Regular Article

Tanshinone IIA Can Inhibit Angiotensin II-Induced Proliferation and Autophagy of Vascular Smooth Muscle Cells *via* Regulating the MAPK Signaling Pathway

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To examine the effect of tanshinone IIA on Angiotensin II (Ang II)-induced proliferation and autophagy in vascular smooth muscle cells (VSMCs) and the related mechanism. VSMCs were treated with Ang II with or without tanshinone IIA (1, 5 and 10 μ g/mL), and the proliferation, apoptosis in cells with different treatment were examined by methylthiazolyl tetrazolium (MTT) and flow cytometry methods. Moreover, the expression of autophagy related proteins and mitogen-activated protein kinase (MAPK) signaling molecules were examined by RT-quantitative (q)PCR and Western blot methods. Ang II induced significantly increase in the proliferation and autophagy of VSMCs, and the MAPK signaling was activated. Tanshinone IIA can attenuate Ang II-induced effects *via* down-regulating the MAPK signaling pathway. Tanshinone IIA can inhibit Ang II-induced proliferation and autophagy of VSMCs *via* regulating the MAPK signaling pathway.

Key words tanshinone IIA; Angiotensin II (Ang II); autophagy; vascular smooth muscle cell (VSMC); mitogen-activated protein kinase (MAPK)

INTRODUCTION

In recent years, increasing evidences indicated that abnormal proliferation of vascular smooth muscle cells (VSMCs) were involved in the pathogenesis of cardiovascular disease, for example atherosclerosis (AS), hyperplasia, in-stent restenosis.^{1–3)} Thus, the attenuation of aberrant proliferation of VSMCs could be a potential novel pharmacotherapeutic strategy to prevent the processes that can induce the occurrence and development of cardiovascular diseases.⁴⁾

Angiotensin II (Ang II) is the primary effector hormone of the renin–angiotensin system, and results of previous studies suggested that Ang II may play a key role in the development of the cardiovascular system.^{5,6)} Autophagy, or type-II programmed cell death, as a activity of cells: on one hand, acts as a protective mechanism for cell survival and growth in normal state; on the other hand, performed aberrant when the cells were activated by certain stimulus.^{7–9)} It was observed that Ang II can increase the autophagy of VSMCs,¹⁰⁾ however, the related mechanism is still unclear.

Tanshinone IIA is the main active component that isolated from danshen, a traditional Chinese medicine for treating cardiovascular disease in China for many years.^{11–13)} It was reported that tanshinone IIA can inhibit the proliferation of VSMCs proliferation and intimal hyperplasia^{14,15)}; however, whether tanshinone IIA can inhibit Ang II-induced proliferation and autophagy of VSMCs is still unknown. Thus, in the present study, we will explore the effects of tanshinone IIA on Ang II-induced proliferation and autophagy of VSMCs and the related mechanism.

MATERIALS AND METHODS

Cell Culture and Treatment Primary VSMCs separated from the thoracic aortas of Sprague-Dawley rats (5–8 weeks old) were incubated in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco, China) and 1% penicillin/streptomycin (Solarbio, China) with 5% CO₂ at 37°C. Cells were subgrouped: 1) Untreated cells (control group); 2) Cells treated with Ang II (Ang II group); 3) Cells treated with 1 μ g/mL of Tanshinone IIA (Tanshinone IIA low group); 4) Cells treated with 5 μ g/mL of Tanshinone IIA (Tanshinone IIA medium group); 5) Cells treated with 10 μ g/mL of Tanshinone IIA (Tanshinone IIA high group).

Cell Viability Assay The cell viability was examined with methylthiazolyl tetrazolium (MTT) assay (Invitrogen, U.S.A.) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates (5 $\times$ 10³ cells/well). Later, cells were treated with 10 μ L MTT at 37°C for 4 h. Subsequently, the absorbance at the wavelength of 490 nm with a microplate reader. Each independent experiment was performed in triplicate.

Cell Apoptosis Analysis Cells was stained with the Annexin V/propidium iodide (PI) apoptosis detection kit (Invitrogen) at 48-hour post-transfection. Cells were harvested and suspended with annexin-binding buffer. After this, cells were stained with AnnexinV-fluorescein isothiocyanate (FITC) and PI for 15 min in shade. Then the apoptosis rate was calculated with BD FACSVerse flow cytometer (BD Biosciences, U.S.A.).

Quantitative (q) Real-Time PCR After 48 h, total RNAs were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. ReverTra Ace- α -kit (Toyobo Life Science, Japan) was applied to reversely transcribe RNAs to cDNA at 25°C for 10 min, 37°C for 100 min

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Fig. 1. Effect of Tanxinone IIA on the Survival of Ang II Treated VSMCs *in Vitro*

Ang II, Angiotensin IIA; VSMCs, vascular smooth muscle cells. * $p < 0.05$, ** $p < 0.01$ vs. Ang II group, *** $p < 0.001$ vs. Ang II group. Each experiment was performed in triplicate ($n = 3$).

