Berberine alleviates liver fibrosis via inhibition of the PI3K/Akt/NF-κB signaling pathway in rats

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Abstract: Fibrosis is the most important pathological feature of chronic liver disease, and it is also an early step in the progression of chronic hepatitis and cirrhosis, eventually developing into hepatocellular carcinoma (HCC). In present study, we aimed at elucidating the therapeutic effects of inhibiting PI3K/Akt/NF-κB signaling pathway in rat hepatic fibrosis by using berberine, an isoquinoline alkaloid which is known for its therapeutic effect on inflammation, diabetes, hyperlipidemia and tumor. A model of hepatic fibrosis was established in SD rats by intraperitoneal injection of thioacetamide, and explored the effect of berberine in the treatment of liver fibrosis. hematoxylin-eosin and sirius red staining were examined to determine histo-pathological changes in liver fibrosis. Immunohistochemical staining (IHC) were used to examine the relative levels of α‑smooth muscle actin (α−SMA) and collagen type I in the liver of rats. In addition, the relative protein expression levels of PI3K/Akt/NF-κB signaling pathway were measured by western blotting, and the relative levels of ALT, Hyp and liver index in groups were evaluated. We observed a massive increase of the relative levels of α‑smooth muscle actin (α−SMA) and collagen type I in the liver of thioacetamide-induced rats compared with the normal group, whereas berberine treatment reversed these changes. The relative levels of ALT, Hyp and liver index in the berberine-treated group were significantly lower than those in the model rats with injection of thioacetamide. Importantly, we demonstrated that berberine could reverse thioacetamide-induced liver fibrosis through the PI3K/Akt/NF-κB pathway *in vivo*. In conclusion, berberine is believed to have a

therapeutic effect in treatment with hepatic fibrosis, probably by inhibiting the PI3K/Akt/NF-κB signaling pathway in rats.

Key words: berberine, liver fibrosis, signaling pathway

Introduction

Liver fibrosis is a pathophysiologic outcome of chronic liver injury characterized by the remodeling and excessive deposition of extracellular matrix (ECM) in liver (1). Various types of chronic hepatic disease could cause liver fibrosis, an early step in the progression of liver cirrhosis, which eventually lead to liver failure and hepatocellular carcinoma (2, 3). Despite all of these extensive efforts, the mechanisms underlying hepatic fibrosis pathogenesis remain unclear, and there is still a lack of effective treatment strategies associated with limited side effects (4, 5). Therefore, reversing or inhibiting the occurrence of hepatic fibrosis has become a focus on controlling the chronic hepatic diseases.

The activation of hepatic stellate cells (HSC) has been considered as an essential role in the formation and development of liver fibrosis (6, 7). In recent years, it has been reported that many cytokine pathways involved in and regulate HSC apoptosis, including TGF‑β/Smads pathway (8), ERK pathway (9), JAK/ STAT pathway (10), Fas/FasL pathway (11). To investigate the signal pathway activated by HSC could provide more effective guidance for the treatment of liver fibrosis. PI3K/Akt signaling pathway is a classic signaling pathway, which plays an important role in many physiological and pathological processes such as cell growth, cell survival and differentiation, movement and apoptosis (12, 13).

It has been confirmed that HSC is the main source of ECM, characterized by the expression of alpha-smooth muscle actin (a-SMA) and collagens (14). A wide array of cytokines have been found to be activated and secreted by hepatocytes, astrocytes, endothelial cells and other cells (15, 16). These factors regulate the function of HSC through various signaling pathways, especially in PI3K/Akt signaling pathway (17).

Berberine (BBR) is a kind of isoquinoline alkaloid extracted from Chinese herbal medicine *Coptis chinensis*, had been used in traditional Chinese medicine for more than a thousand years (18). Previous studies have demonstrated that berberine is believed to have a therapeutic effect in treatment of various diseases, including inflammation, diabetes, hyperlipidemia and tumor (19, 20). Zhang *et al* reported that

berberine could attenuate liver fibrosis by inhibiting HSC proliferation and regulating the expression of a-SMA (21). Suggesting that it is a potent drugs for preventing acute and chronic liver injury and treating liver fibrosis. However, whether PI3K/Akt/NF-κB has a beneficial effect on the hepatoprotective activity of berberine in rat hepatic fibrosis, or involved in its molecular mechanisms remains to be determined. Evaluation of the mechanisms of PI3K/Akt/NF-κB signaling in fibrosis may prove to be a promising and critical step towards the development of novel and specific hepatoprotective agents.

