Tanshinone IIA alters the transforming growth factor-β1/Smads pathway in angiotensin II-treated rat hepatic stellate cells

Guo-wei Wei¹*, Ke-yue Li²*, Ke-li Tang² and Cheng-Xian Shi²

Abstract
Objective: To investigate the effects of tanshinone IIA on the transforming growth factor-β1 (TGF-β1)/Smads signaling pathway in angiotensin II-treated hepatic stellate cells (HSCs).
Methods: HSCs were cultured and treated with angiotensin II (10 µM) or angiotensin II (10 µM) plus tanshinone IIA (3, 10, or 30 µM). Cells were incubated for 48 hours and proliferation was determined with the Cell Counting Kit-8. The relative mRNA expression of TGF-β1, Smad4, and Smad7 was measured by quantitative real-time PCR, and the relative protein expression levels were investigated by western blotting.
Results: After angiotensin II treatment, cell proliferation was significantly accelerated. Furthermore, both the mRNA and protein expression of TGF-β1 and Smad4 was significantly up-regulated, while the mRNA and protein expression of Smad7 was significantly down-regulated compared with the control cells. Tanshinone IIA inhibited the observed effects of angiotensin II in a concentration-dependent manner, with significant inhibition exerted by tanshinone IIA at 10 and 30 µM.
Conclusions: Angiotensin II promotes the proliferation of HSCs, possibly by regulating the expression of components along the TGF-β1/Smads signaling pathway. Tanshinone IIA inhibits the angiotensin II-induced activation of this pathway, and may, therefore, have preventive and therapeutic effects in liver fibrosis.

¹Department of Comprehensive Ward, Guizhou Provincial People’s Hospital, Guiyang, Guizhou Province, China
²Department of Hepatobiliary Surgery, Guizhou Provincial People’s Hospital, Guiyang, Guizhou Province, China

*These authors contributed equally to this work.

Corresponding author:
Ke-Yue Li, Department of Hepatobiliary Surgery, Guizhou Provincial People’s Hospital, 83 Zhongshan East Road, Guiyang, Guizhou Province 550002, China.
Emails: keyuelee@sohu.com; keyuelee@qq.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
Keywords
Hepatic stellate cells, TGF-β1, Smad proteins, angiotensin II, liver fibrosis, tanshinone IIA

Introduction
Liver fibrosis refers to the proliferation of the extracellular matrix (especially collagen) in the liver and is the response of the liver to various forms of chronic injury, such as that caused by alcohol.1 If untreated, the early reversible cirrhosis stage of fibrosis progresses into decompensated cirrhosis, with the emergence of various end-stage liver disease complications.2

Hepatic stellate cells (HSCs) play an important role in the pathogenesis of liver fibrosis.3 Angiotensin II activates HSCs and upregulates transforming growth factor (TGF)–β1, which is involved in the development of liver fibrosis.4 Similarly, the TGF-β1/Smads pathway has crucial functions in HSC activation, proliferation, and liver fibrosis.5,6 Tanshinone IIA is a fat-soluble phenanthrenequinone compound extracted from the traditional Chinese medicine, Salvia miltiorrhiza, which was previously shown to have an anti-fibrotic effect on tissues.7 However, the role of tanshinone IIA in the TGF-β1/Smads pathway of HSCs after angiotensin II intervention remains unclear.

In the present study, we explored the effect of tanshinone IIA on the TGF-β1/Smads pathway of HSCs after angiotensin II intervention. We specifically investigated changes in cell proliferation and the mRNA and protein expression of TGF-β1, Smad4, and Smad7 in HSCs.

Materials and methods

Cells and key reagents
The rat HSC-T6 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The primer sequences used for PCR were: TGF-β1 forward primer, 5’-TGGCGTTACCTTGTAACCCACC-3’, reverse primer, 5’-GGTGTGAAACCATCCTTCAGG-3’; Smad4 forward primer, 5’-CTTTTGCTTGGGCAAATCTC-3’, reverse primer, 5’-CGGGCTACGCATATATTTCC-3’; Smad7 forward primer, 5’-TCAGGTGCCGGATCTCA-3’, reverse primer, 5’-GGTTGATCTCTCCCTGAAGATTCA-3’; β-actin forward primer, 5’-AGGCCATGCACGTTAGCCATGTCGTTAGGAGTCTCTC-3’, reverse primer: 5’-GATCTCTCCCTCCCTGAAGATTCA-3’ (all from Life Technologies, Carlsbad, CA, USA). Angiotensin II was purchased from Sigma-Aldrich (St Louis, MO, USA), sodium tanshinone IIA sulfonate from Shanghai No. 1 Biochemical & Pharmaceutical Co., Ltd. (Shanghai, China), trypsin-ethylenediaminetetraacetic acid from Sangon Biotech Co., Ltd. (Shanghai, China), Dulbecco’s Modified Eagle’s medium (DMEM) from HyClone Laboratories, Inc. (Logan, UT, USA), and fetal bovine serum (FBS) from Gibco Cell Culture (Carlsbad, CA, USA).