Fig. 2. Effect of Tanxinone IIA on the Apoptosis of Ang II Treated VSMCs *in Vitro*

Ang II, Angiotensin IIA; VSMCs, vascular smooth muscle cells. ** $p < 0.01$ vs. Ang II group, *** $p < 0.001$ vs. Ang II group. Each experiment was performed in triplicate ($n = 3$).

and 90°C for 5 s and 4°C for 5 min. Power SYBR Green (TaKaRa, Japan) was used for qPCR analyses. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. PCR was performed under the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. The data were evalu-

ated with $2^{-\Delta\Delta C_q}$ method. The sequences of the primers were listed as follows: p38, forward: 5'-TTCGCA TGA ATGATG GACTGA A-3', reverse: 5'-GAACAAGAC AATCTGGGA GGTG-3'; c-myc, forward: 5'-ATTCTCTGCTCTCCTCGA CG-3' and reverse: 5'-CTGTGA GGAGGT TGTCTGTG-3'; c-fos, forward: 5'-CGAGCCCTTTGA TGA CTTCTCT-3', re-

Fig. 3. Effect of Tanshinone IIA on the Autophagy of Ang II Treated VSMCs *in Vitro*

Ang II, Angiotensin IIA; VSMCs, vascular smooth muscle cells. * $p < 0.05$ vs. Ang II group, ** $p < 0.01$ vs. Ang II group, *** $p < 0.001$ vs. Ang II group. Each experiment was performed in triplicate ($n = 3$).

verse: 5'-GGA GCGGGCTGTCTCAGA-3'; GAPDH, forward: 5'-AGCCTCCCGCTTCGCTCTCT-3', reverse: 5'-GCGCCCAATACGACCAATCCGT-3'.

Western Blot After 48h, total protein was isolated from cells using a radioimmunoprecipitation assay lysis buffer kit (Santa Cruz Biotechnology, U.S.A.) at 4°C for 30min. Cells were lysed with RIPA buffer (Biomed, China). Thirty micrograms of protein were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then protein were moved onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, U.S.A.). Later the protein were blocked with 5% skimmed milk. After this, the membranes were incubated with following primary antibodies overnight at 4°C: anti-p38 (ab170099, 1:1000, Abcam, U.S.A.), anti-LC3-I/II (ab128025, 1:1000, Abcam), anti-Beclin-1 (ab207612, 1:2000, Abcam), and GAPDH (ab181602, 1:10000, Abcam). Next day, the protein were incubated with the secondary antibody (ab6721, 1:2000, Abcam) at room temperature for 1h. Protein bands captured with ECL kit (Beyotime, Beijing, China) and determined with Image J 1.6 (National Institutes of Health, U.S.A.).

Statistical Analysis Data were presented as mean values $\pm$ standard deviation (S.D.) and analyzed with SPSS11.0. ANOVA was performed to determine the difference among multi-groups. $p < 0.05$ was considered as statistic significant.

RESULTS

Tanshinone IIA Inhibited the Proliferation of Ang II-Treated VSMCs First of all, the effect of tanshinone IIA and Ang II on the proliferation of VSMCs was examined by MTT methods. As shown in Fig. 1, Ang II increased the proliferation of VSMCs after 24 and 48h ($p < 0.05$ and $p < 0.01$, respectively); furthermore, the effect of tanshinone IIA on the proliferation of Ang II treated VSMCs were also examined. It was observed that tanshinone IIA significantly decreased the proliferation of Ang II treated VSMCs *in vitro* in a dose-dependent manner.

Tanshinone IIA Inhibited the Ang II-Induced Proliferation of VSMCs Moreover, the effect of tanshinone IIA on

the apoptosis of Ang II treated VSMCs was examined by flow cytometry method. It was observed that Ang II significantly decreased the apoptosis of VSMCs after 48h of treatment (Fig. 2, $p < 0.001$, respectively); while on the other hand, medium and high dose of tanshinone IIA markedly increased the apoptosis of Ang II treated VSMCs in a dose-dependent manner (Fig. 2, $p < 0.01$). Low dosage of tanshinone IIA had no significant effect on the apoptosis of Ang II treated VSMCs *in vitro*.

Tanshinone IIA Can Inhibit Ang II-Induced Autophagy of VSMCs Next, the effect of tanshinone IIA and Ang II on the autophagy of Ang II treated VSMCs was examined. As shown in Fig. 3, compared with un-treated cells, Ang II significantly increased the expression of the autophagy marker LC-3 and Beclin-1 in VSMCs (Fig. 3, $p < 0.001$); meanwhile, medium and high dose of tanshinone IIA can decrease the expression of both LC-3 and Beclin-1 in Ang II treated VSMCs in a dose-dependent manner (Fig. 3, $p < 0.05$). Low dosage of tanshinone IIA had no significant effect on the autophagy of Ang II treated VSMCs *in vitro*.