In present study, we evaluated the antifibrotic effects of berberine in SD rats by intraperitoneal injection of thioacetamide, a thiono−sulfur containing compound, which is usually used to induce the model of liver fibrosis. This study aimed to further investigate the effect of PI3K/Akt/NF-κB pathway on the hepatoprotective activity of berberine in rat hepatic fibrosis. Therefore, changes to the relative protein expression levels of PI3K, Akt, and NF-κB were analyzed, and its relationship with the expression of α‑smooth muscle actin (α‑SMA) and collagen type I.

Materials and methods

Reagents and rats

Berberine hydrochloride and thioacetamide (TAA), a thiono-sulfur containing compound, were purchased from SigmaAldrich (USA). Male SD rats of 220~250 g body weight were purchased from Guangdong Medical Laboratory Animal Centre, Guangzhou, Guangdong Province, China. Animals were housed at 25 ± 2 °C, with a 12 h light cycle, starting at 06:00, and were provided free access to standard laboratory chow and water. All experiments were approved by the ethics committee of the University of Hong Kong and complied with international guidelines.

Rats in sham and model groups

received 10 mL/kg of distilled water per day by oral administration. Rats in BBR treatment group received 120 mg/kg/day berberine dissolved in distilled water orally. All treatment lasted for seven weeks.

Biochemical Analysis

At the end of the experiment, animals were sacrificed by i.p. injection of 200 mg/kg pentobarbitone. Blood was collected and serum was separated by centrifugation at 3000 g for 5 min. Serum AST, ALT, and HyP were quantified by a biochemical autoanalyzer. The tissue hydroxyproline (HyP) level was examined with a HyP detection kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Histopathology

Samples were obtained from the same liver lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylineosin (HE) or sirius red staining. The degree of liver fibrosis was evaluated on HE-stained sections as described previously (22). The collagen content of the sections was also determined on VG-stained sections by a computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics, China). Five random fields were chosen in each section and the amount of total collagen was detected in the area stained by VG, and expressed as percentage relative to the total area (23).

Immunohistochemistry.

Liver tissue was fixed in 10% neutral buffered formalin for one week at 25‑27˚C, dehydrated in a 70‑100% gradient of ethyl alcohol, washed in xylene and embedded in paraffin. Paraffin‑embedded liver tissue samples were cut into 4‑5 µm thick sections. For general histology, tissue sections were subsequently stained with hematoxylin for 5 min at room temperature followed by eosin staining for 5 min at room temperature. Morphological changes were observed via Van Gieson's (VG) staining for 2 min at room temperature. Pathological changes were observed under a light microscope (magnification, x200). Collagen content was analyzed using the Image‑Pro Plus analysis software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis

Western blotting was performed using primary antibodies against phosphorylated

PI3K (1:1,000), phosphorylated Akt (1:1,000), p65 (1:1,000), β-actin (1:3,000). Cells were lysed with RIPA buffer with the complete cocktail proteinase inhibitor (Roche, USA) and phosphatase inhibitor (1mM Na3VO4 and 1mM NaF) on ice for 30 min followed by centrifugation at 14,000 rpm at 4◦C for 15min. Supernatants were transferred and protein concentrations were determined by BSA assay (Bio-Rad, USA). Equal yield of protein was separated on SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF, Bio-Rad). The membrane was then blocked in buffer containing 5% BSA, tris (10mmol/L, pH 7.4), NaCl (150mmol/L), and tween-20 (1%) at room temperature for 1 hr with gentle shaking. The membrane was then incubated with primary antibodies (Cell Signaling, MA, USA) at 4 ℃ overnight followed by incubation with appropriate secondary antibody (Abcam, UK) at room temperature for 1 hr. The immunoreactivities were detected using ECL advanced kit (GE Healthcare, UK) and visualized using a chemiluminescence imaging system (Bio-Rad, USA). Western blots were developed using ECL western blotting substrate and quantified using ImageJ software.

