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Inhibitory effect of reinioside C on vascular smooth muscle cells proliferation induced by angiotensin II via inhibiting NADPH oxidase-ROS-ERK1/2-NF-κB-AP-1 pathway

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The proliferation of vascular smooth muscle cells (VSMCs) induced by angiotensin II (Ang II) plays a vital role in the pathogenesis of arteriosclerosis and restenosis. In the present study, the effect of reinioside C, a main active ingredient of *Polygala fallax* Hemsl, on proliferation of VSMCs induced by Ang II was investigated. It was found that Ang II (1 μM) markedly stimulated proliferation of VSMCs. Pretreatment of reinioside C (3, 10 or 30 μM) concentration-dependently inhibited the proliferative effect of Ang II. To determine the possible mechanism, NADPH oxidase subunits (Nox-1, Nox-4) mRNA expression, intracellular ROS level, phosphorylation of ERK1/2, NF-κB activity, and mRNA expression of AP-1 subunits (c-fos, c-jun) and c-myc were measured. The results demonstrated that reinioside C attenuated Ang II-induced NADPH oxidase mRNA expression, generation of ROS, ERK1/2 phosphorylation, activation of NF-κB, and mRNA expression of AP-1 and c-myc in VSMCs in a concentration-dependent manner. The effects of Ang II were also inhibited by diphenyleneiodonium (DPI, the NADPH oxidase inhibitor), PD98059 (the ERK1/2 inhibitor) and pyrrolidine dithiocarbamate (PDTC, the NF-κB inhibitor). These results suggest reinioside C attenuates Ang II-induced proliferation of VSMCs by inhibiting NADPH oxidase-ROS-ERK1/2-NF-κB-AP-1 pathway.

1. Introduction

The proliferation of vascular smooth muscle cells (VSMCs) is an important pathophysiological event in many cardiovascular diseases such as hypertension, arteriosclerosis, and after percutaneous trans-luminal coronary angioplasty (Rudijanto 2007). Angiotensin II (Ang II), the main peptide hormone of the renin–angiotensin system (RAS), is involved in the restructuring of the arterial wall in both atherogenesis and hypertension. Ang II has been found to stimulate the growth of VSMCs *in vitro* and to enhance the neointimal hyperplasia *in vivo*, when its level is elevated in circulation. Dyslipidemia and hypertension are two powerful risk factors leading to cardiovascular diseases, and they often co-exist (Goode et al. 1995). Dyslipidemia, particularly oxidized low-density lipoprotein (ox-LDL), up-regulates the RAS. The activation of the RAS seems to be intricately involved in the atherosclerotic process, promoting the release of reactive oxygen species (ROS), up-regulating LDL receptors and stimulating the uptake of ox-LDL into macrophages, smooth muscle cells, and endothelial cells. Ang II and ox-LDL thereby influence in a dual manner each other's synthesis and effects on various target cells (Weiss et al. 2001).

It has been found that Ang II enhances the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity, and resultantly induces the generation of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPKs), which leads to proliferation of VSMCs (Blanc et al. 2003; Frank and Eguchi 2003). MAPKs pathway includes p42/p44 extracellular signal-regulated kinases 1/2(ERK1/2), c-Jun N-terminal protein kinase (JNK), p38 MAPK and ERK5. ERK1/2 activates nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and c-myc, thus leading to proliferation of VSMCs (Li and Fukagawa 2010). Previous studies have demonstrated that both antioxidants and angiotensin-converting enzyme inhibition effectively inhibit the proliferation of VSMCs by suppressing the ERK1/2 activation (Heeneman et al. 2007; Satoh et al. 2010). *Polygala fallax Hemsl*, a commonly used Chinese medicinal herb, has been used to treat some diseases such as infective inflammation and hypercholesterolemia. Reinioside C (Fig. 1) is a main component extracted from *Polygala fallax Hemsl* (Xu et al. 2006). Recently, it has been reported that reinioside C exerts hypolipidemic effects in hyperlipidemic mice (Li et al. 2008). Our previous study has shown that reinioside C attenuates the production of tumor necrosis factor-alpha induced by exogenous asymmetric dimethylarginine through the inhibition of ROS/NF-κB pathway in monocytes (Zhang et al. 2008). We have also found that reinioside C attenuates the oxidized low-density lipoprotein induced expression of adhesion molecules and the adhesion of monocytes to endothelial cells by inhibiting the NADPH oxidase/ROS/NF-κB pathway (Bai et al. 2009).

