proliferation significantly compared with the control. However, ginsenoside Rg1 inhibited VSMC growth at doses as low as 20 mg/L (Figure 1). This observation was not a result of cell necrosis as ginsenoside Rg1-treated cells remained greater than 90% viable after Trypan blue staining. The effects of ginsenoside Rg1 on cell cycle progression were determined by flow cytometry (Table 1). Quiescent VSMC was induced to enter S phase by stimulation with TNF- α . The population of G₀/G₁ cells decreased with a significant concomitant rise in S phase cells. Ginsenoside Rg1

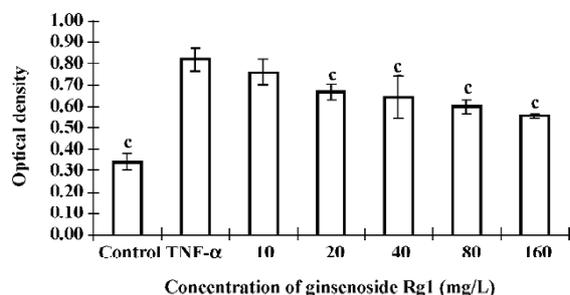


Figure 1. Dose-dependent effects of ginsenoside Rg1 to inhibit proliferation of VSMC. VSMC proliferation was determined by a modified MTS assay. $n=5$ experiments. Mean \pm SEM. $^cP < 0.01$ vs TNF- α treated control.

Table 1. The effects of ginsenoside Rg1 on cell cycle distribution in vascular smooth muscle cells from rat aortic arteries. $n=3$. Mean \pm SD. $^bP < 0.05$, $^cP < 0.01$ vs Normal control. $^eP < 0.05$, $^fP < 0.01$ vs TNF- α stimulation.

Group	G ₀ /G ₁	G ₂ /M	S	PI (%)
Normal control	86.77 \pm 0.48	6.53 \pm 0.23	6.70 \pm 0.32	13.2 \pm 0.4
TNF- α	84.81 \pm 0.26 ^e	6.18 \pm 1.60	9.01 \pm 1.35 ^b	15.2 \pm 0.2 ^c
Ginsenoside Rg1+TNF- α	87.40 \pm 0.80 ^f	6.47 \pm 0.39	6.13 \pm 0.58 ^e	12.6 \pm 0.7 ^f

significantly inhibited VSMC from G₁ to S progression, as shown by the increase in G₀/G₁ cells accompanied by a concurrent decrease in S phase cells. Therefore, ginsenoside Rg1 not only inhibited VSMC proliferation, but also arrested VSMC in G₀/G₁.

Protein expression in normal, TNF- α -stimulated and ginsenoside Rg1-treated VSMC Protein samples extracted from normal, TNF- α -stimulated and ginsenoside Rg1-treated VSMC were loaded on IPGphor gel strips for IEF. Vertical SDS-PAGE was performed immediately following IEF. This experiment was repeated three times. A representative batch of gels is shown in Figure 2. Two dimensional gels of normal, TNF- α -stimulated and ginsenoside Rg1-treated VSMC were analyzed for the purpose of quantitative spot comparisons. Analyzed results showed 845 \pm 22 spots in a normal cell map, 892 \pm 242 spots in a TNF- α -stimulated cell map, and 879 \pm 21 spots in a ginsenoside Rg1-treated cell map. The gels of TNF- α stimulated cells were selected as reference gels, the gels of normal and ginsenoside Rg1 treated cells were matched to reference gels. Differential analysis showed 24 protein spots were different in intensity, including 17 spots that were increased and 7 spots that were decreased after TNF- α stimulation. Ginsenoside Rg1 treatment could prevent this change or reverse it to some degree (Figure 2, Table 2).