Statistical analysis.

Data were presented as the mean ± standard deviation. All statistical analyses were performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). One‑way analysis of variance followed by Student‑Newman‑Keuls test was used to analyze differences among treatment groups. $P<0.05$ was considered to indicate a statistically significant difference.

Result

Berberine ameliorated TAA-induced histological damage and fibrosis in hepatic fibrosis rats.

Using H&E and sirius red staining, we observed that the liver tissue in normal control rats showed normal lobular architecture with central veins and radiating hepatic cords. However, liver sections taken from rats in the model group exhibited more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous

septa compared with the normal control rats after 8 weeks of TAA treatment. In contrast, berberine treatment markedly ameliorated these histopathological changes (Fig.1 A-B).

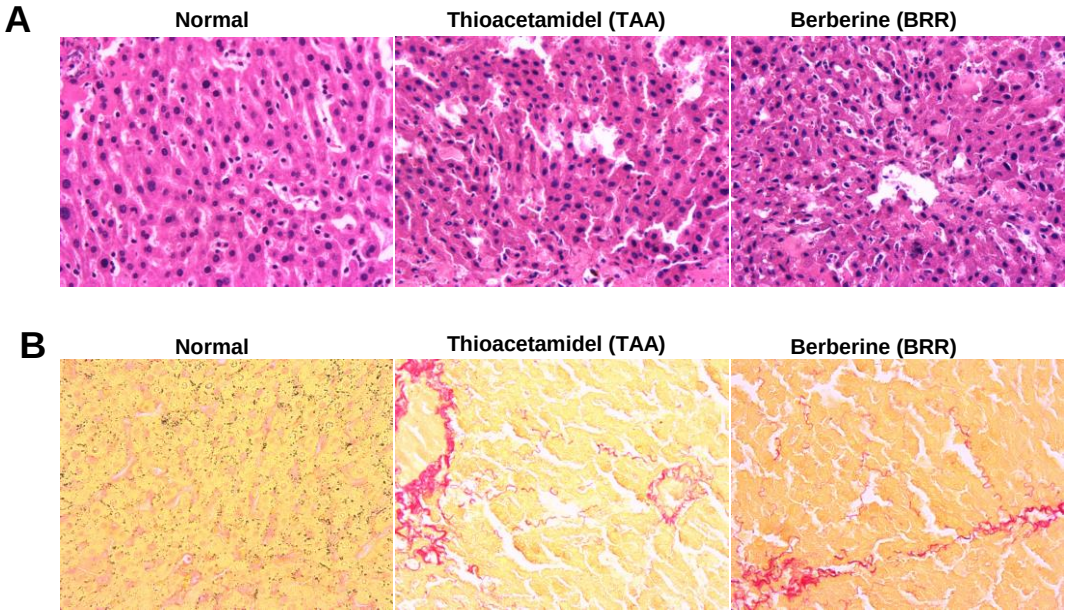

Fig.1 **Representative pathological changes in liver sections in groups**

(A: HE, ×400; B: Sirius red, ×400)

The presence of collagen type I and α−SMA in the liver tissue was observed by IHC staining. In the TAA group, the level of collagen fibers and α−SMA were markedly increased with the formation of fibrous septa surrounding the hepatic lobules compared with the control group. In addition, the collagen type I and α−SMA deposition in the berberine group was less compared with the TAA group (Fig.2 A-B). These results suggest that treatment with berberine may alleviate TAA-induced histological damage and fibrosis in liver fibrosis.