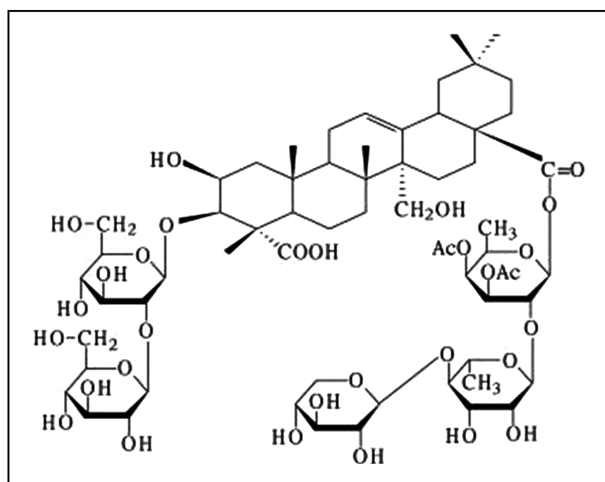

Fig. 1: Chemical structures of reinioside C.

Based on the important effect of the proliferation of VSMCs induced by Ang II in atherosclerosis and restenosis, in the present study we investigated the effect of reinioside C on Ang II-induced proliferation of VSMCs and whether this effect is related to the regulation of NADPH oxidase-ROS-ERK1/2-NF-κB/AP-1 pathway.

2. Investigations and results

2.1. *Effect of reinioside C on Ang II-induced proliferation of A10 VSMCs*

As expected, the treatment of A10 VSMCs with Ang II (1 μM) for 24 h significantly induced cell proliferation which was reflected as an increase both in DNA synthesis ability and in the proportion of cells in the S + G2/M phase, and a decrease in the percentage in the G0/G1phase ($P < 0.01$; Fig. 2a, b). The pretreatment with reinioside C (3, 10, or 30 μM) markedly weakened the stimulation of cell proliferation induced by Ang II in a concentration-dependent manner, which significantly decreased the DNA synthesis ability of A10 VSMCs, and increased the percentage of G0/G1 phase, while the proportion of cells in the S + G2/M phase decreased markedly ($P < 0.01$). The pretreatment with DPI (10 μM), a specific NADPH inhibitor, also markedly attenuated the stimulation of cell proliferation induced by Ang II. Reinioside C (30 μM) itself had no effect on the proliferation of A10 VSMCs, and DPI (10 μM) had no effect on the proliferation of A10 VSMCs (data not shown).

2.2. *Effect of reinioside C on NADPH oxidase subunits mRNA expression and ROS production induced by Ang II*

NADPH oxidases are a major source of ROS generation in vascular cells (Paravicini and Touyz 2006). Reinioside C inhibits NADPH oxidase/ROS pathway in endothelial cells (Bai et al. 2009). In the present study, we therefore measured the effect of reinioside C on NADPH oxidase subunits mRNA expression and ROS production induced by Ang II in A10 VSMCs. The results showed that the treatment with Ang II (1 μM) significantly increased NADPH oxidase subunits (both Nox-1 and Nox-4) mRNA expression and intracellular ROS production while the pretreatment with reinioside C (3, 10 or 30 μM) significantly attenuated these effects of Ang II in a concentration-dependent manner ($P < 0.01$; Fig. 3a b). Ang II-induced NADPH oxidase expression and intracellular ROS production were also inhibited by the pretreatment with DPI (10 μM). Reinioside C (30 μM) itself had no effect on NADPH oxidase expression and intracellular ROS production in A10 VSMCs. DPI (10 μM) also had no such an effect (data not shown).

2.3. *Effect of reinioside C on ERK1/2 phosphorylation induced by Ang II*

ROS-mediated activation of MAPKs is a prerequisite for Ang II-induced proliferation of VSMCs (Blanc et al. 2003; Frank and Eguchi 2003). We therefore investigated the effect of reinioside C on the phosphorylation of ERK1/2 induced by Ang II. As shown in Fig. 4, the treatment with Ang II (1 μM) had no effect on the expression of total ERK1/2, but significantly increased the expression of phosphorylated ERK1/2. The pretreatment with reinioside C significantly inhibited the phosphorylation of ERK1/2 induced by Ang II in a concentration-dependent manner; the same effect was also observed when the cells were pretreated with DPI (10 μM) or the ERK1/2 inhibitor PD98059 (40 μM). Reinioside C (30 μM) or PD98059 (40 μM) had no effect on ERK1/2 phosphorylation. DPI (10 μM) also had no such effect (data not shown).