Identification of proteins associated with different expressions in normal and ginsenoside Rg1-treated VSMC compared with TNF- α -stimulated VSMC by PMF The differently expressed spots were excised from stained Coomassie Brilliant R-250 gels and submitted to in-gel tryptic digestion. The resulting peptide mixtures were analyzed by MALDI-TOF-MS. The resulting peptide mass maps were used to search against a comprehensive protein sequence database, typical peptide mass maps were listed in Figure 3. Table 3 lists the proteins expressed differently in normal and ginsenoside Rg1-treated VSMC compared with TNF- α -stimulated VSMC. We identified 8 proteins by database searching according to identified standards^[12]. Other proteins need

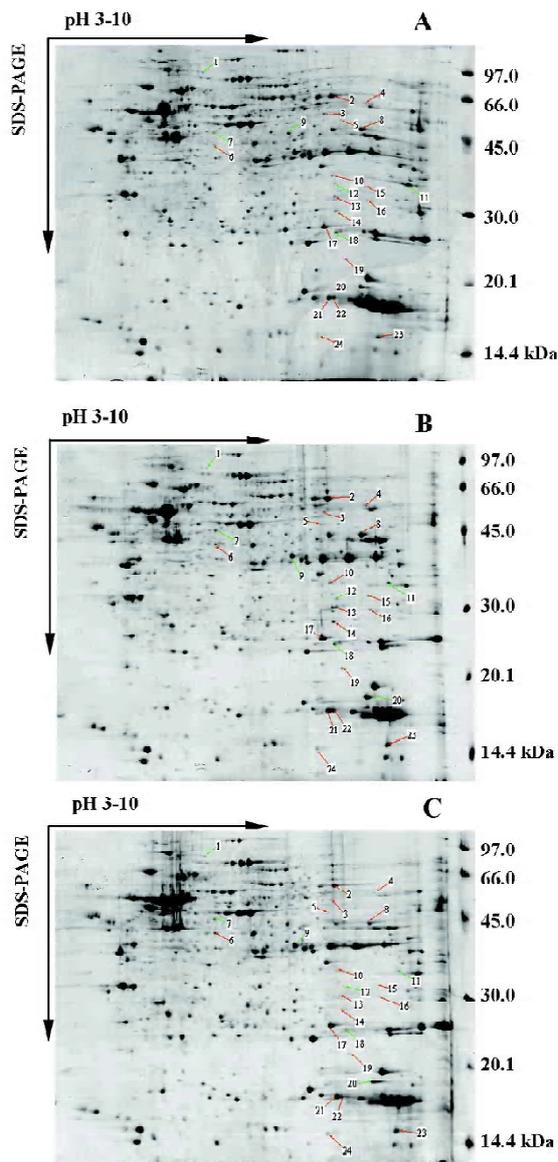


Figure 2. Two dimensional maps of proteins from normal (A), TNF- α control (B) and ginsenoside Rg1 treated VSMC (C). The spots upregulated by TNF- α and reversed by ginsenoside Rg1 are marked with red arrows, the spots downregulated by TNF- α and reversed by ginsenoside Rg1 are marked with green arrows.

to be investigated further because of their poor score. The expression of G-protein coupled receptor kinase, PKC- ζ , N-ras protein were increased, while cycle related protein p21, was decreased by TNF- α compared with normal VSMC. Ginsenoside Rg1 could restore the expression levels of these proteins, at least in part, to basic levels of untreated cells.

Effects of ginsenoside Rg1 on mRNA expression in VSMC TNF- α down-regulated more than 60% of p21 mRNA expression compared with normal VSMC, while ginsenoside

Table 2. Proteins differently expressed in normal, TNF- α -stimulated and ginsenoside Rg1-treated vascular smooth muscle cells from rat aortic arteries. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs Normal control. ^e $P<0.05$, ^f $P<0.01$ vs TNF- α stimulation.