Tanshinone IIA Inhibited Ang II-Induced Activation of Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway MAPK signaling pathway has been reported to be involved in the process of cell proliferation and autophagy.^{16,17} Finally, to further explore the underlying mechanism of tanshinone IIA induced biological effects on Ang II treated VSMCs, the expressions of MAPK signaling molecules p38, c-myc and c-fos were examined. As shown in Fig. 4, Ang II had no significant effect on the expression of p38 on both mRNA and protein levels, while the expressions of phosphorylated (p)-p38, c-myc and c-fos were significantly increased on both mRNA (Fig. 4A, $p < 0.01$) and protein (Fig. 4B, $p < 0.01$) levels. Furthermore, medium and high dose of tanshinone IIA can decreased the expression of p-p38, c-myc and c-fos in Ang II treated VSMCs in a dose-dependent manner (Fig. 4, $p < 0.05$) and low dosage of tanshinone IIA had no significant effect on the expressions of p-p38, c-myc and c-fos in VSMCs *in vitro*.

The Expression of p-38 Moreover, Western blot was used to determine the expression of p-38 at 0, 12, 24, 48h. As

Fig. 4. Effect of Tanshinone IIA on the mRNA (A) and Protein (B) Expressions of p38, p-p38, c-myc and c-fos Ang II Treated VSMCs *in Vitro*

Ang II, Angiotensin IIA; VSMCs, vascular smooth muscle cells. * $p < 0.05$ vs. Ang II group, ** $p < 0.01$ vs. Ang II group. Each experiment was performed in triplicate ($n = 3$).

showed in Fig. 5, the expression of p-38 was decreased in a time-dependent manner after exposed to tanshinone IIA (medium concentration).

DISCUSSION

In the present study, we observed that tanshinone IIA can inhibit Ang II-induced increase in the proliferation and autophagy of VSMCs *via* down-regulating the MAPK signaling pathway, suggesting that tanshinone IIA may be a potential novel medication for the treatment cardiovascular disease that associated with the dysfunction of VSMCs.

The process of vascular injury, which refers to the pathological changes and the local inflammatory effect, can further lead to the incidence of different cardiovascular disease, for example atherosclerosis, ischemic heart disease, hypertension *etc.*¹⁸⁾ Results of previous studies indicated that vascular injury was strongly associated with the increased proliferation, calcification, migration and autophagy of VSMCs, which may mainly be caused by the increased expression of Ang II.¹⁹⁾ In the present study, we observed that Ang II can increase the proliferation and autophagy, and decrease apoptosis of VSMCs *in vitro*, which was consistent with previous observations, suggesting that Ang II was a key mediator during the process of vascular injury.

The protective roles of tanshinone IIA in the cardiovascular

Fig. 5. The Expression of p-p38

The expression of p-p38 was decreased in a time-dependent manner after the treatment of tanshinone IIA compared with p38 level.

system has been discussed in many previous studies.^{14,15,20,21)} For example, it has been reported that tanshinone IIA can inhibit the proliferation of VSMCs in the rat carotid balloon-injured model²²⁾; moreover, tanshinone IIA may inhibit aberrant proliferation and migration of VSMCs cells treated by advanced glycation end products.¹⁴⁾ However, the effects of tanshinone IIA on proliferation and autophagy of Ang II treated VSMCs remain unclear. In the present study, we first explored the that treatment of 5 and 10 $\mu\text{g/mL}$ tanshinone IIA can decrease the proliferation and autophagy, and increase the apoptosis of Ang II treated VSMCs *in vitro*, suggesting that tanshinone IIA can alleviate Ang II induced abnormality in VSMCs.

Results of previous studies indicated that activation of the

MAPK signaling pathway was involved in process of VSMC growth, migration and autophagy.²³⁾ Moreover, Beclin-1 regulates the expression of MAPK in the differentiation of osteoclast.²⁴⁾ However, it remains unclear whether tanshinone IIA can alleviate Ang II induced abnormality in VSMCs *via* regulating the MAPK signaling pathway. Previous study showed that tanshinone IIA suppressed the expression of MAPK *via* regulating miR-124.²⁵⁾ In the present study, we observed that Ang II induced increased expression of LC-3 I/II and Beclin-1 and p-p38, while 5 and 10 $\mu\text{g/mL}$ tanshinone IIA inhibited the phosphorylation of p38 in Ang II treated VSMCs in a dose dependent manner. Moreover, c-fos and c-myc were known as the downstream molecules of the p38 signaling pathway,^{26,27)} increased expressions of c-fos and c-myc may promote the proliferation and autophagy of different type of cells. In the present study, we reported that Ang II increased the expression of both c-fos and c-myc VSMCs, and tanshinone IIA can inhibit the expressions of c-fos and c-myc in Ang II treated VSMCs. Taken together, these results indicated that tanshinone IIA may inhibit the migration and autophagy of VSMCs *via* suppressing the Beclin-1/p38 signaling pathway.

Our study has limitations. We performed only cell studies to examine the effects of tanshinone IIA on Ang II treated VSMCs. In future studies, the roles of tanshinone IIA on Ang II induced effects should also be evaluated using *in vivo* animal models of cardiovascular diseases.

In conclusion, we reported for the first time that tanshinone IIA can inhibit Ang II-induced proliferation and autophagy of VSMCs *via* down-regulating the MAPK signaling pathway. Our results proposed the potential clinical application of tanshinone IIA for the treatment cardiovascular disease that associated with VSMCs dysfunction.

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Conflict of Interest The authors declare no conflict of interest.

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