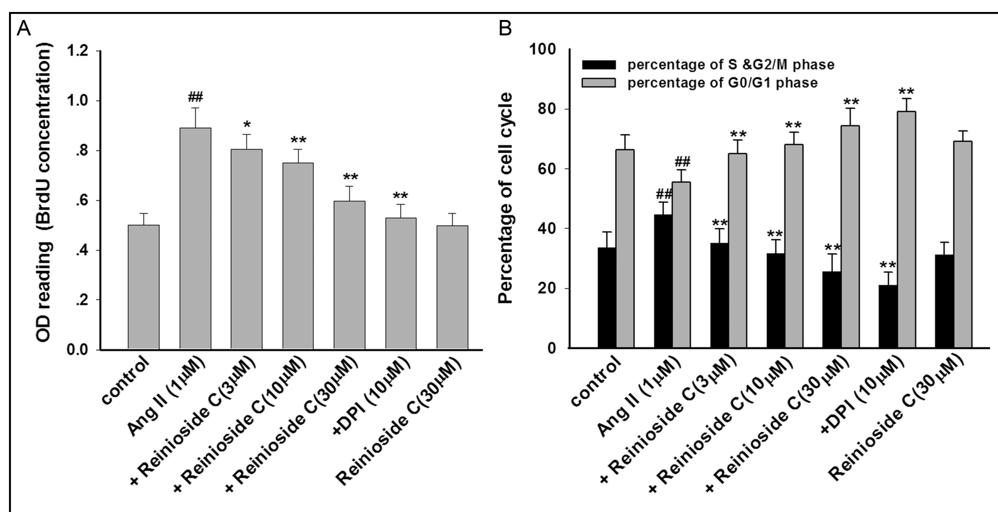

Fig. 2: Effect of reinioside C on Ang II -induced proliferation of A10 VSMCs. (a) The DNA synthesis ability measured by BrdU cell proliferation assay. (b) Cell cycle analysis by flow cytometry. A10 VSMCs were pre-treated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. DPI: diphenyleneiodonium; Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells. Data are expressed as means ± SEM, n = 3. Compared with control, $^{\#\#}P < 0.01$; compared with Ang II, $^{*}P < 0.05$, $^{**}P < 0.01$.

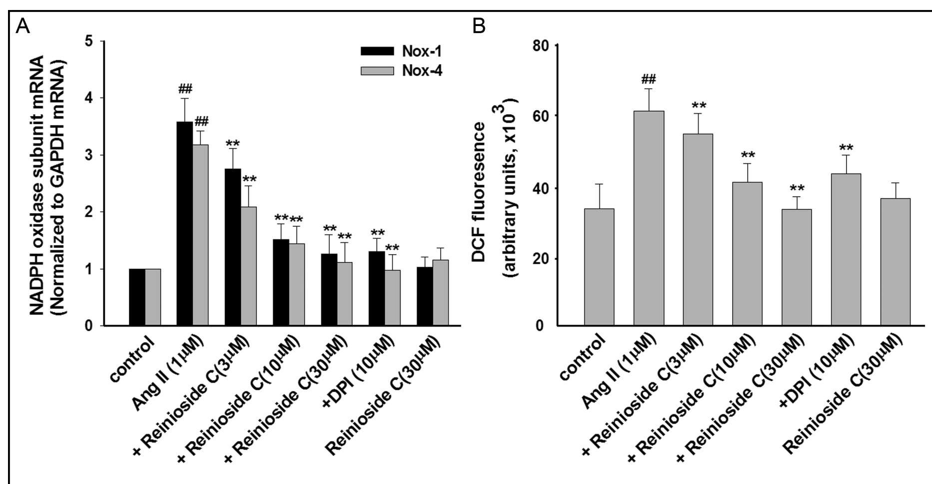

Fig. 3: Effect of reinioside C on NADPH oxidase subunit mRNA expression and ROS production induced by Ang II in A10 VSMCs. (a) The mRNA expression of NADPH oxidase subunits (Nox-1 and Nox-4) by real-time PCR analysis. (b) The level of intracellular ROS as shown by DCF fluorescence. A10 VSMCs were pre-treated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. DPI: diphenyleneiodonium; Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells. Data are expressed as means ± SEM, n = 3. Compared with control, $^{\#\#}$ $P < 0.01$; compared with Ang II, $^{**}P < 0.01$.

2.4. *Effect of reinioside C on activation of NF-κB /AP-1 pathway induced by Ang II*

As ERK1/2 downstream targets, nucleus transcription factors and early growth response genes play an important role in the proliferation of VSMCs (Chakraborti and Chakraborti 1998; Han et al. 2010; Dhar et al. 2002). In the present study, we therefore measured the effect of reinioside C on the DNA-binding activity of transcription factors, NF-κB, and on the expression of phosphorylated IκB-α, AP-1 subunits (c-fos and c-jun) and early growth response gene c-myc. As shown in Fig. 5 and Fig. 6, Ang II (1 μM, 24 h) stimulation markedly increased IκB degradation, the DNA-binding activity of NF-κB, and the mRNA expression of c-fos, c-jun and c-myc. The pretreatment of cells with reinioside C (3, 10, or 30 μM) significantly attenuated these effects of Ang II in a concentration-dependent manner. The pretreatment with PDTC (10 μM), PD98059 (40 μM) or DPI (10 μM) also inhibited NF-κB activation, IκB degradation and the mRNA expression of c-fos, c-jun and c-myc induced by Ang II. Reinioside C (30 μM) itself had no effect on NF-κB activation, IκB degradation and the mRNA expression of c-fos, c-jun and c-myc.