No	pI/M _r (kDa)	Normal control	TNF- α	Ginsenoside Rg1+TNF- α
1	5.877/73.575	0.033 \pm 0.010	0.018 \pm 0.006 ^b	0.034 \pm 0.016 ^e
2	8.012/65.799	0.231 \pm 0.112	0.389 \pm 0.056 ^b	0.171 \pm 0.054 ^e
3	7.945/62.526	0.009 \pm 0.004	0.050 \pm 0.009 ^b	0.017 \pm 0.003 ^e
4	8.688/64.028	0.013 \pm 0.012	0.046 \pm 0.011 ^b	0.013 \pm 0.001 ^e
5	7.878/59.254	0.007 \pm 0.001	0.021 \pm 0.002 ^c	0.009 \pm 0.003 ^f
6	6.031/54.365	0.031 \pm 0.014	0.052 \pm 0.004 ^b	0.043 \pm 0.013
7	6.029/57.676	0.049 \pm 0.010	0.019 \pm 0.005 ^b	0.049 \pm 0.013 ^e
8	8.614/56.636	0.299 \pm 0.026	0.537 \pm 0.089 ^b	0.169 \pm 0.049 ^e
9	7.379/51.401	0.352 \pm 0.003	0.255 \pm 0.013 ^c	0.315 \pm 0.005 ^e
10	8.050/44.663	0.021 \pm 0.008	0.046 \pm 0.007 ^b	0.013 \pm 0.001 ^e
11	9.080/44.625	0.088 \pm 0.012	0.023 \pm 0.018 ^b	0.039 \pm 0.001 ^e
12	8.163/40.621	0.026 \pm 0.006	0.009 \pm 0.004 ^b	0.019 \pm 0.004 ^e
13	8.160/38.850	0.003 \pm 0.004	0.045 \pm 0.014 ^b	0.006 \pm 0.008 ^e
14	8.109/34.885	0.016 \pm 0.015	0.054 \pm 0.013 ^b	0.012 \pm 0.002 ^f
15	8.727/41.391	0.012 \pm 0.001	0.035 \pm 0.024 ^b	Undetected
16	8.757/38.157	0.020 \pm 0.003	0.030 \pm 0.003 ^b	0.010 \pm 0.003 ^f
17	7.917/30.920	0.291 \pm 0.054	0.480 \pm 0.072 ^b	0.304 \pm 0.056
18	8.130/29.380	0.067 \pm 0.020	0.032 \pm 0.005	0.050 \pm 0.003 ^e
19	8.263/23.374	Undetected	0.033 \pm 0.013	Undetected
20	8.706/16.252	0.461 \pm 0.072	0.186 \pm 0.141 ^b	0.345 \pm 0.115 ^e
21	8.019/12.710	0.129 \pm 0.058	0.259 \pm 0.108 ^b	0.144 \pm 0.083 ^e
22	8.114/12.824	0.056 \pm 0.027	0.105 \pm 0.051	0.057 \pm 0.011 ^e
23	9.062/4.356	0.135 \pm 0.084	0.232 \pm 0.037 ^b	0.112 \pm 0.094 ^e
24	7.820/3.394	0.017 \pm 0.004	0.052 \pm 0.012 ^b	0.031 \pm 0.006 ^e

Rg1 upregulated more than 50% of p21 mRNA expression compared with TNF- α stimulated VSMC. The effects of ginsenoside Rg1 on PKC- ζ mRNA expression in VSMC had contrary changes. TNF- α upregulated PKC- ζ to more than 40% of mRNA expression compared with normal VSMC, while ginsenoside Rg1 down-regulated PKC- ζ to more than 17% of mRNA expression compared with TNF- α stimulated VSMC (Figure 4).

Discussion

VSMC in healthy vessel walls have low mitogenic activity. During the early stages of arterial wall injury or atherosclerosis, VSMC may undergo a phenotypic transformation^[13]. They become activated and then proliferate and migrate to the intimal layer where they accumulate lipids and participate in plaque formation atherosclerosis^[14]. The inhibition of this process is considered to be of great benefit in the maintenance of vascular homeostasis and in the prevention and development of atherosclerosis. Both ischemic and

