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Curcumin modulates cannabinoid receptors in liver fibrosis *in vivo* and inhibits extracellular matrix expression in hepatic stellate cells by suppressing cannabinoid receptor type-1 *in vitro*

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ABSTRACT

Activation of hepatic stellate cells (HSCs) is a pivotal event leading to extracellular matrix (ECM) overproduction during hepatic fibrogenesis. Compelling evidence indicates that cannabinoid receptors (CBRs) play an important role in chronic liver disease. Antagonism of hepatic CBR type 1 (CBR_1) could be a novel therapeutic strategy for liver fibrosis. Our previous studies have demonstrated that curcumin has potent antifibrotic activity, but the mechanisms remain to be elucidated. The current work was to examine the curcumin effect on CBRs system and its relevance to inhibition of ECM expression in HSCs. Our *in vivo* data demonstrated that curcumin ameliorated fibrotic injury, and downregulated CBR_1 but upregulated CBR_2 at both mRNA and protein levels in rat fibrotic liver caused by carbon tetrachloride. The subsequent *in vitro* investigations showed that curcumin reduced the mRNA and protein abundance of CBR_1 in cultured HSCs and decreased the expression of three critical ECM proteins. Further analyses revealed that CBR_1 agonist abrogated the curcumin inhibition of ECM expression, but CBR_1 antagonist mimicked and reinforced the curcumin effects. Autodock simulations predicted that curcumin could bind to CBR_1 with two hydrogen bonds. Collectively, our current studies revealed that curcumin reduction of liver fibrosis was associated with modulation of CBRs system and that antagonism of CBR_1 contributed to curcumin inhibition of ECM expression in HSCs.

1. Introduction

Hepatic fibrosis is a serious healthcare problem with high morbidity and mortality. It is the result of wound-healing responses to repeated liver injury irrespective of etiology. With the development of the disease, excessive extracellular matrix (ECM) components are deposited in the liver, leading to portal hypertension, cirrhosis or hepatocellular carcinoma (Hernandez-Gea and Friedman, 2011). Numerous studies have established that following liver injury, quiescent hepatic stellate cells (HSCs) undergo profound morphological and functional changes, and transdifferentiate into proliferative, contractile and chemotaxic myofibroblast-like cells, which function as the main ECM-producing cells contributing to the pathogenesis of liver fibrosis (Friedman, 2008). Reduction of ECM components expressed by HSCs is thus considered to be a primary therapeutic strategy for the treatment of hepatic fibrosis.

Continuing understanding of pathology of liver fibrosis has revealed that the endocannabinoid system plays a critical role in HSC pathobiology and pathogenesis of chronic liver injury (Tam et al., 2011). The endocannabinoid system comprises endocannabinoids such as arachidonoyl ethanolamide and 2-arachidonoylglycerol, and their corresponding receptors, namely cannabinoid receptors type 1 and 2 (CBR_1 and CBR_2). Compelling evidence indicates that CBR_1 expression is confined to HSCs and vascular endothelium, whereas CBR_2 is expressed by inflammatory cells in liver (Caraceni et al., 2008). Studies showed that CBR_1 knockout mice were resistant to fibrogenesis induced by carbon

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tetrachloride (CCl_4), thioacetamide or bile duct ligation (Teixeira-Clerc et al., 2006), whereas CBR_2 knockout mice exhibited more progressive fibrosis after CCl_4 treatment (Julien et al., 2005), suggesting that CBR_1 and CBR_2 mediate opposite effects in liver fibrogenesis. Clinically, patients with chronic hepatitis C and daily cannabis consumption displayed more severe fibrosis progression than non- or occasional consumers (Hezode et al., 2005, 2008; Ishida et al., 2008). These discoveries strongly suggest CBRs as new therapeutic targets for chronic liver diseases.

Currently, research identifying antifibrogenic agents that are innocuous is urgently needed. We previously documented that curcumin, the yellow pigment of turmeric in curry derived from the rhizome of the plant *Curcuma longa*, disrupted transforming growth factor-β (TGF-β) signaling and inhibited connective tissue growth factor expression, leading to inhibited HSC proliferation (Zheng and Chen, 2006, 2007). However, the underlying mechanisms remain incompletely understood. Interestingly, a recent investigation showed that curcumin exerted the antidepressant activity by targeting CBR_1-mediated endocannabinoid signaling and brain nerve growth factor (Hassanzadeh and Hassanzadeh, 2012). Given the therapeutic potential of CBR_1 antagonism for liver fibrosis (Teixeira-Clerc et al., 2006), we thus hypothesized that modulation of CBRs system could contribute to curcumin reduction of liver fibrosis and inhibition of ECM production in HSCs. We performed *in vivo* and *in vitro* experiments to test the hypothesis.