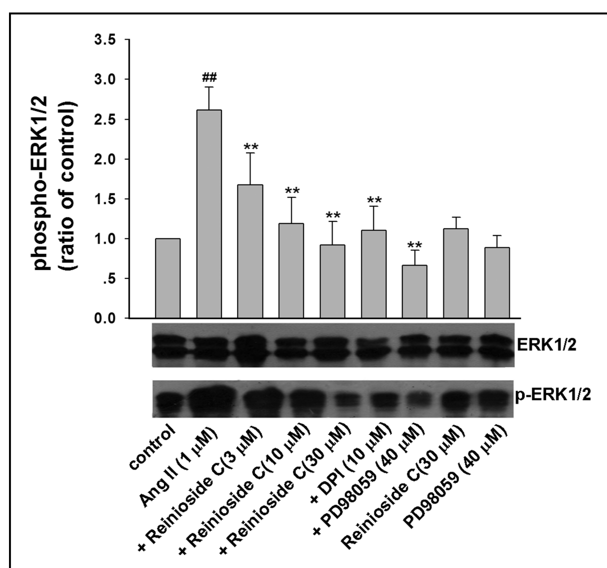

Fig. 4: Effect of reinioside C on ERK1/2 phosphorylation induced by Ang II in A10 VSMCs. The cells were pre-treated with reinioside C (3, 10 or 30 μM), DPI (10 μM) or PD98059 (40 μM) for 1 h, then cultured with Ang II (1 μM) for 2 h, the phosphorylation of ERK1/2 was determined by western blotting analysis. DPI: diphenyleneiodonium; Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells. Data are expressed as means ± SEM, n = 3. Compared with control, $^{\#\#}$ $P < 0.01$; compared with Ang II, $^{**}P < 0.01$.

3. Discussion

The major findings of the present study are as follows: (1) reinioside C significantly inhibits the proliferation of VSMCs induced by Ang II; (2) the effects of reinioside C are related to the inhibition of NADPH oxidase-ROS-ERK1/2-NF-κB/AP-1 pathway.

The pathogenesis of atherosclerosis involves endothelium dysfunction, smooth muscle cell proliferation, hyperlipidemia, infiltration of monocytes and the activation of monocytes into macrophages (Hessler et al. 1979). The synthetic phenotype of VSMCs, migration and proliferation, is the important element in atherosclerosis and restenosis (Rudijanto 2007). Ang II, a principal effector of the renin-angiotensin system, is not only a vasoactive substance but also a mitogen that mediates the proliferation of VSMCs (Heeneman et al. 2007; Sprague and Khalil 2009). In consistence with previous studies (Cain et al. 2006), we confirmed that Ang II treatment induces the proliferation of VSMCs. Inhibition of the vascular smooth muscle cell hyperproliferation is a pharmacological strategy for the prevention of atherosclerosis. Reinioside C is the main active ingredient of the Chinese folk medicine *Polygala fallax Hemsl*. Previous research has suggested that reinioside C could be a potential therapy for atherosclerosis. It has a hypolipidemic effect *in vitro*, and it can inhibit the monocytes adhesion to endothelial cells induced by ox-LDL. And our results further demonstrated that reinioside C can also inhibit the proliferation of VSMCs induced by Ang II, which supports the hypothesis that reinioside C has an inhibitory effect on the proliferation of VSMCs.

A number of studies have demonstrated that Ang II-mediated vascular injuries in artherosclerosis are mainly mediated by NADPH oxidases-derived ROS (Touyz and Briones 2011). Recently, ROS has been considered as the second messenger leading to NF-κB activation in response to extracellular stimuli, and then promoting the proliferation of VSMCs. Some investigations have shown that antioxidants attenuate Ang II-induced proliferation of VSMCs by inhibiting the generation of ROS (Hong et al. 2004; Li et al. 2005). In the present study, we also found that Ang II significantly increases ROS generation, the mRNA expression of NADPH oxidase subunits, and the proliferation of VSMCs. These effects of Ang II are inhibited by DPI,

