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SCIENCE DIRECT
Journal of Ethnopharmacology 105 (2006) 69–75
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Traditional Chinese medicine Bao Gan Ning increase phosphorylation of CREB in liver fibrosis in vivo and in vitro

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Received 30 December 2004; received in revised form 24 September 2005; accepted 30 September 2005
Available online 15 November 2005

Abstract

Previous studies have demonstrated that traditional Chinese medicine Bao Gan Ning, which contains six different drugs: *Trionyx sinensis* Wiegmann shell, *Prunus persica* (L.) Batsch seed, *Salvia miltiorrhiza* Bge. root, *Mallotus opelta* (Lour.) Muell-Arg root, *Astragalus membranaceus* (Fisch.) Bge. var. *mongho-licus* (Bge.) Hsiao root and *Scutellaria baicalensis* Georgi root, was able to protect liver against fibrosis in CCL_4 models. In an effort to elucidate molecular mechanisms by which Bao Gan Ning exerts its anti-fibrosis activity, effects of Bao Gan Ning on liver fibrosis and cAMP response element binding protein (CREB), an important transcription factor involved in liver fibrosis, were evaluated in animal and cell models in this work. Results showed that Bao Gan Ning (2.16 or 4.32 g/kg/day) significantly decreased alanine aminotransferase (ALT) and hyaluronidase levels and reversed liver fibrosis in rat liver fibrosis models. The proliferation of HSC-T6, a hepatic stellate cell line, was also significantly inhibited by incubation with serums that were prepared from rats fed with Bao Gan Ning. Most interestingly, results from Western blot, immunohistochemistry and electrophoretic mobility shift assay (EMSA) showed that Bao Gan Ning up-regulated CREB phosphorylation both in rat liver fibrosis models and in HSC-T6 cells, but did not affect protein level of CREB and the DNA binding activity of CREB. These results suggested that up-regulation of CREB phosphorylation may be involved in anti-fibrosis activity of Chinese medicine Bao Gan Ning.

Keywords: Traditional Chinese medicine; Liver fibrosis; Hepatic stellate cell; CREB; Phosphorylation

1. Introduction

Liver fibrosis is a major medical problem with high morbidity and mortality. Fibrosis is a type of wound healing response to various chronic stimuli that is characterized by an excessive deposition of extracellular matrix proteins (predominantly type I collagen) (Gâbele et al., 2003). The hepatic stellate cells play a key role in the pathogenesis of hepatic fibrosis (Friedman et al., 1985; Maher and McGuire, 1990). Numerous in vivo and in vitro studies indicate that in response to liver injury stellate cells undergo an "activation" process in which they lose Vitamin A, become highly proliferative, and synthesize "fibrotic" matrix rich in type I collagen (Reeves and Friedman, 2002).

The cAMP response element binding protein (CREB) mediates transcriptional activation in response to the cAMP signalling pathway. CREB binds as a dimer to a conserved cAMP response element (CRE) found in the promoters of numerous eukaryotic genes (Montminy, 1997). Phosphorylation of serine 133 is a critical event in CREB activation (Yamamoto et al., 1988) and induces an increase in CREB trans-activation potential by allowing the recruitment and binding to co-activators such as CREB-binding protein (CBP) (Kwok et al., 1994; Chrivia et al., 1993). It has been established that CREB is an important transcription factor involved in liver fibrosis.

Our previous studies have demonstrated that Bao Gan Ning was able to inhibit the production of collagen I (Zhang et al., 2003) and induce regression of liver fibrosis in CCl_4 models (Zhang et al., 2003; Zhao et al., 2002). In this work, we investigate the involvement of CREB phosphorylation in proliferation

Abbreviations: ALT, alanine aminotransferase; CRE, cAMP response element; CREB, cAMP response element binding protein; EMSA, electrophoretic mobility shift assay; PKA, protein kinase A

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doi:10.1016/j.jep.2005.09.040

of activated stellate cells and repression of liver fibrosis by Bao Gan Ning in an effort to elucidate molecular mechanisms by which Bao Gan Ning exerts its anti-fibrosis activity.

2. Materials and methods

2.1. Bao Gan Ning preparation

Bao Gan Ning is obtained from School of Traditional Chinese Medicine of Southern Medical University. The components of Bao Gan Ning are: *Trionyx sinensis* Wiegmann shell, *Prunus persica* (L.) Batsch seed, *Salvia miltiorrhiza* Bge. root, *Mallotus opelta* (Lour.) Muell-Arg root, *Astragalus membranaceus* (Fisch.) Bge. var. *mongho-licus* (Bge.) Hsiao root and *Scutellaria baicalensis* Georgi root. The six traditional Chinese medicines have a long history of medical use in China and their uses were documented formally in the Qing dynasty Bencao Congxin (New Compilation of Materia Medica) in 1757. Twenty grams of each of the above components were primarily washed. *T. sinensis* Wiegmann shell was decocted in water, then crushed and sifted, added eight times water, extracted for 6 h. *S. baicalensis* Georgi root was decocted two times in water and the two extracts combined. The rest four components were decocted two times in water. Then double volume of ethanol was added and the precipitate was removed. The decoction without the precipitate was concentrated by solubility starch and protein sugar. Then Bao Gan Ning granule was ultimately produced.