Grouping and treatment
HSCs were randomly divided into five groups: blank control group, angiotensin II treatment group (10 μM angiotensin II), and three groups treated with a combination of angiotensin II (10 μM) and different concentrations of tanshinone IIA (3, 10, or 30 μM).5,9 Cells were cultured in DMEM containing 10% (v/v) FBS, 100 U/mL penicillin (Sangon Biotech), and 100 mg/mL streptomycin (Sangon Biotech) at 37°C in a humidified incubator at 5% CO₂.
All cells were passaged using trypsin-ethylenediaminetetraacetic acid. HSCs from passages 4–6 were used for experiments, and cell culture aliquots were stored in liquid nitrogen.

Cells were transferred into 6-well or 96-well plates at a density of $2 \times 10^5$/well (6-well plates) or $2 \times 10^4$/well (96-well plates) and cultured in DMEM containing 10% FBS. All cells were starved in serum-free medium overnight, then cultured for 48 hours in DMEM supplemented with the relevant treatment for each treatment group.

**Proliferation assay**

Cells were transferred into 96-well plates, then cultured in 100 μL DMEM containing 10% FBS. After being treated for 48 hours, 10 μL of the Cell Counting Kit-8 (Hyclone Laboratories) solution was added to each well, and the plates were cultured at 37°C for a further 2.5 hours. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to read the optical densities in the wells at 450 nm (OD$_{450}$).

**Detection of TGF-β1, Smad4, and Smad7 mRNA expression**

Total RNA was extracted from the cells with RNAiso Plus (Takara Bio, Inc., Shiga, Japan), then reverse-transcribed into cDNA by reverse-transcription PCR using SYBR Premix Ex Taq (Takara Bio).

Quantitative real-time PCR was performed with the ABI StepOne system (Life Technologies). The fold induction method and the threshold cycle were used to calculate the relative quantities of target mRNA. Melting curves were adopted to confirm the amplification of specific transcripts. β-actin mRNA (Life Technologies) was used as an internal control.

**TGF-beta1, Smad4 and Smad7 protein expression**

Protein concentrations were assessed with the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Cell lysates (13 μg) were separated on a 10% to 12% gradient Bis-Tris polyacrylamide gel (Life Technologies) under reducing conditions, then transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich). Membranes were incubated with anti-rat primary antibodies (ab92486, ab40759, ab216428, and ab6276) from Abcam PLC (Cambridge, UK), followed by IgG-horseradish peroxidase secondary antibodies to detect TGF-β1, Smad4, Smad7, and β-actin expression. Chemiluminescence was detected with an ECL Western Blotting Detection Kit (Amersham, Little Chalfont, UK) and quantified with a Box gel imaging system (Syngene, Frederick, MD, USA) and Image Lab 3.0 software (Bio-Rad). β-actin protein (Abcam PLC) was used as an internal control.

**Statistical analysis**

All experiments were performed 3 to 5 times. Data are given as the mean ± standard deviation. Data were compared using one-way analysis of variance (with the Bonferroni post hoc test). Statistical analyses were conducted using SPSS for Windows, Version 16.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was deemed statistically significant.

**Results**

**Cell proliferation**

The proliferation of cells treated with angiotensin II was significantly increased compared with the control ($P < 0.0001$). However, this proliferation-promoting effect of angiotensin II was inhibited by tanshinone IIA in a dose-
dependent manner, with 10 μM and 30 μM tanshinone IIA having a significant effect (all \( P < 0.0001 \); Table 1).

**Relative mRNA expression of TGF-β1, Smad4, and Smad7**

Compared with the control cells, angiotensin II significantly up-regulated the mRNA expression of TGF-β1 and Smad4 and significantly down-regulated the mRNA expression of Smad7 (all \( P < 0.0001 \)). However, these effects were blocked by tanshinone IIA, as it inhibited the angiotensin II-mediated up-regulation of TGF-β1 and Smad4 and the angiotensin II-mediated down-regulation of Smad7 mRNA expression in a dose-dependent manner, with 10 μM and 30 μM tanshinone IIA having a significant effect (all \( P < 0.0001 \); Table 2).

