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Parthenolide inhibits proliferation of vascular smooth muscle cells through induction of G_0/G_1 phase cell cycle arrest*

Shao-xiang WENG†[1], Mei-hua SUI[2,3], Shan CHEN[1], Jian-an WANG[1], Geng XU[1], Ji MA[1], Jiang SHAN[1], Lu FANG[1]

([1]*Department of Cardiology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, China*)

([3]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, USA*)

†E-mail: wengshaox@sina.com

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Abstract: Objective: This study is to determine the effect of the natural product parthenolide, a sesquiterpene lactone isolated from extracts of the herb *Tanacetum parthenium*, on the proliferation of vascular smooth muscle cells (VSMCs). Methods: Rat aortic VSMCs were isolated and cultured in vitro, and treated with different concentrations of parthenolide (10, 20 and 30 μmol/L). [^{3}H]thymidine incorporation was used as an index of cell proliferation. Cell cycle progression and distribution were determined by flow cytometric analysis. Furthermore, the expression of several regulatory proteins relevant to VSMC proliferation including IκBα, cyclooxygenase-2 (Cox-2), p21, and p27 was examined to investigate the potential molecular mechanism. Results: Treatment with parthenolide significantly decreased the [^{3}H]thymidine incorporation into DNA by 30%~56% relative to control values in a dose-dependent manner (P<0.05). Addition of parthenolide also increased cell population at G_0/G_1 phase by 19.2%~65.7% (P<0.05) and decreased cell population at S phase by 50.7%~84.8% (P<0.05), which is consistent with its stimulatory effects on p21 and p27. In addition, parthenolide also increased IκBα expression and reduced Cox-2 expression in a time-dependent manner. Conclusion: Our results show that parthenolide significantly inhibits the VSMC proliferation by inducing G_0/G_1 cell cycle arrest. IκBα and Cox-2 are likely involved in such inhibitory effect of parthenolide on VSMC proliferation. These findings warrant further investigation on potential therapeutic implications of parthenolide on VSMC proliferation in vivo.

Key words: Parthenolide, Vascular smooth muscle cell (VSMC), Cell proliferation, IκBα, Cyclooxygenase-2 (Cox-2), p21
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INTRODUCTION

The proliferation of vascular smooth muscle cells (VSMCs) is a central event during the pathogenesis of vascular lesions, including postangioplasty restenosis, transplant arteriosclerosis and vein graft occlusion (Rivard and Andres, 2000; Hedin *et al*., 2004). Identification of novel drugs that may inhibit the proliferation of VSMCs has become an area of great interest in both research and clinical settings (Spyridopoulos and Andres, 1998; Weissberg *et al*., 1993). The natural product parthenolide, a sesquiterpene lactone isolated from extracts of the Mexican-Indian medicinal herb *Tanacetum parthenium*, has been commonly used for the treatment of various infectious and inflammatory conditions such as arthritis, asthma and migraines for many years (Heinrich *et al*., 1998). Furthermore, an increasing number of studies have indicated that parthenolide could inhibit tumor growth (Oka *et al*., 2007; Ross *et al*., 1999), exert beneficial effects in myocardial reperfusion injury (Zingarelli *et al*., 2002), and interfere with a number of cellular processes including oxidative phosphorylation, platelet aggregation, and histamine and serotonin release, as well as neutrophil chemotaxis (Groenewegen and Heptinstall, 1990; Sheehan *et al*., 2002). Parthenolide has also been found

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to inhibit activation of transcription factor nuclear factor-κB (NF-κB) (Zingarelli *et al*., 2002; Sheehan *et al*., 2002; Hehner *et al*., 1998; 1999).

In this study, we determined the effects of parthenolide on the proliferation and cell cycle dynamics of rat aortic VSMCs. Moreover, the regulatory effects of parthenolide on p21 and p27 were also examined, since p21 and p27 are two important members of Cip/Kip family that negatively regulates G_1/S phase and inhibits cell proliferation (Maddika *et al*., 2007; Hengst and Reed, 1998). Previous studies have shown that alpha inhibitor of nuclear factor-κB (IκBα) may mediate the anti-inflammatory effect of parthenolide (Zingarelli *et al*., 2002; Hehner *et al*., 1998; 1999) and that cyclooxygenase-2 (Cox-2) is regulated by NF-κB (Dubois *et al*., 1998; Moon *et al*., 2004). Both IκBα and Cox-2 may play roles in VSMC proliferation (Young *et al*., 2000; Tzeng *et al*., 2007); therefore, the effects of parthenolide on these two molecules were also investigated.