2. Materials and methods

2.1. Reagents and antibodies

Curcumin, *N*-arachidonoyldopamine (NADA), and AM251 were from Sigma (St Louis, MO, USA). All these compounds were dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for experiments. Primary antibodies against α-SMA, α(I) procollagen, fibronectin were from Epitomics (San Francisco, CA, USA). Primary antibodies against CBR_1, CBR_2, and β-actin were from Bioworld Technology (Nanjing, China).

2.2. Experimental animal procedures

Male Sprague-Dawley rats (180–220 g body weight) were obtained from Nanjing Medical University (Nanjing, China). A mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (w/v)] was used to induce liver fibrosis in rats. Thirty rats were randomly divided into five groups (six rats/group). Group 1 was the vehicle control in which rats were not administrated CCl_4 or curcumin but intraperitoneally (i.p.) injected with olive oil. Group 2 was the CCl_4 group in which rats were i.p. injected with CCl_4 without curcumin treatment. Groups 3–5 were treatment groups in which rats were i.p. injected with CCl_4 and orally given curcumin at 100, 200, and 400 mg/kg, respectively. Rats in groups 2–5 were i.p. injected with CCl_4 every other day for 8 weeks. Curcumin was suspended in 1% sodium carboxyl methyl cellulose and given once daily by gavage during weeks 5–8. At the end of experiment, rats were sacrificed after being anesthetized by i.p. pentobarbital (50 mg/kg).

2.3. Liver histopathology

Liver histology and collagen deposition were examined using hematoxylin–eosin (HE) staining and masson staining, respectively, as we previously described (Fu et al., 2008). Representative views of liver sections were shown and quantified with Image J software.

2.4. Cell isolation and culture conditions

Primary HSCs were isolated from male Sprague-Dawley rats. Briefly, portal vein perfusion was begun after heparin administration. Phosphate buffered saline (PBS) at 37 °C was perfused at 10 ml/min to adequately blanch the liver. This was followed by 0.1% Pronase for 3–4 min and then 0.03% collagenase for 30 min with reperfusion at 5 ml/min of the latter from the inferior vena cava. The liver was subsequently removed, minced in 0.02% Pronase, and incubated in a shaking 37 °C water bath for 20–30 min with deoxyribonuclease (10 μg/ml). The mixture was then centrifuged at 50*g* for 2 min to remove dead hepatocytes and undigested debris. The supernatant containing hepatic nonparenchymal cells was washed four times in PBS and then layered over a 25% preformed (30,000*g* for 15 min) Percoll gradient and centrifuged at 800*g* for 30 min. This gradient produced a top layer of yellowish white oily debris with a band of cells immediately beneath which contained HSCs. The HSC band was then washed two times and placed on a 45% unformed Percoll gradient and centrifuged at 15,000*g* for 20 min. This latter centrifugation removed potential bacterial contamination, leaving the cell band at the top of the tube. The cells were then washed two times in PBS and resuspended in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials, Hangzhou, China). This culture medium was routinely used throughout primary and secondary cultures, and then changed into Dulbecco's modified eagle medium (DMEM; Invitrogen, Grand Island, NY, USA) with 10% FBS, 1% antibiotics, and grown in a 5% CO_2 humidified atmosphere at 37 °C. HSCs at passages 4–8 were used in experiments.

2.5. Immunofluorescence staining

Staining of liver sections with primary antibodies against CBR_1 and CBR_2 was performed as we previously reported (Fu et al., 2008). For staining with cells, HSCs were seeded in 6-well plates and cultured in DMEM with 10% FBS for 24 h. HSCs were then treated with DMSO (0.02%, w/v) or curcumin at indicated concentrations for 24 h. Staining was performed as we previously described (Zhang et al., 2013) and Hoechst 33342 reagent (Beyotime Institute of Biotechnology, Haimen, China) was used to stain the nucleus. Representative micrographs are shown.

2.6. Real-time PCR

Total RNA was isolated from liver tissues or treated HSCs, and real-time PCR was performed as we previously described (Zhang et al., 2012). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the invariant control. The following primer sequences were used in experiments: CBR_1: (forward) 5′-CTGATCCCACACC CTTTCAT-3′, (reverse) 5′-TTAAGGGCTCTGACGCTCAT-3′; CBR_2: (forward) 5′-TTCCCCCTGATCCCCAACGACTA-3′, (reverse) 5′-CTCTCCAC TCCGCAGGGCATAAAT-3′; GAPDH: (forward) 5′-GGCCCCTCTGGAA AGCTGTG-3′, (reverse) 5′-CCGCCTGCTTCACCACCTTCT-3′.

2.7. Western blot analyses

Total proteins were prepared from liver tissues or treated HSCs as we previously described (Zhang et al., 2013) with corresponding primary antibodies or β-actin as loading control. The levels of protein bands were densitometrically determined using Quantity One 4.4.1 (Bio-Rad Laboratories, Hercules, CA, USA). Representative blots from three independent experiments are shown.