### Table 1. Assay of cell proliferation in the different treatment groups.

| Group     | OD\(_{450}\) Mean ± SD | \( P \) value |
|-----------|-------------------------|---------------|
| Control   | 0.309 ± 0.004           | <0.0001       |
| Ang       | 0.399 ± 0.006           | –             |
| Ang + 3 μM STS | 0.386 ± 0.007   | 0.013         |
| Ang + 10 μM STS | 0.370 ± 0.005      | <0.0001       |
| Ang + 30 μM STS | 0.354 ± 0.006      | <0.0001       |

Ang, Angiotensin II; STS, sodium tanshinone IIA sulfonate. Cell proliferation was determined with the Cell Counting Kit-8. Data represent the mean ± standard deviation, \( n = 5 \). \( P \) values determined by one-way analysis of variance in comparison with Ang.

### Table 2. The relative mRNA expression of TGF-β1, Smad4, and Smad7 in the different treatment groups.

| Gene | Group     | Expression (fold-change) Mean ± SD | \( P \) value |
|------|-----------|-----------------------------------|---------------|
| TGF-β1 | Control   | 1.001 ± 0.035                     | <0.0001       |
|       | Ang       | 4.032 ± 0.089                     | –             |
|       | Ang + 3 μM STS | 3.835 ± 0.038   | 0.013         |
|       | Ang + 10 μM STS | 3.459 ± 0.040      | <0.0001       |
|       | Ang + 30 μM STS | 3.089 ± 0.114      | <0.0001       |
| Smad4 | Control   | 1.000 ± 0.050                     | <0.0001       |
|       | Ang       | 3.500 ± 0.065                     | –             |
|       | Ang + 3 μM STS | 3.367 ± 0.024   | 0.036         |
|       | Ang + 10 μM STS | 2.856 ± 0.078      | <0.0001       |
|       | Ang + 30 μM STS | 2.349 ± 0.037      | <0.0001       |
| Smad7 | Control   | 1.003 ± 0.037                     | <0.0001       |
|       | Ang       | 0.483 ± 0.023                     | –             |
|       | Ang + 3 μM STS | 0.548 ± 0.020   | 0.030         |
|       | Ang + 10 μM STS | 0.670 ± 0.025      | <0.0001       |
|       | Ang + 30 μM STS | 0.725 ± 0.021      | <0.0001       |

Ang, Angiotensin II; STS, sodium tanshinone IIA sulfonate. The level of mRNA expression was determined by real-time PCR and normalized to β-actin mRNA levels in the same sample. Data represent the mean ± standard deviation, \( n ≥ 3 \). \( P \) values determined by one-way analysis of variance in comparison with Ang.
down-regulation of Smad7 protein expression in a dose-dependent manner, with 10 µM and 30 µM tanshinone IIA having a significant effect for TGF-β1 and Smad4 (all \( P < 0.0001 \)) and 30 µM tanshinone IIA having a significant effect for Smad7 (\( P < 0.0001 \); Table 3 and Figure 1).

**Discussion**

HSCs, also known as Ito cells, vitamin A storage cells, or sinus cells, account for 5% to 8% of all intrahepatic cells. Under normal physiological conditions, these cells are involved in the storage of fat and vitamin A and the maintenance of the hepatic sinus extracellular matrix balance and hepatic sinus micro-ecological system.\(^{11}\) When the liver is stimulated by physical, chemical, or biological factors, stationary HSCs are “activated”, and provide the main source of extracellular matrix during liver fibrosis.\(^{3,12}\) Liver fibrosis is a common chronic liver disease characterized