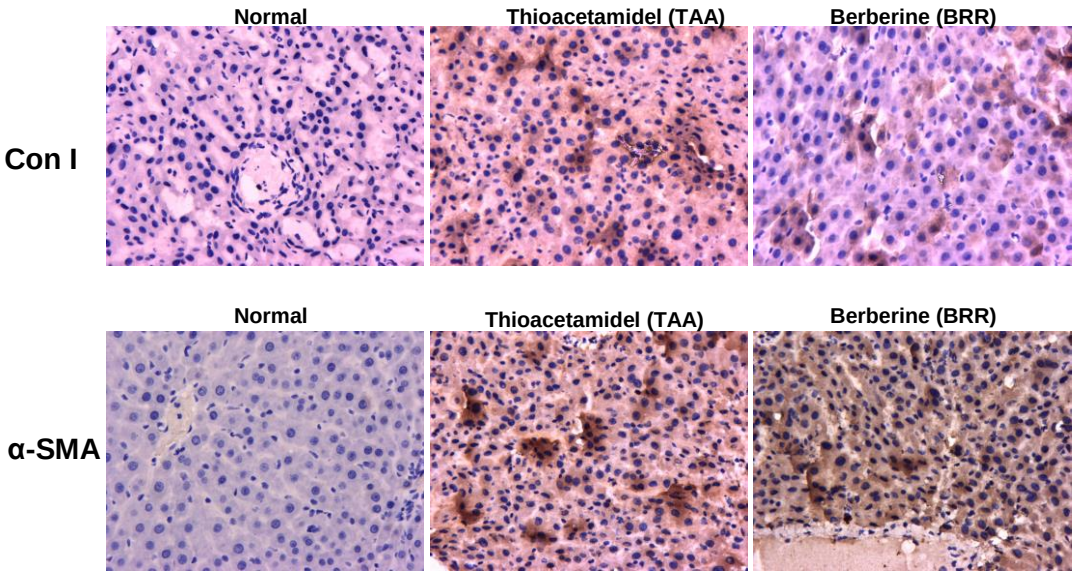

Fig.2 **Immunohistochemistry for collagen type I and α -SMA protein (brown stain) in liver sections in groups**

(collagen type I × 400; α-SMA, ×400).

Berberine reduces the Serum levels of hepatic function index in a TAA-induced liver fibrosis rats.

To investigate the potential anti-liver fibrosis activity of berberine in vivo, the present study used a TAA-induced rat model of liver fibrosis. Serum levels of ALT, AST and Hyp were determined in animals treated with or without TAA and berberine. As shown in Table 1, TAA-induced group had significantly higher levels of ALT and AST when compared with those in the untreated control group. Berberine treatment decreased the levels of ALT, AST and Hyp in a dose-dependent manner in the TAA-induced rats. These results suggest that treatment with berberine may alleviate TAA-induced functional damage in liver fibrosis.

Table 1 **The serum Levels of AST, ALT and Hyp in different treatment groups (mean ± SD)**

Groups	n	ALT (U/L)	AST (U/L)	Hyp(μg/g)
Normal	7	117.30±7.26	201.38±9.80	168.24±21.58
TAA	6	233.78±20.15^{b}	412.03±18.69^{b}	552.91±93.45^{b}
BRR	7	186.53±14.30^{bd}	339.04±15.51^{bd}	387.46±72.50^{bd}

[a]$P < 0.05$, [b]$P < 0.01$ vs normal control group; [c]$P < 0.05$, [d]$P < 0.01$ vs model group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Hyp: hydroxyproline.

Berberine ameliorated TAA-induced hepatic fibrosis via inhibition of the PI3K /Akt/NFκB signaling pathway in rats

To define further the molecular mechanism of berberine in hepatic fibrosis rats, we evaluated the potential role of the PI3K /Akt/NFκB signaling pathway. The expression levels of p-PI3K, p-Akt and p65 were measured by western blotting. Similarly, western blot analysis demonstrated that the relative protein expression levels of p-PI3K, p-Akt and p65 were significantly upregulated in the livers of rats in the TAA group compared with the control group. However, the protein expression levels of p-PI3K, p-Akt and p65 were significantly downregulated in the TAA group compared with the TAA group (P<0.05; Fig. 3). In summary, these results established a connection among berberine, PI3K-pAkt activation and the NFκB signaling pathway to ameliorated TAA-induced hepatic fibrosis in rats.