2.2. Establishment of rat cirrhosis model and its biochemical and morphological identification

Female Wistar rats (Grade III, Certificate No. 99B025, 120–150 g), obtained from the Laboratory Animal Center of Southern Medical University, with free access to water were randomly divided into five groups. Animals were housed in steel cages and maintained under standard conditions (12 h light/12 h dark cycle; 25 ± 3 °C; 35–60% relative humidity). For control group ($n = 8$), saline was used for immunological primary and second injection instead of BSA, the others were the same as those in model groups. For model group ($n = 8$), 0.5 ml of BSA Freund' incomplete adjuvant was injected hypodermically at days 1, 15, 22, 29, 36 for primary sensitization. Some 7 days after the fifth injection, BSA antibody in rats' serum was detected by double agar diffusion method. Various concentrations (5.00–10.00 g/l) of 0.4 ml BSA in normal saline were administered once through caudal vein for second injection in BSA antibody-positive rats, with twice a week for 15 times. At the same period, these rats were given orally 10 ml/kg/day normal saline. All animals were killed 7 days after the last injection. Then the liver was subjected to biochemical detection and morphological observation. For Bao Gan Ning group ($n = 8$), Bao Gan Ning granule (2.16 g/kg/day, solved in saline) was given orally once a day in the second injection period. The whole time of drug treatment was 60 days. For Bao Gan Ning double group ($n = 8$), Bao Gan Ning granule (4.32 g/kg/day) was given orally in the second injection period. Fu Fang Bie Jia Ruan Gan Pian, a new Chinese medicine troche recognized by China (Batch No. Z19991011) that mainly contains *Cordyceps sinensis* (Berk.) Sacc., *S. miltiorrhiza* Bge. root, *Panax notoginseng* (Burk.) FHchen root and so on, was used as a positive control in our experiments. It has recognized anti-liver fibrosis effect through inhibiting the proliferation of *Ito* cell and decreasing the secretion of collagen protein. For Fu Fang Bie Jia Ruan Gan Pian group ($n = 8$), Fu Fang Bie Jia Ruan Gan Pian (0.108 g/kg/day, solved in saline) was given orally once a day in the second injection period. Levels of alanine aminotransferase (ALT) and hyaluronidase in rat blood were determined by Bergman's method (Zhu et al., 1993). Furthermore, livers were embedded with paraffin and stained with hematoxylin–eosin. According to the quality and quantity of liver histological features, pathological liver changes were classified into five grades, expressed as the average of the total rank sum in each group and analyzed by *K* independent samples test.

2.3. Immunohistochemistry staining

Slides with liver sections were blocked with blocking buffer (5% goat serum in PBS) for 1 h at room temperature. After being blocked, sections were incubated with the phospho-CREB primary antibody (Cell Signaling Technology, USA) in a humidified chamber at 4 °C overnight. Then, washed three times for 5 min each time in PBS and incubated with secondary antibody (Boster Biological Technology Co. Ltd., China) at room temperature for 30 min; washed again and incubated with ABC reagent (add 5 ml PBS + 1 drop A + 1 drop B, mix thoroughly and set aside until use, Boster Biological Technology Co. Ltd., China) at room temperature for 30 min; added DAB reagent (peroxidase substrate) to each section and monitor staining closely until desired stain; after dehydrated sections, used neutral gum to mount slides. Images were obtained on an Olympus 3.3 × 40 microscope and results were determined as described previously (Wang et al., 2004).

2.4. Prepare serum with Bao Gan Ning ingredients

Male or female Wistar rats (Grade III, Certificate No. 99B025, 120–150 g), were obtained from Laboratory Animal Center of Southern Medical University, and divided into four groups that were fed with saline, Bao Gan Ning granule, Fu Fang Bie Jia Ruan Gan Pian or colchicines (Batch No. H53021369, 0.108 mg/kg/day), respectively, for 7 days. The serum with or without Bao Gan Ning or Fu Fang Bie Jia Ruan Gan Pian ingredients was prepared 1 h after the last feeding.

2.5. Cell culture and MTT assay

HSC-T6 cells, donations of Hepatopathy Research Institute, Shanghai University of Traditional Chinese Medicine, were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and adjusted to 6×10^4 cell/ml. The cells were inoculated into 96-well plate, 0.2 ml/well, 3-well/group and treated separately with serum containing saline, Bao Gan Ning, Fu Fang Bie Jia Ruan Gan Pian and colchicine for 48 h. The serum was removed, and 20 μl MTT was added in each well for