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Activation of PPARγ is required for hydroxysafflor yellow A of *Carthamus tinctorius* to attenuate hepatic fibrosis induced by oxidative stress

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ABSTRACT

Oxidative stress caused hepatic fibrosis by activating hepatic stellate cells (HSCs), which were implemented by depressing PPARγ activation. Hydroxysafflor yellow A (HSYA) as a nature active ingredient with antioxidant capacity was able to effectively attenuate oxidative stress mediated injury. So it will be very interesting to study effect of HSYA on HSCs activation and liver fibrosis, and reveal the role of PPARγ. CCl_4 and H_2O_2 were used to mimic oxidative stress mediated hepatic injury *in vitro* and *in vivo* respectively. The anti-fibrosis effects of HSYA were evaluated and its mechanisms were disclosed by applying western blot, histopathological analysis, flow cytometry, RT-PCR and ELISA. Our results showed that HSCs activation and proliferation could be induced by oxidative stress, and the expressive levels of TGF-β1 and TIMP-1, the serum levels of ALT, AST, HA, LN, III-C and IV-C were also enhanced by oxidative stress, which is correlated with liver fibrosis ($p < 0.05$ or $p < 0.01$). HSYA was able to effectively inhibit oxidative stress mediated hepatic injury by increasing the activities of antioxidant enzymes, up regulating the expression of PPARγ and MMP-2, and down regulating the expression of TGF-β1 and TIMP-1, and reducing α-SMA level. The protective effect of HSYA can be significantly attenuated by GW9662 *via* blocking PPARγ ($p < 0.05$ or $p < 0.01$). Taken together, these results demonstrate that HSYA is able to significantly protect the liver from oxidative stress, which requires for HSYA to stimulate PPARγ activity, reduce cell proliferation and suppress ECM synthesis.

Introduction

Hepatic fibrosis is a chronic pathological process, which was induced by many different factors. Among of them, oxidative stress is one of the primary factors and plays an important role. Carbon tetrachloride (CCl_4), a hepatotoxin, has been used extensively to induce oxidative stress which results in the production of reactive oxygen species (ROS) (Basu 2003). Study showed ROS, as a key trigger, can stimulate hepatic stellate cell (HSC) activation, cause cell proliferation and overproduction of extracellular matrix (ECM), and finally exacerbate hepatic damage and lead to liver fibrosis. PPARγ is essential for HSC quiescence (Hazra et al. 2004; She et al. 2005). HSC activation coincides with a dramatic reduction in the peroxisome proliferator-activated receptor (PPARγ), in addition the process is coupled with the sequential up-expression of TGF-β1 and the contractile protein α-smooth-muscle actin (α-SMA) and excessive secretion of ECM and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Marra et al. 2000; Miyahara et al. 2000; Friedman 1993).

Traditional Chinese medicine (TCM) has been used for thousands of years in China, previous studies have shown that Chinese herbs and herb extracts with antioxidant and anti-inflammatory activities can slow down the progress of hepatic fibrosis (Thyagarajan et al. 2002; Chor et al. 2005). *Carthamus tinctorius* L., as a well-known TCM which is able to active blood circulation, remove blood stasis and relieve swelling and edemas, is extensively used for the treatment of cirrhosis, cerebrovascular and cardiovascular diseases (Wagner et al. 2011; Tien et al. 2010; Wang et al. 2011). Hydroxysafflor yellow A (HSYA) (Fig. 1-1) extracted from the flower of *Carthamus tinctorius* L., as a flavonoid compound, can effectively increase the activities of antioxidant enzyme and reduce oxidative stress mediated damage. Previous studies had shown that HSYA was able to effectively inhibit hepatic stellate cell (HSC) activation, protect rat liver from CCl_4-mediated fibrogenesis (Li et al. 2012; Zhang et al. 2011). We hypothesize that HSYA alleviate or mitigate oxidative stress-mediated hepatic fibrosis *via* up regulating PPARγ expression and inhibiting HSC activation. In