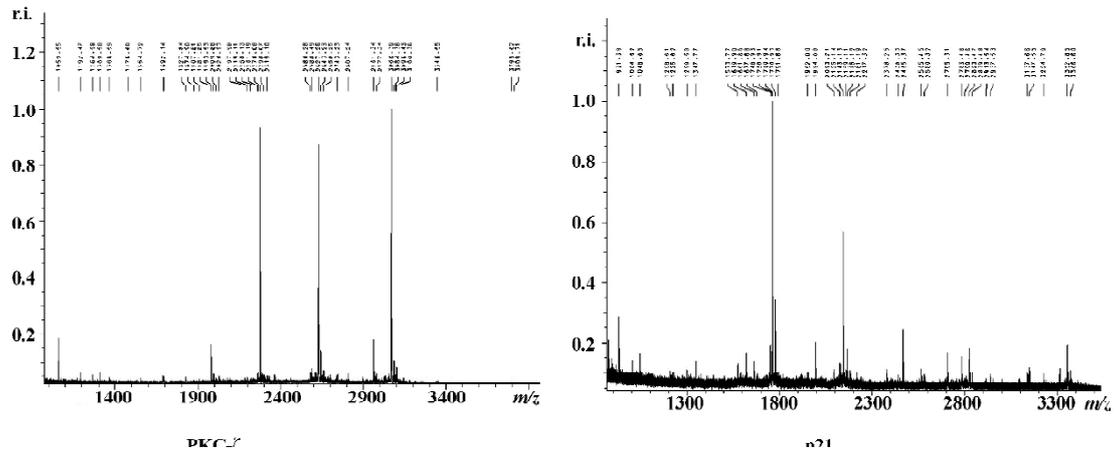


Figure 3. Identification of differentially expressed proteins in 2D gels with peptide mass fingerprint spectrum. Peptide mass fingerprint spectrum obtained from crude peptide mixture after in-gel tryptic digest of proteins in 2D gels. After database searching, the protein score is significant ($P < 0.05$).

Table 3. The search results by matrix internet of proteins that were analyzed with MALDI-TOF-MS.

No	Match No	Score	Peptide matches	pI/M _r (kDa)	Sequence coverage	Description
2	KPY2	88	8/16	7.40/56869	23%	Pyruvate kinase
3	Q9CZN7	82	7/11	8.73/55900	28%	Serine hydroxymethyltransferase
8	PGK2	103	8/15	7.52/44603	29%	Phosphoglycerate kinase
9	LURT1	94	7/10	6.97/38965	26%	Annexin-I
10	Q05513	142	13/37	7.60/35168	67%	Activated PKC- ζ
18	Q8CFC3	75	12/42	6.54/4446	97%	Cyclin dependent kinase inhibitor p21
23	P97913	71	10/41	4.43/10935	68%	N-ras protein
24	Q9EPB9	67	3/6	9.54/28486	31%	G-protein coupled receptor kinase

direct vascular injury result in the elaboration of proinflammatory substances, including TNF- α , which regulate VSMC proliferation and promote vessel stenosis^[15]. Other investigators have suggested that TNF- α induces VSMC proliferation^[16]. In our present study, we found that TNF- α stimulated VSMC proliferation. Ginsenoside Rg1 is a major ingredient used in many traditional Chinese medicines to cure atherosclerosis. It has biphasic regulation and little adverse effect. Our results suggest that ginsenoside Rg1 inhibits the proliferation of VSMC in a dose-dependent manner and this action is not due to a cell-toxicity effect.

There have been no previous studies to report that ginsenoside Rg1 has the action to inhibit proliferation of VSMC. Cell-cycle analysis using flow cytometry confirmed the results of MTS methods. Flow cytometry results showed that ginsenoside Rg1 arrested the VSMC cell cycle at G₁ in growing-phase cells and inhibited the cell cycle progression

from G₀/G₁ to S phase in G₀-arrested cells. These results suggest that the inhibitory effect of ginsenoside Rg1 is exerted at a point in the G₁ phase. It has been reported that cyclin dependent kinase (CDK) promote G₁/S transition and that p21 is an intrinsic inhibitor of the kinase activity of CDK^[17]. At 40 mg/L, ginsenoside Rg1 increased the p21 protein level while producing G₁ arrest. RT-PCR results suggested ginsenoside Rg1 increased p21 mRNA levels. These results indicate that the induction of p21 by ginsenoside Rg1 is due to an induced increase in its mRNA level. It has recently been reported that the migration of VSMC can be inhibited by an overproduction of p21 in cultured VSMC^[18], thus the induction of p21 by ginsenoside Rg1 may be one of the mechanisms by which ginsenoside Rg1 exerts its action on migration activity.

