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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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Summary

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 μg/ml, 5 μg/ml and 10 μg/ml), and the proliferation, apoptosis in cells with different treatment were examined by MTT and flow cytometry methods. Moreover, the expression of autophagy related proteins and MAPK signaling molecules were examined by RT-qPCR and WB 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.

Keywords: Tanshinone IIA, Ang II, autophagy, VSMCs, 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\(^1\text{--}^3\). 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\(^7\text{--}^9\). 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 form danshen, a traditional Chinese medicine for treating cardiovascular disease in China for many years\(^11\text{--}^13\). 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.
Material 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 µg/ml of Tanshinone IIA (Tanshinone IIA low group); 4) Cells treated with 5 µg/ml of Tanshinone IIA (Tanshinone IIA medium group); 5) Cells treated with 10 µg/ml of Tanshinone IIA (Tanshinone IIA high group).

Cell viability assay. The cell viability was examined with MTT assay (Invitrogen, USA) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates (5×10³ cells/well). Later, cells were treated with 10 µl methylthiazolyl tetrazolium (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 apoptosis detection kit (Invitrogen, USA) at 48-hour post-transfection. Cells were harvested and suspended with annexin-binding buffer. After this, cells were stained with AnnexinV-FITC and PI for 15 min in shade. Then the apoptosis rate was calculated with BD FACSVerse flow cytometer (BD Biosciences, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR). After 48 hours, total RNAs were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. ReverTra Ace-α-kit (Toboyo Life Science, Japan) was applied to reversely transcribe RNAs.
to cDNA at 25°C for 10 min, 37°C for 100 min and 90°C for 5 sec and 4°C for 5 min. Power SYBR Green (Takara, Japan) was used for qPCR analyses. 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 sec, 60°C for 30 sec, 72°C for 30 sec. The data were evaluated with 2^{-ΔΔCq} method. The sequences of the primers were listed as follows: p38, forward: 5’-TTGCATGAATGGACGACTGAA-3’, reverse: 5’-GAACAAGACAATCTGGGGAGGTG-3’; c-myc, forward: 5’-ATTCTGTCTCTCTCGACG-3’ and reverse: 5’-CTGTGAGGAGGTTGTGTGAGT-3’; c-fos, forward: 5’-CAGCGCGCTCTCTCGAC-3’, reverse: 5’-GGAGCGGGCTGTCTCACA-3’; GAPDH, forward: 5’-AGCCTCCCGCTTCGCTCTCT-3’, reverse: 5’-GCGCCCAATACGACCAAATCCGT-3’.

**Western blot.** After 48 hours, total protein was isolated from cells using a radioimmunoprecipitation assay lysis buffer kit (Santa Cruz Biotechnology, USA) at 4°C for 30 min. Cells were lysed with RIPA buffer (Biomed, China). 30 µg of protein were separated with 10% SDS-PAGE. Then protein were moved onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, USA). 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, USA), anti-LC3-I/II (ab128025, 1:1000, Abcam, USA), anti-Beclin-1 (ab207612, 1:2000, Abcam, USA), and GAPDH (ab181602, 1:10000, Abcam, USA). Next day, the protein were incubated with the secondary antibody (ab6721, 1:2000, Abcam, USA) at room temperature for 1 h. Protein bands captured with ECL kit (Beyotime, Beijing, China) and determined with Image J 1.6 (National Institutes of Health, USA).
Statistical analysis. Data were presented as mean values ± standard deviation (SD) 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 Figure 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 (Figure 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 (Figure 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 Figure 3, compared with un-treated cells, Ang II significantly increased the expression of the autophagy marker LC-3 and Beclin-1 in VSMCs, (Figure 3, p<0.001); meanwhile, medium and high dose of tanshinone IIA can decreased the expression of both
LC-3 and Beclin-1 in Ang II treated VSMCs in a dose-dependent manner (Figure 3, p<0.05). Low dosage of tanshinone IIA had no significant effect on the autophagy of Ang II treated VSMCs \textit{in vitro}.

\textit{Tanshinone IIA inhibited Ang II-induced activation of MAPK signaling pathway}. MAPK signaling pathway has been reported to be involved in the process of cell proliferation and autophagy\textsuperscript{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 Figure 4, Ang II had no significant effect on the expression of p38 on both mRNA and protein levels, while the expressions of p-p38, c-myc and c-fos were significantly increased on both mRNA (Figure 4A, p<0.01) and protein (Figure 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 (Figure 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 \textit{in vitro}.

\textit{The expression of p-38}

Moreover, western blot was used to determine the expression of p-38 at 0, 12, 24, 48 h. As showed in Figure 5, the expression of p-38 was decreased in a time-dependent manner after exposed to tanshinone IIA (medium concentration).

\textbf{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 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 in the rat carotid balloon-injured model\(^{22}\); 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 μg/ml and 10 μg/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 previously 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 phosphorylated-p38, while 5 \(\mu\)g/ml and 10 \(\mu\)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 \textit{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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Figure 1. Effect of tanshinone 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).
Figure 2. Effect of tanshinone IIA on the apoptosis 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. Each experiment was performed in triplicate (n=3).
Figure 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).
Figure 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).
Figure 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.