MATERIALS AND METHODS

Cell culture

Rat aortic smooth muscle cells were isolated from the thoracic aorta of 2- to 3-month-old Harlan Sprague-Dawley rats by using the explant technique according to a modified method (Thyberg *et al*., 1983). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. The purity of VSMCs was evaluated by morphology and immunocytochemistry staining with monoclonal antibodies against α-smooth muscle actin (SM-α actin). Prior to the experimental treatments, cells were grown to ~80% confluency followed by incubation with serum-free medium for 24 h to make cells quiescent. VSMCs from passages 4~6 were used in this study.

Reagents

DMEM, FBS and penicillin/streptomycin were obtained from Gibco Life Technologies Inc. (Rockville, MD, USA). Parthenolide was purchased from Jingmei Biotech. Co. Ltd. (Shenzhen, China). [^{3}H]thymidine was supplied by Chinese Academy of Sciences Shanghai Atomic Institute (Shanghai, China). Polyvinylidine difluoride transfer membranes were purchased from Boehringer Mannheim Corp. (Indianapolis, IN, USA). Anti-IκBα, Cox-2, p21, p27 and β-actin antibodies, as well as enhanced chemiluminescence (ECL) kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SM-α actin monoclonal antibody and horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies were purchased from Beijing Chemical Corp. (Beijing, China). All other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA).

Determination of DNA synthesis

[^{3}H]thymidine incorporation into DNA synthesis was used as an index of cell proliferation (Nilsson *et al*., 1983). Cells were seeded in 24-well plates and grown to ~80% confluency. After serum starvation for 24 h, cells were treated with parthenolide at different concentrations (10, 20, and 30 μmol/L) in DMEM containing 10% FBS for 24 h. 1 μCi/ml [^{3}H]thymidine was coincubated with the cells for the last 8 h of treatment. Incubations were terminated by removing the culture medium and washing the cells three times with cold phosphate buffer saline (PBS), followed by the addition of ice-cold 10% (w/v) trichloroacetic acid for 20 min at 4 °C to remove the unincorporated [^{3}H]thymidine and protein precipitation. Next, the cells were washed three times with ice-cold water, solubilized with 1 ml of 0.5 mol/L NaOH, and incubated for 30 min at 37 °C. After solubilization, 0.5 ml/well aliquot samples were removed, transferred to scintillation vials with 10 ml of scintillation cocktail. The radioactivity of incorporated [^{3}H]thymidine was quantified by a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA). Three independent experiments were performed with triplicate samples for each group in each experiment.

Flow cytometric analysis

Cell cycle progression and distribution were determined by flow cytometric analysis. After serum starvation for 24 h, cells were stimulated with different concentrations of parthenolide (10, 20 and 30 μmol/L) in medium supplemented with 10% FBS for 24 h. At the end of time-point, all the cells were harvested by trypsinization, washed with ice-cold PBS,

and fixed in 70% ice-cold ethanol diluted in PBS. Around 1×10^5 VSMCs were incubated in PBS containing 100 g/ml RNase and 40 g/ml propidium iodide at room temperature for 0.5~1.0 h before flow cytometry analysis. Cell cycle distribution and DNA content were determined by using a Berkman Coulter cytometer (Fullerton, CA, USA).

Western blots

After VSMCs were stimulated with 20 μmol/L of parthenolide in medium containing 10% FBS for 2, 6, 12, and 24 h, cells were harvested and cellular protein was isolated with a protein extraction buffer. Protein concentrations were determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Milan, Italy). Equal amounts (30 g/lane) of proteins were fractionated on 10%~12% (w/v) sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with anti-IκBα, Cox-2, p21 and p27 primary antibodies (all in 1:400 dilution), respectively, overnight or with anti-actin (1:1000) for 1 h. After washing with PBS containing 0.1% (v/v) Tween 20, the membranes were incubated with corresponding horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1000) for 1 h, followed by exposure using enhanced chemiluminescent reagent. The images of Western blots were scanned and quantitated using the software Quantity One (Bio-Rad Laboratories). Results were normalized by the respective level of β-actin.

