Aspirin attenuates liver fibrosis by suppressing TGF-β1/Smad signaling

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Abstract. Aspirin reduces the liver fibrosis index and inflammation in patients and rats. However, the specific mechanism underlying the effects of aspirin are yet to be elucidated. The present study aimed to investigate the effects of aspirin on thioacetamide (TAA)-induced liver fibrosis in rats and hepatic stellate cells (HSCs) via the TGF-β1/Smad signaling pathway. Liver fibrosis was induced in Sprague Dawley rats by intraperitoneal injection of 200 mg/kg TAA twice weekly for 8 weeks. Aspirin (30 mg/kg) was administered to rats by gavage once every morning over a period of 8 weeks. Masson’s trichrome and H&E staining were used to detect and analyze the pathological changes in liver tissues. Western blot analysis and immunohistochemistry were applied to determine the protein expression levels of α-smooth muscle actin (α-SMA), collagen I, TGF-β1, phosphorylated (p)-Smad2 and p-Smad3. In addition, reverse transcription-quantitative PCR was performed to detect the mRNA expression levels of α-SMA, collagen type I α1 chain (COL1A1) and TGF-β1. The results demonstrated that treatment with aspirin significantly reduced the serum levels of alanine aminotransferase, aspartate aminotransferase and hydroxyproline in the TAA + aspirin compared with that in the TAA group. In the rat liver fibrosis model, pathological changes in liver tissues were improved following treatment with aspirin. Similarly, a marked decrease was observed in protein expression levels of α-SMA, collagen I, TGF-β1, p-Smad2 and p-Smad3. Furthermore, aspirin administration decreased the mRNA levels of α-SMA, COL1A1 and TGF-β1. In addition, HSCs were treated with different concentrations of aspirin (10, 20 and 40 mmol/l), and the protein expression levels of α-SMA, collagen I, TGF-β1, p-Smad2 and p-Smad3 were reduced in a dose-dependent manner. Overall, the present study showed that aspirin attenuated liver fibrosis and reduced collagen production by suppressing the TGF-β1/Smad signaling pathway, thus revealing a potential mechanism of aspirin in the treatment of liver fibrosis.

Introduction

Liver fibrosis is the result of a healing response to a variety of injury factors, such as chronic hepatitis, cholestasis, alcohol and drugs. It has been reported that liver fibrosis serves a significant role in the development of liver cirrhosis and liver cancer (1,2). When the liver is damaged, the injured hepatocytes, Kupffer cells or inflammatory cells produce several endogenous profibrotic cytokines or proinflammatory factors, which in turn activate resting hepatic stellate cells (HSCs). Activated HSCs undergo collagen overproduction, while collagen degradation is inhibited, eventually leading to the accumulation of extracellular matrix (ECM). ECM is considered to be the main pathophysiological process leading to liver fibrosis (3). Liver fibrosis has recently become the main focus in numerous studies. Unfortunately, safe and effective anti-fibrotic drugs are still lacking (4). Therefore, further studies on the development of effective drugs for treating liver fibrosis are urgently required.

TGF-β1 plays an important role in liver fibrosis (5-7). Activated HSCs secrete TGF-β1 and differentiate into myofibroblasts, which in turn promote the synthesis and accumulation of ECM-related proteins leading to liver fibrosis. Additionally, TGF-β1 can also activate HSCs, which further increase the synthesis and secretion of TGF-β1, thereby promoting the development of liver fibrosis (8). Furthermore, another study revealed that TGF-β1 enhanced ECM by regulating the TGF-β1/Smad signaling pathway to further promote the development of fibrosis (6).

Aspirin is a common non-steroidal anti-inflammatory drug, also known as acetylsalicylic acid; it is most commonly used to decrease fever, relieve pain and attenuate inflammatory responses (9). However, it is also used to improve the cardiovascular system (10), central nervous system (11) and

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rats (age, 6 weeks), weighing 200–220 g, were obtained from Animals. was purchased from Bayer AG.