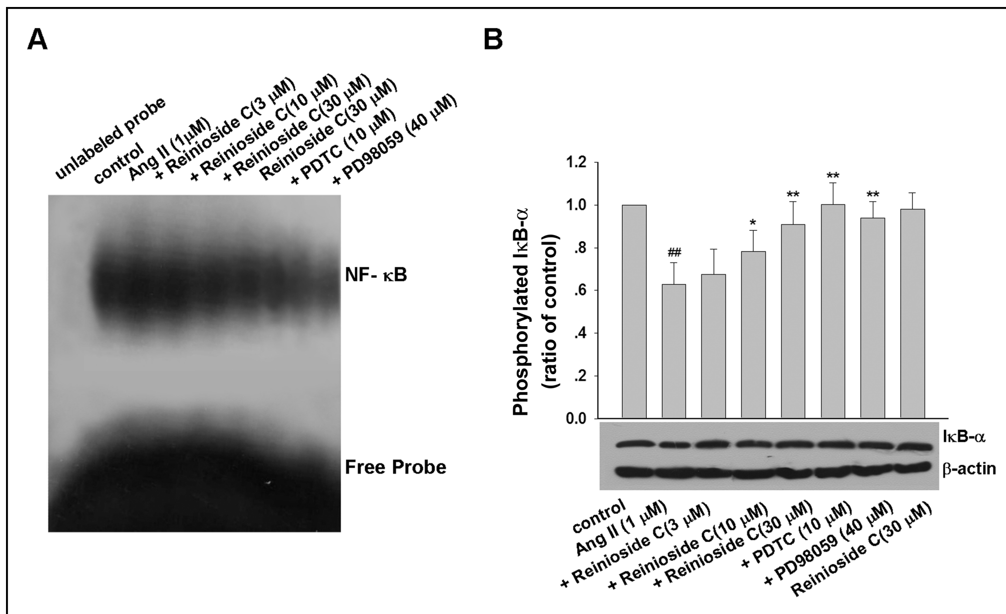

Fig. 5: Effect of reinioside C on Ang II-induced NF-κB/DNA-binding activity (a) and the phosphorylation status of IκB-α (b) in A10 VSMCs. The cells were pre-treated with reinioside C (3, 10 or 30 μM), DPI (10 μM), PD98059 (40 μM) or PDTC (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. Competition experiment where unlabeled NF-κB probe was added at 100-fold excess concentrations to the γ-^{32P}-labeled probe was performed. PDTC: pyrollidine dithiocarbamate; Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells. Data are expressed as means ± SEM, n = 3. Compared with control, ## $P < 0.01$; compared with Ang II, * $P < 0.05$, ** $P < 0.01$.

a specific inhibitor of NADPH oxidase. Our previous studies have also demonstrated that reinioside C inhibits NADPH oxidase *via* its antioxidant property (Zhang et al. 2007; Bai et al. 2009). In the present study, reinioside C was found to markedly inhibit the proliferative effect induced by Ang II via reducing the mRNA expression of NADPH oxidase subunits (both Nox-1 and Nox-4) and intracellular ROS generation in VSMCs.

The proliferation of VSMCs is controlled by diverse signal transduction pathway, such as ERK1/2 (Blanc et al. 2003). ERK1/2, the classic MAPK signal transduction pathway, is easily activated by the stimulation of Ang II. It has been found that ERK1/2 activation caused by Ang II is dependent on ROS production (Frank and Eguchi 2003). In the present study, we found that treatment with Ang II significantly increases the ROS production and enhances ERK1/2 phosphorylation in VSMCs. Pretreatment with reinioside C markedly suppressed the ERK1/2 phosphorylation while attenuating the ROS production. These results suggest that the suppression of ERK1/2 activation might be associated, at least partly, with the antioxidant property of reinioside C.

Accumulating evidence suggests that ERK1/2 activates NF-κB, AP-1 and c-myc, leading to the proliferation of VSMCs (Chakraborti and Chakraborti 1998; Li and Fukagawa 2010). It has been found that NF-κB and AP-1 play an important role in the proliferation of VSMCs induced by Ang II (Li and Fukagawa 2010). C-myc, an early growth response gene, also plays a role in the proliferation of VSMCs (Bennett et al. 2010). In the present study, we found that incubation with Ang II significantly enhances NF-κB DNA-binding activity, IκB degradation, and the mRNA expression of AP-1 subunits (c-fos and c-jun) and c-myc. It also increases the proliferation of VSMCs. However, reinioside C significantly inhibits these effects of Ang II. Pretreatment with PDTC or PD098059 could also inhibit the proliferative effect of Ang II by inhibiting NF-κB DNA-binding activity, IκB degradation and the mRNA expression of AP-1 and c-myc. These findings suggest that the inhibitory effect of