Statistical analysis

Data are presented as mean±*SD* of three independent experiments. Student's *t*-test was used to determine the statistical difference between various experimental and control groups. Differences were considered statistically significant at a level of $P<0.05$.

RESULTS

Identification and characterization of VSMCs

VSMCs isolated by using the explant method in this study grew in the characteristic hill and valley pattern. As shown in Fig.1b, the purity of the VSMCs was determined by immunostaining for monoclonal anti-SM-α-actin. Primary cultures with less than 95% VSMCs were discarded. Cellular viability was determined by cell counting, morphology examinations, and trypan blue exclusion, and the concentrations of parthenolide (10, 20, and 30 μmol/L) used in this study were not toxic to VSMCs.

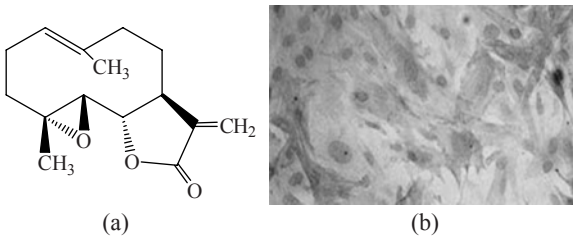

Fig.1 (a) Chemical structure of parthenolide; (b) Characterization of cultured rat aortic smooth muscle cells by immunohistochemical staining with anti-α-smooth muscle actin mouse monoclonal antibody

Effects of parthenolide on DNA synthesis in VSMCs

First, we determined the effect of parthenolide on VSMC proliferation assessed by determination of [^{3}H]thymidine incorporation. As indicated in Fig.2, the incubation of 10, 20 and 30 μmol/L of parthenolide decreased the [^{3}H]thymidine incorporation into DNA by 30.3%, 35.93%, and 56.1%, respectively,

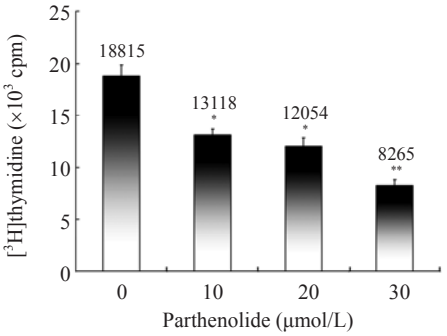

Fig.2 Effect of parthenolide on the incorporation of [^{3}H]thymidine into vascular smooth muscle cells (VSMCs) VSMCs were serum-starved for 24 h to arrest the cells at G_0/G_1 phase. Then cells were stimulated with different concentrations of parthenolide (0, 10, 20 and 30 μmol/L) in DMEM containing 10% FBS for 16 h, followed by pulsing cells with 1 μCi/ml [^{3}H]thymidine for an additional 8 h. The radioactivity of incorporated [^{3}H]thymidine was quantified as counts per minute (cpm) by a liquid scintillation counter. Data are the representative of three independent experiments with similar results and are expressed as mean±*SD* (n=3). $^*P<0.05$ and $^{**}P<0.01$, compared with the corresponding control group

relative to respective control levels (P<0.05 or P<0.01). Thus, the treatment of parthenolide resulted in significant and dose-dependent inhibition on DNA synthesis in VSMCs cultured under 10% FBS.

Effects of parthenolide on cell cycle progression in VSMCs

Next, we determined the effect of parthenolide on cell cycle progression in VSMCs by flow cytometry. Treatment with 10, 20, and 30 μmol/L parthenolide significantly increased the cell population at G_0/G_1 phase by 19.2%, 45.3%, and 65.7%, respectively, and decreased the cell population at S phase by 50.7%, 76.2%, and 84.8%, respectively (P<0.05 or P<0.01 compared with the control group) (Fig.3 and Table 1). These results indicate that parthenolide induces cell cycle arrest at G_0/G_1 phase and decreases the cell population at S phase, which is consistent with the finding that treatment of parthenolide inhibits [^{3}H]thymidine from being incorporated into DNA in VSMCs.