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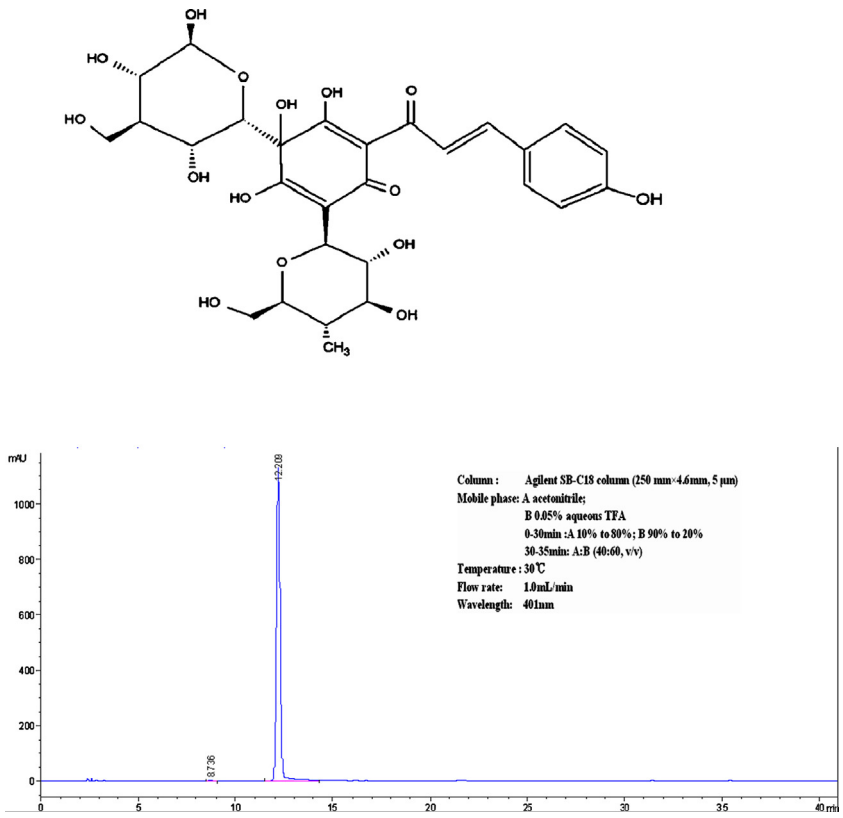

Fig. 1. Structure of HSYA.

study, we investigate the protective effect of HSYA on oxidative stress induced liver injury and its mechanisms *in vivo* and *in vitro*, and further extend our opinion.

Materials and methods

Reagents

Hydroxysafflor yellow A (HSYA, Fig. 1-1) extracted from *C. tinctorius* (Asteraceae) is the yellow amorphous powder generously provided by Dr. Da Lei Zhang, Shandong Natural Drugs Research & Development Center, Shandong Province, China. HSYA was separated and analyzed by using Agilent 1100 HPLC system equipped with quaternary solvent delivery system, autosampler and VWD detector, separation parameters as follow: Agilent SB-C18 column (250 mm × 4.6 mm, 5 μm); mobile phase acetonitrile (A) and 0.05% aqueous TFA (B); flow rate 1.0 ml/min; column temperature 30 °C; detection wavelength 401 nm. The gradient elution was applied according to the procedure: 0–30 min, linear gradient from A:B (10:90, v/v) to A:B (20:80, v/v); 30–35 min, linear gradient to A:B (40:60, v/v). As shown in Fig. 1-2, HSYA is water soluble compound with purity of more than 98%, whose molecular formula is $C_{27}H_{32}O_{16}$ with a molecular weight of 611.16 Da. All cell culture media and related reagents including fetal bovine serum were obtained from HyClone. The specific antibodies analyzed target protein were supplied by Santa Cruz Biotechnologies, USA.

Animals

Forty male Sprague–Dawley rats with weighing 200–250 g were purchased from Experimental Animal Department of Shandong Luye-Pharmcautical Co. Ltd., Shandong Province, and bred in a temperature controlled animal facility with a 12-h light–dark cycle. Rats were randomly divided into the following five groups: (1) normal group (olive oil), (2) model group (1.0 ml/kg of 40% CCl_4), (3) HSYA group (1.0 ml/kg of 40% CCl_4 + HSYA 10 mg/kg), (4) combination group (1.0 ml/kg of 40% CCl_4 + HSYA 10 mg/kg + GW9662 3 mg/kg) and (5) GW9662 group (1.0 ml/kg of 40% CCl_4 + GW9662 3 mg/kg). Except for control group, all rats were given intraperitoneal injection of 40% CCl_4 (dissolved in soybean oil, v/v) 1.0 ml/kg twice weekly for 8 wk, with the first dosage doubled. Rats in HSYA group or GW9662 group were injected intraperitoneally daily with HSYA or GW9662 in the doses indicated above, respectively and the rats in combination group were simultaneously administrated with HSYA and GW9662 *via* the same drug delivery described above. At the end of 8 wk, all rats were briefly anesthetized and sacrificed by bleeding from abdominal aorta. Blood and livers were collected for further examinations. All animals were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of Binzhou Medical University.

Cell culture

The primary HSCs were isolated from normal rats by the method previously described (Siegmund et al. 2005). Briefly, the liver was perused with pronase/collagenase (Sigma), and then minced with scissors. After further incubation with pronase and DNase (Sigma) at 37 °C for 30 min, the digested liver was filtered through a 100 μm nylon mesh, and parenchymal cells were removed by low speed centrifugation, further HSCs was further purified by Nycodenz gradient (Sigma, St. Louis, MO, USA) centrifugation. The purity of isolated HSC was identified by their typical light microscopic appearance and autofluorescence of the stored retinoides. Primarily, HSCs were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 3.7 g/l $NaHCO_3$. The cells