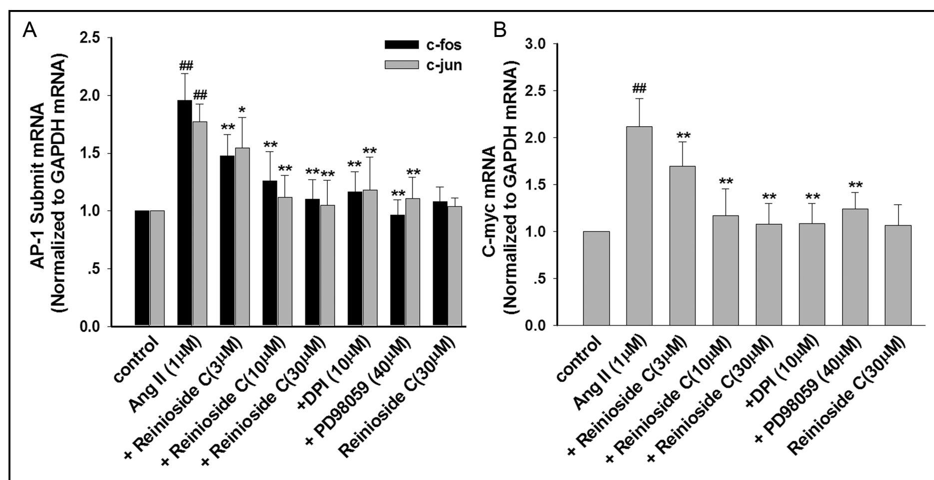

Fig. 6: Effect of reinioside C on mRNA expression of AP-1 subunits and c-myc induced by Ang II in A10 VSMCs. (a) mRNA expression of AP-1 subunits c-fos and c-jun. (b) mRNA expression of c-myc. The cells were pre-treated with reinioside C (3, 10 or 30 μM), DPI (10 μM), or PD98059 (40 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. DPI: diphenyleneiodonium. Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells. Data are expressed as means ± SEM, n = 3. Compared with control, ## $P < 0.01$; compared with Ang II, ** $P < 0.01$.

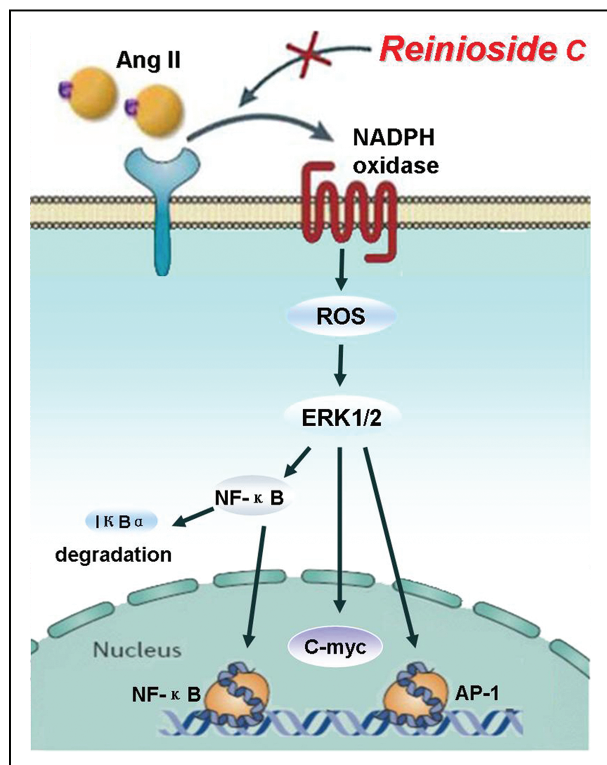

Fig. 7: The proposed pathway of reinioside C in inhibiting Ang II-induced proliferation of VSMCs. Ang II up-regulated the expression of NADPH oxidase subunit Nox-1 and Nox-4, and resultantly increased intracellular ROS production and the phosphorylation of ERK1/2, which in turn activates NF-κB/AP-1/c-myc -dependent signaling pathway to mediate Ang II-induced proliferation of A10 VSMCs. Reinioside C inhibited all these effects of Ang II. Ang II: angiotensin II; A10 VSMCs: rat aortic smooth muscle cells.

reinioside C on the proliferation of VSMCs induced by Ang II is due to inhibition of ERK1/2/NF-κB/AP-1/c-myc pathway.

In conclusion, the present study demonstrates that reinioside C significantly inhibits Ang II-induced proliferation of VSMCs *via* inhibiting NADPH oxidase-ROS-ERK1/2-NF-κB-AP-1 pathway. The proposed pathway of reinioside C in inhibiting Ang II-induced proliferation of VSMCs is summarized in Fig. 7. Reinioside C may be a potential anti-proliferative compound, but we could not identify the target inhibited by reinioside C in this study, maybe AT1 receptor or other upstream pathways of NADPH oxidase, so further studies are needed to define its targets and its effect *in vivo*.