Effects of parthenolide on expression of IκBα, Cox-2, p21 and p27 proteins

To investigate the potential molecular mechanisms that may be involved in the inhibitory effect of parthenolide on VSMCs, we examined several regulatory proteins that are associated with cell proliferation, differentiation, and cell cycle progression, including IκBα, Cox-2, p21 and p27. As shown in Fig.4, 10% FBS induced degradation of IκBα in the control group at 12 and 24 h of incubation. However, the treatment with 20 μmol/L parthenolide completely blocked the serum-induced IκBα degradation at 12 and 24 h incubation (P<0.01). In contrast, 10% FBS alone induced up-regulation of Cox-2, particularly, at 6 h of incubation, but the presence of parthenolide signifycantly reduced the protein levels of Cox-2 at 2, 6, and 12 h of incubation (P<0.01). Moreover, while there were no remarkable changes on protein levels of p21 and p27 in the control group, the treatment of parthenolide significantly up-regulated both proteins at 12 and 24 h of incubation (P<0.01).

DISCUSSION

Parthenolide is a sesquiterpene lactone found as

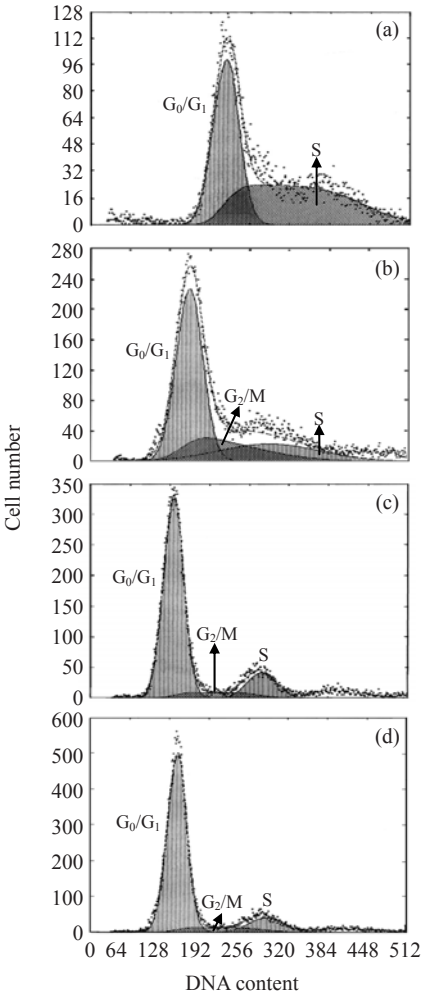

Fig.3 Flow cytometric analyses of cell cycle distribution in vascular smooth muscle cells (VSMCs) with or without the treatment of parthenolide

After serum starvation, VSMCs were stimulated with different concentrations of parthenolide ((a) 0, (b) 10, (c) 20, and (d) 30 μmol/L) in medium supplemented with 10% FBS for 24 h. Cells were harvested and DNA contents were stained with propidium iodide for flow cytometric analysis. The cell populations corresponding to G_0/G_1, G_2/M and S phases of the cell cycle are summarized in Table 1

Table 1 Effects of parthenolide on cell cycle distribution in vascular smooth muscle cells

Parthenolide (μmol/L)	Cell cycle (%)		
	G_0/G_1	S	G_2/M
0	49.5±0.8	48.7±3.3	1.9±3.3
10	59.0±2.4*	24.0±6.5*	16.1±8.4
20	71.9±5.4*	11.6±8.8*	16.5±9.8
30	82.0±1.7**	7.4±0.4**	10.4±1.2

Data are the representative of three independent experiments with similar results and are expressed as mean±SD (n=3). $^*P$<0.05 and $^{**}P$<0.01, compared with the corresponding control group

