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Inhibitory effect of tetrahydrocurcumin on dimethylnitrosamine-induced liver fibrosis in rats

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ABSTRACT

Chronic liver disease is characterized by an exacerbated accumulation of deposition of collagen, causing progressive fibrosis, which may lead to cirrhosis. We evaluated the inhibitory effect of tetrahydrocurcumin (THC), a major metabolite of curcumin, on liver fibrogenesis both *in vitro*, HSC-T6 cell, and *in vivo*, dimethylnitrosamine (DMN)-induced liver fibrosis in rat. The liver inflammation in HSC-T6 cell was initiated by transforming growth factor-β1 (TGF-β1), and fibrosis in Sprague–Dawley rats was induced by intraperitoneal (*i.p.*) injection of DMN (10 mg/kg) 3 days per week for four consecutive weeks. THC (10 mg/kg) was administered in rats by oral gavage daily. DMN caused hepatic injury as indicated by analysis of liver function, morphology, histochemistry, and fibrotic parameters. Once-daily dosing with THC alleviated liver damage as signified by histopathological examination of the α-smooth muscle actin (α-SMA) and collagen I, accompanied by the reduction TGF-β1 and serum levels of transaminase ($P < 0.05$). These data revealed that the THC exerts hepatoprotective activity in experimental fibrosis, potentially by inhibiting the TGF-β1/Smad signaling.

1. Introduction

Tetrahydrocurcumin (THC), identified both in intestinal and hepatic cytosol of humans and rats (Holder, Plummer, & Ryan, 1978; Naito et al., 2002), is a major colorless metabolite of curcumin (diferuloylmethane, CUR), a yellow pigment derived from *Curcuma longa* L (Sandur et al., 2007). THC has been reported to exhibit stronger anti-oxidative activity than CUR in several *in vitro* systems(Okada et al., 2001; Pari & Murugan, 2004), and to exert a variety of biological activities both *in vivo* and in *vitro* systems including anti-inflammation and anti-cancer (Lai et al., 2011; Murakami et al., 2008; Pan,

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Lin-Shiau, & Lin, 2000; Wu et al., 2011). THC has demonstrated with anti-colon carcinogenesis in mice, and its chemopreventive efficacy is better than CUR (Lai et al., 2011). The studies showed that treatment with THC inhibited HT1080 cellular migration and invasion by down-regulation of matrix metalloproteinase 9 (Yodkeeree, Garbisa, & Limtrakul, 2008).Our previous data also indicated that the anticancer mechanism of THC in human leukemia HL-60 cells is by inducing autophagic cell death (type II programmed cell death) (Wu et al., 2011).

Numerous etiologies of chronic liver diseases, including alcohol abuse, chemical intoxication, viral hepatitis infection like hepatitis B and C and autoimmune disorders, attribute to chronic liver fibrosis, eventually leading to liver cirrhosis, a risk factor in the development of hepatocellular carcinoma (HCC). HCC is the most common primary tumor of the liver and causes of cancer, showing a rising incidence and currently ranks the fifth of cancer incidences worldwide (Lee et al., 2013a). Liver fibrosis from chronic liver injury is a wound healing process characterized by over accumulation of extracellular matrix proteins (ECM), especially collagen types I and III, and consequently, fibrosis or scarring ensues (Lee et al., 2013a; Qian, Niu, Zhai, Zhou, & Zhou, 2012).

