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

Jingping Lu, Jinjun Shan, Ning Liu, Yao Ding, and Pei Wang*

Department of Cardiology, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine; Nanjing 210029, Jiangsu, China; and Jiangsu Key Laboratory of Pediatric Respiratory Disease, Institute of Pediatrics, Affiliated Hospital of Nanjing University of Chinese Medicine; Nanjing 210029, Jiangsu, China.

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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. 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.

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. 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. It was observed that Ang II can increase the autophagy of VSMCs, 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. It was reported that tanshinone IIA can inhibit the proliferation of VSMCs proliferation and intimal hyperplasia, 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.

*To whom correspondence should be addressed. e-mail: Peiwang2012@yandex.com

MATERIALS AND METHODS

Cell Culture and Treatment Primary VSMCs separated from the thoracic aortas of Sprague-Dawley rats 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 Tanshinone IIA (Tanshinone IIA low group); 4) Cells treated with Tanshinone IIA (Tanshinone IIA medium group); 5) Cells treated with 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 and cultured with 5% CO2 at 37°C for 48h. Subsequently, the absorbance at the wavelength of 490 nm with a microplate reader. Each independent experiment was performed in triplicate.

Cell Apoptosis Analysis Cells were 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 48h, total RNAs were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. ReverTra Ace-alpha-kit (Toyobo Life Science, Japan) was applied to reversely transcribe RNAs to cDNA at 25°C for 10min, 37°C for 100min

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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 evaluated with $2^{-\Delta\Delta Cq}$ method. The sequences of the primers were listed as follows: p38, forward: 5’-TTC GCA TGA ATG ATG GAC TGA A-3’/uni2032, reverse: 5’/uni2032- GAA CAA GAC AAT CTG GGA GGT G-3’; c-myc, forward: 5’/uni2032- ATT CTC TGC TCT CCT CGA CG-3’ and reverse: 5’-CTG TGA GGA GGT TTG CTG TG-3’; c-fos, forward: 5’-CGA GCC CTT TGA ATG GAC TGA A-3’/uni2032, reverse: 5’/uni2032- GAA CAA GAC AAT CTG GGA GGT G-3’; c-fos, forward: 5’-CGA GCC CTT TGA ATG GAC TGA A-3’/uni2032, reverse: 5’/uni2032- GAA CAA GAC AAT CTG GGA GGT G-3’.

Fig. 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 ($n=3$).

Fig. 2. Effect of Tanshinone IIA on the Apoptosis of Ang II Treated VSMCs in Vitro
Ang II, Angiotensin IIA; VSMCs, vascular smooth muscle cells. **$p<0.01$ vs. Ang II group, ***$p<0.001$ vs. Ang II group. Each experiment was performed in triplicate ($n=3$).
Tanshinone IIA Inhibited Ang II-Induced Proliferation of 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 was examined. It was observed that tanshinone IIA significantly decreased the proliferation of Ang II treated VSMCs 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.

**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 was 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
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. 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. 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. For example, it has been reported that tanshinone IIA can inhibit the proliferation of VSMCs in the rat carotid balloon-injured model; moreover, tanshinone IIA may inhibit aberrant proliferation and migration of VSMCs cells treated by advanced glycation end products. 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 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 previous studies indicated that activation of the

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

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.
MAPK signaling pathway was involved in process of VSMC growth, migration and autophagy.\textsuperscript{21} Moreover, Beclin-1 regulates the expression of MAPK in differentiation of osteoclast.\textsuperscript{21} 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.\textsuperscript{23} In the present study, we observed that Ang II induced expression of LC-3-I/II and Beclin-1 and p-p38, while 5 and 10wg/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.\textsuperscript{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.

REFERENCES

1. Qiu L, Xu C, Jiang H, Li W, Tong S, Xia H. Cantharidin attenuates the proliferation and migration of vascular smooth muscle cells through suppressing inflammatory response. Biochem. Biophys. Res. Commun., 42, 34–42 (2019).

2. He J, Zhong X, Zhao L, Gan H, JAK2/STAT3/BMP-2 axis and NF-kappaB pathway are involved in erythropoietin-induced calcification in rat vascular smooth muscle cells. Clin. Exp. Nephrol., 23, 501–512 (2019).

3. Schwartz M, Bockmann S, Hinz B. Up-regulation of heme oxygenase-1 expression and inhibition of disease-associated features by cannabinoid in vascular smooth muscle cells. Oncotarget., 9, 34595–34616 (2018).

4. Li M, Qian M, Kylar K, Xu J. Endothelial-sascular smooth muscle cells interactions in atherosclerosis. Front. Cardiovasc. Med., 5, 151 (2018).

5. Eshraghian A, Iravani S, Azimzadeh P. The association between angiotensin II type 1 receptor gene A166C polymorphism and non-alcoholic fatty liver disease and its severity. Middle East J. Dig. Dis., 10, 96–104 (2018).

6. Galatioti J, Caescu CI, Hansen J, Cook JR, Miramontes I, Iyengar R, Ramirez F. Cell type-specific contributions of the angiotensin II type 1a receptor to aorta homeostasis and aneurysmal disease—brief report. Arterioscler. Thromb. Vasc. Biol., 38, 588–591 (2018).

7. Qiu X, Liu K, Xiao L, Jin S, Dong J, Teng X, Guo Q, Chen Y, Wu Y. Alpha-lipoic acid regulates the autophagy of vascular smooth muscle cells in diabetes by elevating hydrogen sulfide level. Biochim. Biophys. Acta. Mol. Basis. Dis., 1864, 3723–3738 (2018).