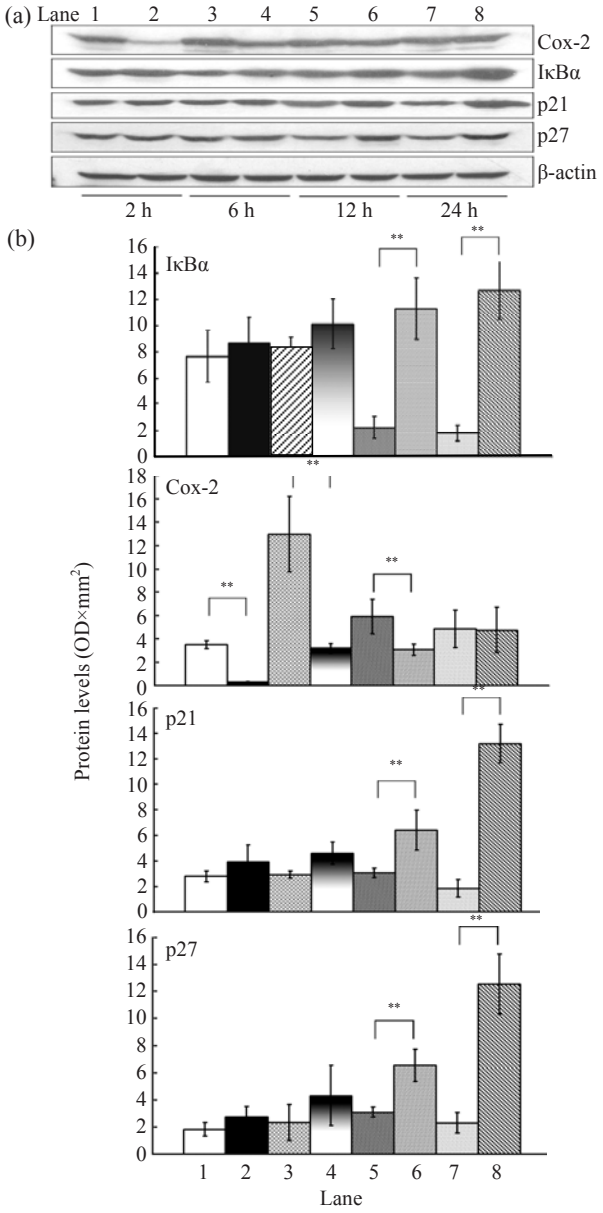

Fig.4 Western blot analyses for the regulatory effects of parthenolide on IκBα, cyclooxygenase-2 (Cox-2), p21 and p27 proteins in vascular smooth muscle cells (VSMCs)
(a) Cells were cultured in 10% FBS and treated with 20 μmol/L of parthenolide after serum starvation. Cellular proteins were extracted from cells after 2, 6, 12 and 24 h of treatment. Equal amounts (30 μg/lane) of cellular proteins were fractionated on 10%~12% SDS-PAGE and transferred to polyvinylidene difluoride membranes, followed by immunoblotting with anti-IκBα, Cox-2, p21 and p27 primary antibodies. β-actin protein was blotted as a control (Lane 1: control 2 h; 2: parthenolide 2 h; 3: control 6 h; 4: parthenolide 6 h; 5: control 12 h; 6: parthenolide 12 h; 7: control 24 h; 8: parthenolide 24 h); (b) The quantification of Western blots was done by densitometry and protein levels were expressed as product of OD and area (mm^2). Data are the representative of three independent experiments with similar results and are expressed as mean±*SD* (n=3). $^{**}P<0.01$, compared with the corresponding control group

the major active component in feverfew (*Tanacetum parthenium*), a herbal medicine that has been used to treat migraine and rheumatoid arthritis for centuries (Heinrich *et al*., 1998; Knight, 1995). Recently, it has been reported that parthenolide possesses antitumor activity in a number of cultured cancer cell lines and in xenograft models (Oka *et al*., 2007; Ross *et al*., 1999). Moreover, parthenolide has been suggested to protect against myocardial ischemia and reperfusion injury in rats (Zingarelli *et al*., 2002), inhibit the proliferation of vascular endothelial cells (Parada-Turska *et al*., 2007; Shanmugam *et al*., 2006), and inhibit inducible nitric oxide synthase gene expression in cultured rat aortic smooth muscle cells (Wong and Menendez, 1999). Thus, interest has arisen in investigating the potential roles of parthenolide in preventing or treating cardiovascular diseases. In the present study, we evaluated the effects of parthenolide on the proliferation of rat aortic smooth muscle cells. We found that treatment of parthenolide, at non-toxic concentrations between 10~30 μmol/L, significantly suppressed the [^{3}H]thymidine incorporation into DNA by 30.3%~56.1% in a dose-dependent manner (Fig.2), and increased the cell population at G_0/G_1 phase while decreased the cell population at S phase (Fig.3). These results suggest that parthenolide inhibits VSMC proliferation by inducing cell cycle arrest at G_0/G_1 phase. We further found that parthenolide increased IκBα, p21, and p27, while decreased Cox-2 in VSMCs, providing some clues on the mechanisms of inhibitory effects of parthenolide on VSMC proliferation.