Hepatic stellate cell (HSC) is the most relevant cell type for the development of liver fibrosis, and its activation is the key step in the process of liver fibrogenesis. Activated HSCs are the main ECM-producing cells in the injured liver (Qian, Niu, Zhai, Zhou, & Zhou, 2012; Tsukamoto, 2005).When activated, HSCs switch from a quiescent, epithelial appearance to an activated, α-smooth muscle actin (α-SMA)-expressing myofibroblastic phenotype (Friedman, 2008; Gressner, Weiskirchen, & Gressner, 2007; Qian, Niu, Zhai, Zhou, & Zhou, 2012). Excessive accumulation of ECM proteins followed by collagen type I is predominantly responsible for scarring (Lee et al., 2013b; Qian, Niu, Zhai, Zhou, & Zhou, 2012), and involved in a series of inflammatory and fibrotic processes. One of the most important cytokines expressed following liver injury is transforming growth factor-β1 (TGF-β1) (Ko et al., 2013). TGF-β1 is considered the most powerful mediator of HSC activation *in vitro* and *in vivo*, and plays a central role in initiating fibrogenic cascade in liver through binding to serine/threonine kinase TGF-β1 receptors (Ko et al., 2013; Qian, Niu, Zhai, Zhou, & Zhou, 2012). In response to activated TGF-β1, the Smad-group of proteins has been shown to be specifically activated by phosphorylation of Smad2 and Smad3 (Receptor-Regulated Smads), which further form heteromeric complexes with Smad4, and then the Smad complexes translocate to the nucleus, where they regulate transcription of target gene expression such as collagen type I. (Cho et al., 2010; Ko et al., 2013). Therefore, measurements of anti-inflammation and anti-fibrogenesis, especially inactivation of HSC and elimination of pro-fibrogenic signaling, are promising strategies to prevent further liver damage.

Dimetylnitrosamine (DMN)-treated animal models are widely used to study the biochemical and pathological manifestations of liver injury (George & Chandrakasan, 1996; George, Rao, Stern, & Chandrakasan, 2001; la-Kokko et al., 1987), since it is a potent hepatotoxin, carcinogen and mutagen, leading to liver damage in rats, which mimics the progression of liver fibrosis and cirrhosis in humans (George, Rao, Stern, & Chandrakasan, 2001).

In this study, the hepatoprotective effects in particular the inhibition of liver fibrosis of THC was investigated using both *in vitro* study by TGF-β1-induced alpha-smooth muscle actin (α-SMA) secretion of HSC-T6 cell and *in vivo* systems by a well-characterised animal model of DMN-induced liver fibrosis. We found that THC improved serum parameters of liver function, inhibited the activation of HSC, reduced the expression of α-SMA and collagen I, and alleviated the progression of liver injury, potentially by inhibiting the TGF-β1/Smad-mediated signaling.

2. Materials and methods

2.1. Reagents and chemicals

THC was obtained from American Medical Holdings Inc. (New York, NY, USA). All reagents and chemicals were purchased from Sigma, Inc. (St. Louis, MO, USA) unless specified otherwise. *N*-Nitrosodimethylamine (dimethylnitrosamine; DMN) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). α-SMA and antibody were obtained from Epitomics, Inc. (Burlingame, CA, USA). β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TGF-β, antibody were purchased from Transduction Laboratories (BD Biosciences, Lexington, KY, USA).

2.2. Cell culture and treatment

HSC-T6 stellate cells were cultured in Waymouth medium supplemented with 10% endotoxin-free, heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, 2 mM L-glutamine (Life Technologies, Grand Island, NY) and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells (2×10^5/mL) were seeded into 10 cm dish and cultured overnight, and then replaced with fresh medium containing 0.5% FBS. After serum-starvation for 24 h, cells were treated TGF-β (1 ng/mL) with or without THC for 24 h. THC was dissolved in dimethylsulfoxide (DMSO as final concentration was 0.05%). Cells were treated with 0.05% DMSO as vehicle control.

2.3. Western blotting

After treatment, HSC-T6 stellate cells were washed with PBS and the total proteins were extracted via addition of ice-cold gold lysis buffer (50 mM Tris–HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10,000×*g* for 30 min at 4 °C. The total proteins were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 10% SDS–polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was then carried out on SDS–polyacrylamide gels. Proteins on the gel were electrotransferred onto an immobile membrane (PVDF;

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