### Table 3. The relative protein expression of TGF-β1, Smad4, and Smad7 in the different treatment groups.

| Gene   | Group          | Expression (fold-change) | \( P \) value |
|--------|----------------|--------------------------|---------------|
| TGF-β1 | Control        | 1.001 ± 0.049            | <0.0001       |
|        | Ang            | 3.100 ± 0.070            | –             |
|        | Ang + 3 µM STS | 2.934 ± 0.076            | 0.039         |
|        | Ang + 10 µM STS| 2.640 ± 0.070            | <0.0001       |
|        | Ang + 30 µM STS| 2.207 ± 0.075            | <0.0001       |
| Smad4  | Control        | 0.998 ± 0.051            | <0.0001       |
|        | Ang            | 2.832 ± 0.093            | –             |
|        | Ang + 3 µM STS | 2.806 ± 0.052            | 1.000         |
|        | Ang + 10 µM STS| 2.545 ± 0.087            | <0.0001       |
|        | Ang + 30 µM STS| 1.814 ± 0.063            | <0.0001       |
| Smad7  | Control        | 1.001 ± 0.042            | <0.0001       |
|        | Ang            | 0.536 ± 0.039            | –             |
|        | Ang + 3 µM STS | 0.596 ± 0.049            | 0.428         |
|        | Ang + 10 µM STS| 0.628 ± 0.026            | 0.040         |
|        | Ang + 30 µM STS| 0.695 ± 0.031            | <0.0001       |

Ang, Angiotensin II; STS, sodium tanshinone IIA sulfonate.
The level of protein expression was determined by western blotting and normalized to β-actin protein levels in the same sample. Data represent the mean ± standard deviation, \( n \geq 3 \). \( P \) values determined by one-way analysis of variance in comparison with Ang.

**Figure 1.** Relative protein expression levels of TGF-β1, Smad4, and Smad7 in the different treatment groups. Ang, Angiotensin II; STS, sodium tanshinone IIA sulfonate.
by the over-deposition of collagen-based extracellular matrix.

The cytokine TGF-β1 is significantly elevated in activated HSCs and promotes their proliferation; however, if it is over-expressed or its expression is sustained, it causes liver fibrosis.\(^2,13\) Smads are important mediators of the TGF-β signaling pathway. The Smad family includes eight members in humans, of which five (1, 2, 3, 5, and 8) are receptor-regulated. Smad4 is a common mediator, while Smad6 and Smad7 are inhibitory proteins.\(^5,14\) The TGF-β/Smads signaling pathway includes TGFβ type I and II serine/threonine kinase receptors, endoglin, TGF-β, Smad2/Smad3, Smad4, and Smad6/Smad7, and is controlled by positive and negative feedback.\(^5,15\) Smad4 functions as a partner of all receptor-regulated Smads, and is required for most gene responses to the TGF-β superfamily,\(^5\) while Smad7 is more potent than Smad6 in inhibiting TGF-β signaling.\(^5\) Regulating the expression of the TGF-β1/Smads signaling pathway in HSCs has become one of the main strategies for treating liver fibrosis.\(^15,16\)

Angiotensin II plays a major role in liver fibrosis by promoting the formation of HSCs and elevating TGF-β1 expression.\(^17\) Previously, angiotensin II expression was shown to be significantly increased in HSCs with liver fibrosis, mainly through the renin–angiotensin system.\(^18\) Therefore, interventions targeted toward this system is an important approach to the treatment of liver fibrosis.\(^19\) Our study showed that angiotensin II promotes the proliferation of HSCs and regulates TGF-β1/Smads signaling in HSCs, confirming that the TGF-β1/Smads signaling pathway and angiotensin II play crucial roles in liver fibrosis.

Tanshinone IIA has attracted increasing attention for its beneficial effects including antioxidant,\(^20\) anti-fibrotic,\(^21\) and anti-tumor properties.\(^22\) Previous studies have found that tanshinone IIA decreases lipopolysaccharide-induced HSC activation,\(^9\) induces apoptosis and S phase cell cycle arrest in activated rat HSCs,\(^23\) and inhibits angiotensin II-induced cell proliferation in rat cardiac fibroblasts.\(^24\) Our results suggest that an appropriate concentration of tanshinone IIA can significantly inhibit the angiotensin II-induced up-regulation of TGF-β1 and Smad4 and the down-regulation of Smad7 along the TGF-β1/Smads pathway of HSCs.

**Conclusion**

Angiotensin II promotes HSC proliferation, possibly via regulation of the TGF-β1/Smads signaling pathway. Tanshinone IIA can inhibit this angiotensin II-induced activation, and may, therefore, have preventive and therapeutic effects in liver fibrosis.

**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

**Funding**

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

**ORCID iD**

Ke-yue Li [https://orcid.org/0000-0001-9970-9383](https://orcid.org/0000-0001-9970-9383)

**References**

1. Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; 275: 2247–2250.
2. Ismail MH and Pinzani M. Reversal of hepatic fibrosis: pathophysiological basis of antifibrotic therapies. *Hepat Med* 2011; 3: 69–80.
3. Puche JE, Saiman Y and Friedman SL. Hepatic stellate cells and liver fibrosis. *Compr Physiol* 2013; 3: 1473–1492.

