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

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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 ($10\,\mu M$) or angiotensin II ($10\,\mu M$) plus tanshinone IIA (3, 10, or $30\,\mu M$). 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 $30\,\mu M$.

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.

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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 antifibrotic 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′-TGGCGTTACCTTGGTAACC-3′, reverse primer, 5′-GGTGTTGAGCCCTTTCCAG-3′; Smad4 forward primer, 5′-CTTTTGCT TGGGTCAACTCTC-3′, reverse primer, 5′-TGGGCTCACGCATATATTT CC-3′; Smad7 forward primer, 5′-TCAGGTGGC CGGATCTCA-3′, reverse primer, 5′-GG TTGATCTTCCCGTAAG ATTCA-3′; β-actin forward primer, 5′-AGGCCATGT ACGTAGCCATCCA-3′, reverse primer: 5′-TCTCCGGAGTCCA TCACAATG-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).[8,9] 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% CO_2.

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 2×10^5/well (6-well plates) or 2×10^4/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.[10] 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

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

Group	OD$_{450}$	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.

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).

TGF-β1, Smad4, and Smad7 protein expression

Compared with the control cells, angiotensin II significantly up-regulated the protein expression of TGF-β1 and Smad4 and significantly down-regulated the protein expression of Smad7 (all $P < 0.0001$). 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

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

Gene	Group	Expression (fold-change)	P value
TGF-β1	Control	1.001 ± 0.035	<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 $\pm$ standard deviation, n $\geq$ 3. P values determined by one-way analysis of variance in comparison with Ang.
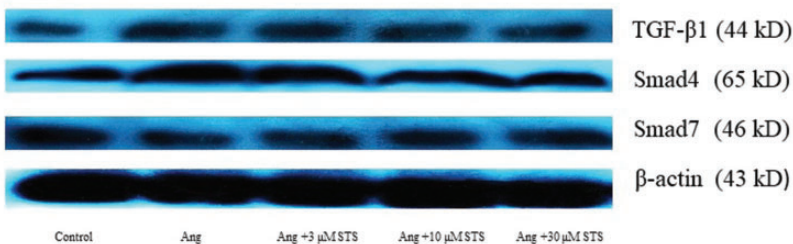

Figure 1. Relative protein expression levels of TGF-β1, Smad4, and Smad7 in the different treatment groups. Ang, Angiotensin II; STS, sodium tanshinone IIA sulfonate.

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.

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