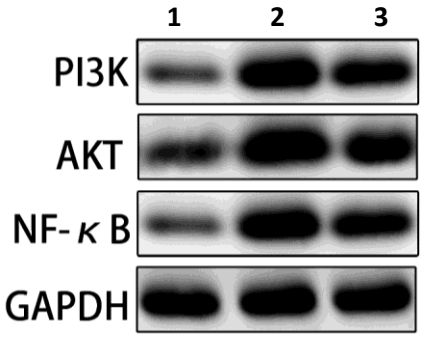

Fig.2 **Relative expression levels of proteins determined by western blot analysis in rat liver tissue samples**

Discussion

Critical role of HSC activation in the early development of liver fibrosis has been revealed by previous studies (2). The activation of HSC initiates its proliferation as well as the production of extracellular matrix (ECM) proteins such as α-SMA and collagens (24). Attempts have been made to explore the use of BBR in the therapy for

fibrosis-related hepatic diseases. Previous studies have shown that BBR could be used for the treatment against hypertyraminemia in patients with liver cirrhosis (25), which was correlated with BBR's capacity of reducing blood lipid in hyperlipidemic patients (26). Experimental studies have been also conducted, the results of which exhibit the potential of BBR in ameliorating hepatic fibrosis with various mechanisms (27). In particular, it was shown that the anti-oxidative activity of BBR contributes to improvement of experimental hepatic fibrosis via stimulating MMP-2 (28). In the present study, our findings showed that significantly lowered the levels of serum ALT, AST and Hyp, reduced histological changes of liver fibrosis, suppressed the expression of several fibrosis indices including α-SMA and collagen I. Furthermore, our previous study showed that the increases of expression levels of p-PI3K, p-Akt and p65 after the TAA injection can be notablely inhibited by BBR treatment. The above findings demonstrated that BBR can effectively prevent TAA-induced hepatic fibrosis in rats by inhibition of the PI3K/Akt/NF-κB signaling pathway in rats and regulate the production of factors correlated with fibrosis.

It has been previously shown that the activity of the PI3K/Akt/NF-κB signalling is a critical factor in the prevention of hepatic fibrogenesis (17, 29,). It was found that the proliferation and activation of HSC were inhibited, apoptosis was increased, and the expression of collagen fibers and some fibrosis indices were significantly reduced by blocking PI3K/Akt signal pathway (30). It is suggested that PI3K/Akt signal pathway can be interfered to prevent and alleviate liver fibrosis. NF-κB is a nuclear transcriptional activator that plays a central role in stress response and inflammation (31). Activation of NF-κB can promote HSC proliferation, reduce HSC apoptosis and increase the production of collagen and inflammatory chemokines in the process of liver fibrosis. But inhibiting the activation of NF-κB can induce apoptosis of HSC (32). Phosphorylation of Akt is essential for NF-κB activation via the stimulation of the IκB kinase complex, which phosphorylates and inactivates IκB, an inhibitor of NF-κB (33). Since PI3K/pAkt are upstream of IκB kinase (IKK) and the IKK complex is central for the activation of NF-κB to regulate collagen-I (34, 35), we

focused on analyzing this signaling pathway. A recent study showed that berberine attenuates myocardial ischemia reperfusion injury by suppressing the activation of PI3K/AKT signaling (36). However, whether activation of PI3K/Akt/NF-κB by berberine is responsible for the improvement of experimental fibrosis remains not clear. Furthermore, whether activation of AMPK by berberine can suppress activated hepatic stellate cells, which majorly mediates fibrogenesis in the liver, was not studied. In our study, we observed that hepatic fibrosis induced by TAA could be attenuated by BBR, which inhibited the activation of PI3K/Akt/NF-κB signaling in TAA treatment and decreased the production of collagen type I and α-SMA. These findings support a central role of PI3K/Akt/NF-κB activation in BBR's effect on HSC activation and subsequent fibrogenesis.

In conclusion, BBR has significant antifibrogenic effects on TAA-induced liver fibrosis in rats. Furthermore, treatment with berberine may alleviate TAA-induced histological damage and fibrosis in liver fibrosis by suppressed the expression of several fibrosis indices including α-SMA and collagen I. In addition, the inhibition of PI3K/Akt/NF-κB activation, as previously reported, is one of the most important factors involved in the preventive effect of berberine on TAA-induced liver fibrosis. The exact molecular mechanisms remain to be explored.

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