Materials and methods

The results of the present study may provide further knowledge for the production, thus uncovering the molecular mechanism underlying the signaling pathway and the effects of aspirin on HSC collagen production, as previously described (16).

Materials and methods

Drugs. TAA was obtained from MilliporeSigma, while aspirin was purchased from Bayer AG.

Animals. A total of 30 healthy male Sprague-Dawley (SD) rats (age, 6 weeks), weighing 200-220 g, were obtained from the Experimental Animal Center of the Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All animals had free access to food and water. The animal experiments were approved by the Ethics Committee of Animal Experiments of the Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The animals were maintained under a 12 h light/dark cycle at room temperature (20-25˚C) and a humidity of 50-60%. Animals were anaesthetized with 200 mg/kg intraperitoneal sodium pentobarbital and sacrificed by cervical dislocation. All animal experiments in the present study were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Blood was collected by cardiac puncture and livers were subsequently removed. Liver and blood samples were collected and stored at -80˚C for further analysis.

Histopathological and immunohistochemical (IHC) analysis. Liver tissues were fixed in 4% paraformaldehyde solution at 4˚C for 24 h and embedded in paraffin. Paraffin-embedded tissue samples were cut into 4-μm thick sections followed by staining with H&E (hematoxylin 5 min, eosin 3 min) and Masson's trichrome (Wuhan Servicebio Technology Co., Ltd.) for 15 min at room temperature according to the manufacturer’s protocol. Subsequently, the tissue sections were deparaffinized with xylene and rehydrated in gradient ethanol at room temperature. For antigen retrieval, the tissue was boiled in a microwave for 15 min and washed with PBS. The tissue sections were blocked with 10% bovine serum albumin (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) for 15 min at room temperature and then incubated overnight at 4˚C with the following primary antibodies: Rabbit anti-TGF-β1 (dilution 1:1,000; cat. no. 21896-1-AP; Wuhan Sanya Biotechnology), rabbit anti-collagen I (dilution 1:500; cat. no. ab32575; Abcam), rabbit anti-α-smooth muscle actin (α-SMA) (dilution, 1:500; cat. no. ab32575; Abcam), rabbit anti-phosphorylated (p)-Smad2 (dilution 1:500; cat. no. 3108; Cell Signaling Technology, Inc.) and rabbit anti-p-Smad3 (dilution 1:500; cat. no. 1880-1; Epitomics; Abcam). Following rinsing in PBS, the tissue sections were incubated with horsedarish peroxidase-conjugated goat anti-rabbit IgG (1:500; cat. no. PV-9001; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) secondary antibody for 1 h at 37˚C, followed by staining with 0.05% diaminobenzidine for 90 sec and counterstaining with hematoxylin for 5 min at room temperature. Finally, the tissue sections were dehydrated again in gradient ethanol and mounted with neutral rubber (17). Representative images in three randomly selected fields per slide were captured under the Nikon Digital ECLIPSE C1 confocal microscope (Nikon Corporation). The positive staining to total area ratios were calculated using the Image-Pro Plus 6.0 imaging software (Media Cybernetics, Inc.). The average value from three ratios was used for statistical analysis.

Biochemical analyses. Serum was isolated from rat blood samples at 1,200 x g for 5 min at 4˚C and the serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were measured using the corresponding AST (cat. no. C010-2-1; Nanjing Jincheng Bioengineering Institute) and ALT (cat. no. C009-2-1; Nanjing Jincheng Bioengineering Institute) commercial kits, according to the manufacturer's instructions. The levels of hepatic hydroxyproline were measured using a hydroxyproline test kit (cat. no. A030-2-1; Nanjing Jincheng Bioengineering Institute), according to the manufacturer's protocol.