4. Munshi MK, Uddin MN and Glaser SS. The role of the renin-angiotensin system in liver fibrosis. *Exp Biol Med (Maywood)* 2011; 236: 557–566.

5. Hu HH, Chen DQ, Wang YN, et al. New insights into TGF-β/Smad signaling in tissue fibrosis. *Chem Biol Interact* 2018; 292: 76–83.

6. Mehta KJ, Coombes JD, Briones-Orta M, et al. Iron enhances hepatic fibrogenesis and activates transforming growth factor-β signaling in murine hepatic stellate cells. *Am J Med Sci* 2018; 355: 183–190.

7. Jiang X, Chen Y, Zhu H, et al. Sodium tanshinone IIA sulfonate ameliorates bladder fibrosis in a rat model of partial bladder outlet obstruction by inhibiting the TGF-β/Smad pathway activation. *PLoS One* 2015; 10: e0129655.

8. Yang L, Zou XJ, Yin Z, et al. [Effect of sodium tanshinone II (A) sulfonate on Ang II-induced atrial fibroblast collagen synthesis and TGF-beta1 activation]. *Zhongguo Zhong Yao Za Zhi* 2014; 39: 1093–1096.

9. Liu YW and Huang YT. Inhibitory effect of tanshinone IIA on rat hepatic stellate cells. *PLoS One* 2014; 9: e103229.

10. Li KY, Shi CX, Huang JZ, et al. Cisplatin plus norcantharidin alter the expression of TGF-β1/Smads signaling pathway in hepatocellular carcinoma. *Bratisl Lek Listy* 2017; 118: 85–88.

11. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* 2001; 21: 311–335.

12. Trautwein C, Friedman SL, Schuppan D, et al. Hepatic fibrosis: concept to treatment. *J Hepatol* 2015; 62: S15–S24.

13. Wu L, Zhang Q, Mo W, et al. Quercetin prevents hepatic fibrosis by inhibiting hepatic stellate cell activation and reducing autophagy via the TGF-β1/Smads and PI3K/Akt pathways. *Sci Rep* 2017; 7: 9289.

14. Blobe GC, Schiemann WP and Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000; 342: 1350–1358.

15. Cheng C, Yu S, Kong R, et al. CTRP3 attenuates hepatic stellate cell activation through transforming growth factor-β/Smad signaling pathway. *Biomed Pharmacother* 2017; 89: 1387–1391.

16. Liu X and Zhao X. Scoparone attenuates hepatic stellate cell activation through inhibiting TGF-β/Smad signaling pathway. *Biomed Pharmacother* 2017; 93: 57–61.

17. Kisseleva T and Brenner DA. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J Gastroenterol Hepatol* 2007; 22: S73–S78.

18. Pereira RM, dos Santos RA, da Costa Dias FL, et al. Renin-angiotensin system in the pathogenesis of liver fibrosis. *World J Gastroenterol* 2009; 15: 2579–2586.

19. Moreno M and Bataller R. Cytokines and renin-angiotensin system signaling in hepatic fibrosis. *Clin Liver Dis* 2008; 12: 825–852, ix.

20. Han D, Wu X, Liu L, et al. Sodium tanshinone IIA sulfonate protects ARPE-19 cells against oxidative stress by inhibiting autophagy and apoptosis. *Sci Rep* 2018; 8: 15137.

21. Cao L, Huang B, Fu X, et al. Effects of tanshinone IIA on the regulation of renal proximal tubular fibrosis. *Mol Med Rep* 2017; 15: 4247–4252.

22. Xu Z, Chen L, Xiao Z, et al. Potentiation of the anticancer effect of doxorubicin in drug-resistant gastric cancer cells by tanshinone IIA. *Phytomedicine* 2018; 51: 58–67.

23. Che XH, Park EJ, Zhao YZ, et al. Tanshinone II A induces apoptosis and S phase cell cycle arrest in activated rat hepatic stellate cells. *Basic Clin Pharmacol Toxicol* 2010; 106: 30–37.

24. Chan P, Liu JC, Lin LJ, et al. Tanshinone IIA inhibits angiotensin II-induced cell proliferation in rat cardiac fibroblasts. *Am J Chin Med* 2011; 39: 381–394.