Cellular proliferation is regulated primarily by regulation of the cell cycle, which consists of four distinct sequential phases (G_0/G_1, S, G_2, and M). As two important members of Cip/Kip family, p21 and p27 negatively regulate G_1/S phase and inhibit cell proliferation (Maddika *et al*., 2007; Hengst and Reed, 1998). Our results indicate that parthenolide significantly increased the expression levels of both p21 and p27 (Fig.4), which is consistent with parthenolide-induced inhibition of DNA synthesis and G_0/G_1 cell cycle arrest in VSMCs.

Although the mechanisms mediating the various effects of parthenolide in different diseases are not entirely clear, several studies have shown that an important part of the anti-inflammatory action of this

compound appears to be related to its activity in inhibiting the NF-κB signal pathway through preventing the degradation of IκBα. NF-κB is a dimeric transcription factor, and genes activated by NF-κB play central roles in cell differentiation, proliferation, inflammation, and apoptosis (Baeuerle and Henkel, 1994; Chen *et al*., 1999). This transcription factor is involved in the pathogenesis of many vascular diseases such as atherosclerosis (Collins and Cybulsky, 2001). IκBα is the specific cytoplasmic inhibitory protein of NF-κB, and degradation of IκBα is generally believed to be the critical step for the activation of NF-κB (Brown *et al*., 1993). A recent study indicated that in vascular smooth muscle cells and monocytes stimulated with lipopolysaccharide (LPS), parthenolide reduced IκBα degradation and NF-κB activation (López-Franco *et al*., 2006). By Western blots, we observed that 10% FBS induced IκBα degradation, which may be related to the activation of NF-κB signal pathway and the proliferation of VSMCs stimulated by enriched serum. However, the presence of parthenolide completely antagonized this effect and resulted in upregulation of IκBα protein, which is in agreement with the previous reports showing parthenolide as an inhibitor of NF-κB (Zingarelli *et al*., 2002; Sheehan *et al*., 2002; Hehner *et al*., 1998; 1999; López-Franco *et al*., 2006).

As an inducible isoform of enzyme, Cox-2 is upregulated in response to inflammatory factors and some peptides (Bishop-Bailey *et al*., 1999). Cox-2 metabolites can regulate a number of cellular processes involved in inflammation and remodeling, including the production of chemokines, matrix metalloproteinase, and angiogenic factors (Bishop-Bailey *et al*., 1999; Marnett *et al*., 1999; Pang *et al*., 1998). Emerging evidence has also shown that Cox-2 enhances VSMC proliferation (Young *et al*., 2000; Tzeng *et al*., 2007). In the present study, we observed that the protein level of Cox-2 was significantly reduced in VSMCs treated with parthenolide for 2~12 h compared with cells cultured with 10% FBS alone (Fig.4). Recent studies have reported inhibitory effect of parthenolide on Cox-2 expression in monocytic cells and lipopolysaccharide-stimulated macrophages and in cancers (Gomez-Hernandez *et al*., 2006; Smolinski and Pestka, 2005). Since the expression of Cox-2 has been shown to be regulated by NF-κB (Dubois *et al*., 1998; Moon *et al*., 2004), our results may suggest that parthenolide upregulates the expression of IκBα protein and subsequently inhibits NF-κB activity, thus reducing the expression of Cox-2 protein. Our results also indicate that NF-κB/IκBα signaling pathway and Cox-2 are likely involved in the inhibitory effect of parthenolide on VSMC proliferation.

In summary, this study has evaluated the effects of parthenolide on proliferation and cell cycle progression in rat aortic VSMCs in vitro. Our results show that the treatment of parthenolide, in a dose-dependent manner, significantly inhibited the proliferation and induced cell cycle arrest at G_0/G_1 phase in VSMCs, which was supported by the finding that parthenolide significantly increased the protein levels of p21 and p27. Furthermore, parthenolide also significantly increased the protein level of IκBα, while reduced the expression of Cox-2 in a time-dependent manner, suggesting that NF-κB/IκBα signaling pathway may play an important role in this process. Taken together, these findings merit further preclinical studies as well as clinical investigations on the potential therapeutic applications of parthenolide on vascular disorders with overgrowth of VSMCs, such as restenosis and atherosclerosis.

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