8. An XR, Li X, Wei W, Li XX, Xu M. Prostaglandin E1 inhibited diabetes-induced phenotypic switching of vascular smooth muscle cells through activating autophagy. Cell. Physiol. Biochem., 50, 745–756 (2018).

9. Ye G, Fu Q, Jiang L, Li Z. Vascular smooth muscle cells activate PI3K/Akt pathway to attenuate myocardial ischemia/reperfusion-induced apoptosis and autophagy by secreting bFGF. Biomed. Pharmacotherapy., 107, 1779–1785 (2018).

10. Yu KY, Wang YP, Wang LH, Jian Y, Zhao XD, Chen JW, Murao K, Zhu W, Dong L, Wang GQ, Zhang GX. Mitochondrial KATP channel involvement in angiotensin II-induced autophagy in vascular smooth muscle cells. Basic. Res. Cardiol., 109, 416 (2014).

11. Zhu Z, Wang Y, Liu W, Li H, Wang D. Effect of various Danshen injections on patients with coronary heart disease after percutaneous coronary intervention: a protocol for a systematic review and network meta-analysis. Medicine, 97, e11062 (2018).

12. Yang K, Dong G, Tian Y, Li J. Effects of compound Danshen injection combined with magnesium sulfate on serum MPO and hs-CRP in patients with severe preeclampsia. Exp. Ther. Med., 16, 167–170 (2018).

13. Yu YG, Yang J, Cheng XH, Shang W, Zhao BH, Zhao F, Chen ZG, Huang ZH. The protection of acute spinal cord injury by subarachnoid space injection of Danshen in animal models. J. Spinal Cord Med., 42, 355–359 (2019).

14. Lu M, Luo Y, Hu P, Dou L, Huang S. Tanshinone IIA inhibits AGEs-induced proliferation and migration of cultured vascular smooth muscle cells by suppressing ERK1/2 MAPK signaling. Iran. J. Basic Med. Sci., 21, 83–88 (2018).

15. Wang B, Ge Z, Cheng Z, Zhao Z. Tanshinone IIA suppresses the progression of atherosclerosis by inhibiting the apoptosis of vascular smooth muscle cells and the proliferation and migration of macrophages induced by ox-LDL. Biol. Open, 6, 489–495 (2017).

16. Obergastegger J, Frappotti G, Pramstaller PP, Hicks AA, Volta M. A new hypothesis for Parkinson’s disease pathogenesis: GTPase-p38 MAPK signaling and autophagy as convergence points of etiology and genomics. Mol. Neurodegener., 13, 40 (2018).

17. Barutcu SA, Grimus N, Verha S, Davis RJ. Role of the MAPK/ERK/H2-terminal kinase signaling pathway in starvation-induced autophagy. Autophagy, 14, 1586–1595 (2018).

18. Luo Y, Utu S, Toyoda M, Spees JL, Umezawa A, Suzuki H. Bone marrow-derived mesenchymal stem cells inhibit vascular smooth muscle cell proliferation and neuroinflammatory hyperplasia after arterial injury in rats. Biochem. Biophys. Rep., 16, 79–87 (2018).

19. Ben P, Hu M, Wu H, Zhang Z, Gao Y, Luo L, Yin Z. L-theanine down-regulates the JAK/STAT3 pathway to attenuate the proliferation and migration of vascular smooth muscle cells induced by angiotensin II. Biol. Pharm. Bull., 41, 1678–1684 (2018).

20. Xing Y, Tu J, Zheng L, Guo L, Xi T. Anti-angiogenic effect of tanshinone IIA involves inhibition of the VEGF/VEGFR2 pathway in vascular endothelial cells. Oncol. Rep., 33, 163–170 (2015).

21. Morton JS, Andersson JJ, Cheung PY, Baker P, Davidge ST. The vascular effects of sodium tanshinone IIA sulphonate in rodent and human pregnancy. PLOS ONE, 10, e0121897 (2015).

22. Li X, Du JR, Yu Y, Bai B, Zheng XY. Tanshinone IIA inhibits smooth muscle proliferation and intimal hyperplasia in the rat carotid balloon-injured model through inhibition of MAPK signaling pathway. J. Ethnopharmacol., 129, 273–279 (2010).
23) Zheng YH, Tian C, Meng Y, Qin YW, Du YH, Du J, Li HH. Osteopontin stimulates autophagy via integrin/CD44 and p38 MAPK signaling pathways in vascular smooth muscle cells. J. Cell. Physiol., 227, 127–135 (2012).

24) Chung YH, Jang Y, Choi B, Song DH, Lee EJ, Kim SM, Song Y, Kang SW, Yoo SY, Chang EJ. Beclin-1 is required for RANKL-induced osteoclast differentiation. J. Cell. Physiol., 229, 1963–1971 (2014).

25) Gong G, Gu Y, Zhang Y, Li L, Li J. Tanshinone IIA alleviates oxidative damage after spinal cord injury in vitro and in vivo through up-regulating miR-124. Life Sci., 216, 147–155 (2019).

26) Kalra N, Kumar V. c-Fos is a mediator of the c-myc-induced apoptotic signaling in serum-deprived hepatoma cells via the p38 mitogen-activated protein kinase pathway. J. Biol. Chem., 279, 25313–25319 (2004).

27) Shang W, Zhao L, Dong X, Zhao ZM, Li J, Zhang BB, Cai HH. Curcumin inhibits osteoclastogenic potential in PBMCs from rheumatoid arthritis patients via the suppression of MAPK/RANK/NFATc1 signaling pathways. Mol. Med. Rep., 14, 2367–2373 (2016).