4. Experimental

4.1. *Plant material*

Reinioside C (the purity was 99.0 % by HPLC and the structure was elucidated on the basis of spectral evidences) was extracted from *Polygala fallax Hemsl* (School of Pharmaceutical Sciences, Central South University, China). Reinioside C was dissolved in distilled water for the experiment.

4.2. *Cell culture*

Rat aortic smooth muscle cells A10 (A10 VSMCs) were obtained from ATCC (CRL-1476^{TM}, Manassas, Virginia). A10 VSMCs were cultured in Dulbecco's modified essential medium (DMEM) with 10% heat inactivated Fetal bovine serum (FBS), 100 U/ml penicillin,100 μg/ml streptomycin and $NaHCO_3$ (3.7 g/L), and were grown at 37 °C in a humidified 5% CO_2 incubator. The forth to sixth passages of the A10 VSMCs in the active growing condition were used for the experiments.

According to our previous studies and the dose response curve of reinioside C, we used three appropriate dosages of reinioside C (3, 10 or 30 μM) in this research. To study the effect of reinioside C on the proliferation of VSMCs induced by Ang II, VSMCs were pretreated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. To examine the role of ROS, cells were pretreated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then exposed to Ang II (1 μM) for 2 h; while NADPH oxidase subunits (Nox-1, Nox-4) and intracellular ROS level were determined. To further examine the role of ERK1/2, cells were pretreated with reinioside C (3, 10 or 30 μM), DPI (10 μM) or PD98059 (40 μM) for 1 h, then cultured with Ang II (1 μM) for 2 h; and the phosphorylation of ERK1/2 was determined. To explore the role of the NF-κB/AP-1 pathway, cells were pretreated with reinioside C (3, 10 or 30 μM), DPI (10 μM), PD98059 (40 μM) or PDTC(10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h; and the NF-κB DNA-binding activity, the phosphorylation status of IκB-α, mRNA expression of AP-1 subunits (c-fos, c-jun) and early growth response of gene c-myc were determined.

4.3. *Chemicals and biochemicals*

DMEM and FBS were obtained from Hyclone. Ang II, diphenyleneiodonium (DPI), pyrollidine dithiocarbamate (PDTC) and PD98059 were purchased from Sigma. The BrdU cell proliferation assay was purchased from Millipore. ROS assay kits and bicinchoninic acid protein assay kits were purchased from Beyotime Company (Jiangsu, China). ERK1/2 antibodies were purchased from Cell Signaling Technology. The antibody for IκB-α was purchased from Santa Cruz Biotechnologies. The RevertAid^{TM} first strand cDNA synthesis kit was obtained from Fermentas. The PCR primer and the SYBR green realtime PCR master mix kit were purchased from Takara. The [γ-^{32}P] ATP was obtained from Furui Biological Engineering Institute (Beijing, China). The Gel shift assay system for determination of NF-κB activity and the probe of NF-κB were obtained from Promega. All other biochemicals used were of the highest available purity.

4.4. *Cell proliferation assays*

As described in our previous study, the cell proliferation was measured by two methods. DNA synthesis and cell cycle were analyzed by BrdU marking and flow cytometry, respectively. For the BrdU incorporation assay, A10 VSMCs were counted and seeded into 96-well culture plates (6×10^3 cells per well). After 24 h, they were placed into DMEM containing 1% FBS and let stand for 24 h. A10 VSMCs were pre-incubated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. BrdU (0.1 L/mL; Cell Proliferation ELISA for BrdU; Roche) was added. Cells were fixed and stained after 12 h according to the manufacturer's instructions; colorimetric analysis was performed with an ELISA plate reader (DTX880, Beckman, Miami, FL, USA).

For cell cycle analysis using flow cytometry A10 VSMCs were counted and seeded into six-well culture plates (1×10^5 cells per well). After 24 h, they were placed into DMEM containing 1% FBS to let stand for 24 h. A10 VSMCs were pre-incubated with reinioside C (3, 10 or 30 μM) or DPI (10 μM) for 1 h, then cultured with Ang II (1 μM) for 24 h. On the next day, the cells were fixed gently with 70% cold alcohol at 4 °C over night. The cells were then treated with 0.25% Triton X-100 for 5 min in an ice bath and re-suspended in 300 mL of phosphate-buffered saline containing 50 mg/mL propidium iodide and 0.1 mg/mL RNase. Cells were incubated in a dark room for 30 min at room temperature and then subjected to cell cycle analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 10000 cells were counted. Data are presented as percentage of cells in a given sub-population.