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protein concentration was measured using BCA and 40 µg protein/lane was separated by SDS-PAGE on a 8-10% gel and then transferred onto a PVDF membrane. Following blocking with 5% skimmed milk in TBS at room temperature for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-TGF-β1 (dilution 1:500; cat. no. 21898-1-AP; Wuhan Sanying Biotechnology), rabbit anti-collagen I (dilution, 1:500; cat. no. ab34710; Abcam), rabbit anti-α-SMA (dilution 1:500; cat. no. ab32575; Abcam), rabbit anti-p-Smad2 (dilution 1:500; cat. no. 1641-1), rabbit anti-p-Smad3 (dilution, 1:1,000; cat. no. 1880-1) and rabbit anti-Smad3 (dilution, 1:500; cat. no. 1735-1) (all from Epitomics; Abcam) and rabbit anti-β-actin (dilution 1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Following washing with TBS containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (dilution 1:5,000; cat. no. ZB-5301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 h at room temperature. The immunoreactive bands were visualised using an ECL reagent (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from liver tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A SMARTA2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to measure RNA absorbance at 260 nm. Next, the PrimeScript® RT reagent kit (Takara Biotechnology Co., Ltd.) was used for reverse transcription. The transcriptional conditions were 37°C for 15 min and 98°C for 5 min, followed by maintenance at 4°C. qPCR was performed using the SYBR Green PCR Master Mix-PLUS kit (Toyobo Life Science) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.). Each 20-µl reaction contained 10 µl SYBR Green Mix-PLUS kit (Toyobo Life Science) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.). Each 20-µl reaction contained 10 µl SYBR Green Mix, 6 µl nuclease-free H₂O, 2 µl cDNA, 1 µl upstream primer and 1 µl downstream primer. The thermocycling conditions for qPCR were as follows: Initial denaturation at 95°C for 60 sec, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and extension at 72°C for 45 sec. The primer sequences were synthesized by Sangon Biotech Co., Ltd. The relative mRNA expression levels were calculated with the 2^{-ΔΔCq} method (18). GAPDH served as an internal reference gene. The primer sequences used are listed in Table I.

Statistical analysis. All experimental data are expressed as the mean ± SEM of three independent experiments. Comparisons among multiple groups were performed with one-way ANOVA followed by Tukey's post hoc test. All graphs were constructed by GraphPad Prism 5.0 software (GraphPad Software, Inc.). All statistical analyses were performed using SPSS 22.0 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Aspirin attenuates TAA-induced liver fibrosis in a rat model. The livers in the sham and aspirin groups of the in vivo rat model appeared bright red, soft, smooth and sharp-edged at the eighth week after treatment (Fig. 1A). By contrast, the livers in the TAA group were dark red, hard, rough with granular nodules and blunt-edged (Fig. 1A). The livers in the TAA + aspirin group were more yellow and slightly harder compared with those in the sham and sham + aspirin group. However, livers in the TAA + aspirin group were softer compared with those in the TAA group, while no granular nodules were observed (Fig. 1A). Subsequently, H&E and Masson's staining were performed to evaluate the degree of liver fibrosis. Liver tissues derived from rats in the TAA group had pathological changes, with hepatic steatosis and fat vacuoles, while the liver lobule structure was abnormal or damaged as shown by H&E staining. Masson's staining showed a marked increase in collagen and the formation of pseudolobules (Fig. 1B). However, the aforementioned damaged tissues and fibrotic areas were marked improved in the TAA + aspirin group (Fig. 1B). To evaluate the effect of aspirin on liver injury, the levels of AST and ALT in the serum were detected. As shown in Fig. 1C, the serum levels of AST and ALT were significantly downregulated again in the TAA + aspirin group compared with that in the sham group. However, the levels of both AST and ALT were significantly decreased in the TAA + aspirin group compared with that in the TAA group indicating that aspirin attenuated TAA-induced liver injury. Furthermore, hydroxyproline, a marker of total hepatic collagen content, was significantly increased in the TAA group compared with that in the sham group, and then decreased in the TAA + aspirin group compared with that in the TAA group (Fig. 1D). Additionally, the results demonstrated that the protein and mRNA expression levels of α-SMA and collagen I were upregulated in the TAA group compared with those in the sham group, and then downregulated again in the TAA + aspirin group compared with those in the TAA group (Fig. 2A-E). Additionally, IHC staining was performed to analyze the expression levels of α-SMA and collagen I in the liver tissue. The results showed enhanced staining of α-SMA and collagen I in the TAA group,