PKC could mediate activation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase

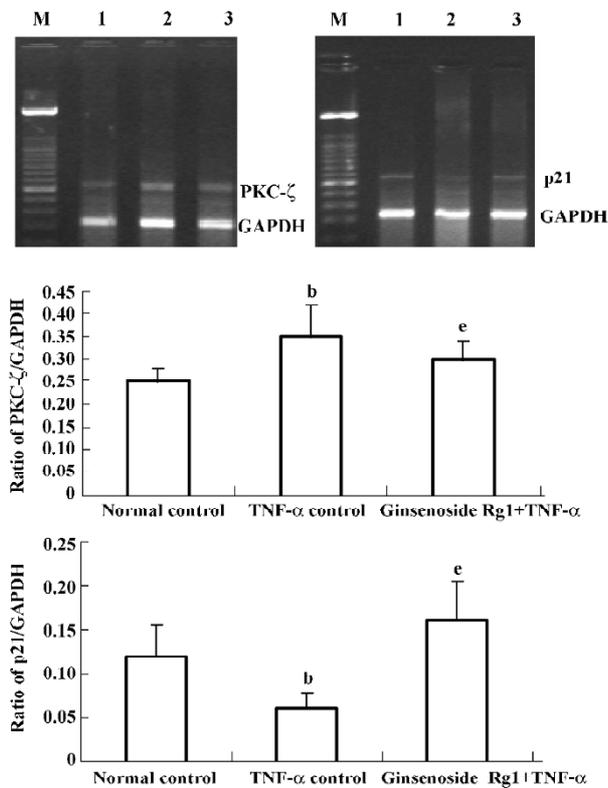


Figure 4. The expression of PKC-ζ, p21 mRNA were detected by RT-PCR. VSMC were incubated with ginsenoside Rg1 and TNF-α for 24 h, and mRNA was extracted and amplified using RT-PCR. The PCR products were subjected to 2% agarose gel electrophoresis. Quantitative data normalized to GAPDH were obtained from a densitometer and analyzed with the included Quantity One 4.4.0 software. Lane 1: normal control; Lane 2: TNF-α control; Lane 3: ginsenoside Rg1 treated. *n*=3 experiments. Mean±SD. ^b*P*<0.05 vs Normal control. ^e*P*<0.05 vs TNF-α stimulation.

(MAPK) in VSMC. The proliferation of VSMC is mediated by the MAPK pathway^[19]. The down-regulation of PKC-ζ by ginsenoside Rg1 may be one of the mechanisms that inhibit proliferation. PKC-ζ, an atypical PKC that are Ca²⁺-independent and do not require diacylglycerol for activation, can phosphorylate and decrease the half-life of p21^[20]. PKC also play a role in p21 mRNA stability^[21], which hints that activation of PKC-ζ results in a decrease of the p21 level. The induction of p21 and inhibition of PKC-ζ may be the mechanism by which ginsenoside Rg1 inhibits VSMC proliferation.

Because upregulation of N-ras activated MAPK pathway and induced inflammation^[22-23], ginsenoside Rg1 could downregulate the N-ras level, which suggests it exerts anti-inflammation and anti-proliferation effects by the N-ras pathway.

The present results suggest that ginsenoside Rg1 inhibits the proliferation of VSMC through an induction of p21 and inhibition of PKC-ζ. The above results suggest that ginsenoside Rg1 is proved to be a valuable drug to cure atherosclerosis.

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