4.5. *Determination of ROS*

Changes of intracellular ROS level were determined by the oxidative conversion of cell permeable 2',7'-dichlorofluorescein diacetate (H_2DCF) to fluorescent dichlorofluorescein (DCF) in fluorospectrophotometer (F4000, Japan). The medium was aspirated and cells were washed twice with phosphate-buffered saline and incubated in 1 ml of the medium without FBS. H_2DCF was added to obtain a final concentration of 10 μM and incubated for 20 min at 37 °C. The fluorescence was monitored under a fluorescence microscope equipped with a FITC filter, and the average intensity values were measured at 200 × magnification in five randomly chosen fields of the nine replicates from four independent experiments.

4.6. *Real-time PCR analysis*

The mRNA expressions of Nox-1, Nox-4, c-fos, c-jun and c-myc were evaluated by real time PCR. Total RNA was isolated from V10 VSMCs by TRIZOL extraction, and 4 μg RNA from each sample were reversely transcribed into cDNA using RevertAid^{TM} first strand cDNA synthesis kit. For PCR amplification, cDNA was amplified in the ABI 7300 real-time PCR system by using SYBR Green Real-time PCR Master Mix kit. 10 μL reaction mixture contains 2 μL cDNA template, 7.6 μL SYBR Master Mix, and 0.2 μL of each primer (0.4 μM). Amplification was carried out with an

Table: The primer sequences for real- time PCR analysis

Name	Sequence (5'-3')
Nox-1	P+: 5'- CTTCCTCACTGGCTGGGATA -3' P-: 5'- AGCCTGCGCAAATGCTGTC -3'
Nox-4	P+: 5'- CAGTAATCAATCATCCCTCAGA -3' P-: 5'- TGTCCCAGTGTATCAGCATTAG -3
c-fos	P+: 5'- AGGAGAATCCGAAGGGAAAGG -3', P-: 5'- TCCGCTTGGAGTGTATCAGTCA -3'.
c-jun	P+: 5'- GGGAACAGGTGGCACAGCTTA -3' P-: 5'- GCAACTGCTGCGTTAGCATGA -3'.
c-myc	P+: 5'- CTCGGTGGTCTTCCCCTAC -3' P-: 5'- AGGAGCCTGCCTCTTTTC -3'.
GAPDH	P+: 5'- CTGCACCACCAACTGCTTAG -3' P-: 5'- AGGTCCACCACTGACACGTT -3'

initial step for 30 s at 94 °C and 40 cycles of the amplification step (94 °C 30 s, 60 °C 60 s, and 72 °C 1 min). All amplification reactions for each sample were carried out in triplicates and the averages of the threshold cycles were used to modifier curves using 7300 System SDS Software. Results were reported as the ratio of Nox-1, Nox-4, c-fos, c-jun and c-myc to GAPDH mRNA, and the value of the control group was defined as 1.
The primer sequences used in the present study were shown in the Table.

4.7. Western blotting analysis

Cells were lysed for 30 min at 4 °C in a lysis buffer. Total cell protein concentration was determined by bicinchoninic acid reagent. Total protein (50 to 100 μg) was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblot analysis. The 1:1,000 dilution primary monoclonal antibodies for total ERK1/2, phosphorylated ERK1/2, phosphorylated IκB-α or β-actin, and horseradish peroxidase-conjugated secondary antibody (Santa Cruz) were used. The bands were visualized using enhanced chemiluminescence reagents and analysed in a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1). All results were representative of at least three independent experiments.

4.8. Electrophoretic mobility shift assay

Nuclear protein extraction was carried out as described previously (Bai et al. 2009). The electrophoretic mobility shift assay for determining the NF-κB DNA-binding activity was performed by incubating aliquots of nuclear extracts containing 15 μg total protein with [γ-^{32}P] ATP-labeled double-stranded NF-κB-specific oligonucleotide probe (sense: 3′-TCAACTCCCCTGAAAGGGTCCG-5′; antisense: 5′-AGTTGAGGGGACTTTCCCAGGC-3′) marked by T4 polynucleotide kinase. The labeled probe was purified through Sephadex G-25. After 10 min of incubation at room temperature, the mixture was reacted on a 4% non-denaturing polyacrylamide gel in 0.5 × Tris base–boric acid–EDTA buffer. After electrophoresis, the gels were dried, and the DNA–protein complexes were detected by autoradiography.

4.9. Statistical analysis

Statistical calculations were operated with the SPSS 13.0 for Windows software package (Statistica). Results are represented as means ± SEM. The data were analyzed by ANOVA followed by Newman-Student-Keuls test for multiple comparisons. The statistical significance was considered when $P < 0.05$.

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