| Gene        | Sequence (5’-3’)| Product size, bp |
|-------------|-----------------|-----------------|
| COL1A1      | GAGAGAGCATGACCGATGGA | 251             |
| Forward     | CGTGTGTAGGGTGAATGCAC |                |
| Reverse     | TGTGCCTAGCTGGAGATG | 148             |
| α-SMA       | GAAGGAATCCCACGCCTAG | 72              |
| Forward     | CGGACTACTACGCCAAGAAGT |              |
| Reverse     | TGGTTTTGTCAAGATTTGCAGT |           |
| GAPDH       | GACATCAGAAGGGTGTTG | 124             |
| Forward     | GCAGATCAGAAGGGTGGAG |                |

α-SMA, α-smooth muscle actin; COL1A1, collagen type I α 1 chain.
which was then decreased in the TAA + aspirin group (Fig. 2F). The aforementioned findings suggested that aspirin could attenuate TAA-induced hepatic fibrosis in rats.

**Aspirin attenuates liver fibrosis by regulating the TGF-β1/Smad signaling pathway.** To explore the mechanisms underlying the effect of aspirin on inhibiting liver fibrosis, the protein expression levels of TGF-β1, p-Smad2, p-Smad3, Smad2 and Smad3 were determined by western blotting and IHC staining (Fig. 3). The results showed that the protein expression levels of TGF-β1, p-Smad2 and p-Smad3 were significantly increased in the TAA group compared with those in the sham group, while they were significantly reduced in the TAA + aspirin group compared with those in the TAA group. No changes were observed in the protein levels of total Smad2 and Smad3 (Fig. 3A-D and F). In addition, treatment with aspirin significantly decreased the mRNA levels of TGF-β1 (Fig. 3E). These findings indicated that aspirin could attenuate liver fibrosis by inhibiting the TGF-β1/Smad signaling pathway.

**In vitro effect of aspirin on HSCs.** To further investigate whether the antifibrotic effect of aspirin was triggered by regulating the TGF-β1/Smad signaling pathway in HSCs, HSCs were treated with different concentrations of aspirin. As shown in Fig. 4, aspirin downregulated the protein expression levels of α-SMA, collagen I, TGF-β1, p-Smad2 and p-Smad3 in a dose-dependent manner. These results were consistent with the observations in the rat model of liver fibrosis.
Liver fibrosis, characterized by increased deposition of ECM, is the repair and healing process during liver injury caused by various factors (19). However, this process is also associated with the transformation of chronic liver diseases into cirrhosis and liver cancer (20). Currently, there are no safe and effective drugs for the treatment of liver cirrhosis. Most drugs can only reduce complications, but they cannot treat the underlying cause of liver fibrosis. Therefore, further studies on the development of novel antifibrotic treatments are urgently needed to prevent and/or reverse liver fibrosis. Although it has been reported that aspirin exhibits antifibrotic effects, the mechanism underlying the effect on hepatic fibrosis remains elusive. The present study revealed that aspirin attenuated liver fibrosis by suppressing TGF-β1/Smad signaling in rats.

Emerging evidence has suggested that the activation of HSCs plays an important role in liver fibrosis. Once the liver is injured, quiescent HSCs are activated and differentiate into myofibroblasts, which express high levels of α-SMA, collagen I and collagen III, thus resulting in an increased deposition of ECM and the development of liver fibrosis (25). Profibrogenic cytokines, such as TGF-β1 and TNF-α, also play a critical role in the progress of liver fibrosis (26). Liver injury induces...
Kupffer cells to produce TGF-β1 (27). In addition, another study showed that HSCs could be activated by TGF-β1 and differentiate into myofibroblasts to further promote the development of fibrosis (28). The present study demonstrated that the expression levels of TGF-β1, p-Smad2, p-Smad3, collagen I and α-SMA were significantly increased in the TAA-induced liver fibrosis model, and then significantly reduced following aspirin treatment. Furthermore, aspirin downregulated the protein expression levels of TGF-β1, p-Smad2, p-Smad3, collagen I and α-SMA in HSCs. The in vitro results were consistent with those observed in vivo. These results suggested that aspirin could attenuate liver fibrosis by inhibiting the TGF-β1/Smad signaling pathway.

A recent study showed that aspirin downregulated TGF-β1 in a carbon tetrachloride-induced chronic liver injury model (29). TGF-β1 is a significant mediator of liver fibrosis. TGF-β1 is secreted by Kupffer cells, activated in HSCs and specific inflammatory cells (30). TGF-β1 activates resting...
HSCs, which in turn secrete more TGF-β1 to maintain the activation status of these cells, thus enhancing ECM deposition (31). TGF-β1 binds to type I and type II TGF-β receptors (TβRs) on the surface of HSCs, while the intracellular signal transduction of TGF-β1 is mediated by the Smad protein family. When TGF-β1 is activated, it binds to TβRII on the
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surface of HSCs to activate TβRI. In turn, activated TβRI primarily induces the phosphorylation of Smad2 and Smad3 in quiescent and activated HSCs, respectively (32). Subsequently, activated Smad2/3 bind to Smad4 and the complex translocates into the nucleus to activate the transcription of downstream genes, inducing liver fibrosis-related genes such as fibronectin, α-SMA and collagen I (33,34). Previous studies suggested that the increased expression of fibronectin, α-SMA and collagen I in liver fibrosis could be associated with the TGF-β1/Smad signaling pathway (35,36). The aforementioned molecules could alleviate liver fibrosis in rats by inhibiting the TGF-β1 signaling pathway (37). The present study explored the effect of aspirin on the TGF-β1/Smad signaling pathway. The results showed that treatment with TAA increased TGF-β1 levels, which were restored following treatment with aspirin. Accordingly, aspirin reduced the expression levels of the downstream molecules p-Smad2 and p-Smad3. Therefore, these results indicated that aspirin could effectively inhibit the TGF-β1/Smad signaling pathway involved in liver fibrosis.

Aspirin alleviated TAA-induced liver fibrosis in rats. Therefore, treatment with aspirin reduced the secretion of TGF-β1 by activated HSCs and Kupffer cells, thus resulting in p-Smad2, p-Smad3, α-SMA and collagen I downregulation by the TGF-β1/Smad signaling pathway. Additionally, reduction of TGF-β1 reduced the activation of HSCs and myofibroblasts and attenuated ECM production (Fig. 5).
The present study was the first to demonstrate that aspirin could exert a potential protective role against liver fibrosis. However, whether Smad 6, as a negative regulator of the TGF-β1/Smad signaling pathway, is involved in the antifibrotic effects of aspirin should be further investigated. Third, the present study revealed that aspirin alleviated TAA-induced liver fibrosis. However, the underlying mechanism of aspirin with regard to improving liver fibrosis was only preliminarily investigated. Therefore, additional studies are required to further investigate the involvement of the TGF-β1/Smad signaling pathway in the antifibrotic effect of aspirin.

In summary, the present study suggested that aspirin could exert a potential protective role against liver fibrosis. The present study was the first to demonstrate that aspirin could ameliorate TAA-induced liver fibrosis by inhibiting the TGF-β1/Smad signaling pathways. These results indicated that aspirin could provide novel insights into the development of new drugs for the prevention and treatment of liver fibrosis. Furthermore, the safety of aspirin in clinical practice and other mechanisms remain to be studied.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YS and BL designed and performed the experiments, designed the figures and drafted the manuscript. JXie, XJ, BX and XH performed the experiments. JXia designed the experiments, analyzed the data, and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript. YS and JXia confirm the authenticity of all the raw data.

Ethics approval and consent to participate
All animal experiments were approved by the Ethics Committee of Animal Experiments of the Tongji Medical College (approval no. TJ-A20150803).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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