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Caffeine protects against alcohol-induced liver fibrosis by dampening the cAMP/PKA/CREB pathway in rat hepatic stellate cells

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ABSTRACT

Alcoholic liver fibrosis (ALF) is characterized by hyperplasia of extracellular matrix under long-term alcohol stimulation. Hepatic stellate cell (HSC) activation plays an important role in promoting hepatic fibrogenesis. Caffeine, as the main active component of coffee and tea, was widely consumed in daily life. It was always a thought that caffeine can reduce the probability of suffering from liver diseases. In this study, we attempt to validate the hypothesis that caffeine inhibits activation of HSCs which were isolated from rat ALF model. The rats were gavaged by ethanol to establish ALF model and then treated with different concentrations of caffeine or colchicine. Serum was collected to measure the contents of serum alanine aminotransferase (ALT), aspartate transaminase (AST), hyaluronic acid (HA), laminin (LN), N-terminal peptide of type III procollagen (PIIINP) and type IV collagen (CIV). Then liver tissues were obtained for hematoxylin-eosin staining and Sirius-red staining. Others were treated through liver perfusion to isolate primary rat HSCs. Interestingly, we found that caffeine significantly decreased ALT, AST, HA, LN, PIIINP and CIV levels and reversed liver fibrosis in rat ALF models. Results of immunohistochemistry, real-time PCR and western blot indicated that caffeine could reduce fibrosis and inhibit cAMP/PKA/CREB signal pathway in HSC. Caffeine has a preventive effect on ALF. The mechanism may be interpreted that caffeine inhibits the cAMP/PKA/CREB signal pathway through adenosine A2A receptors in HSC.

1. Introduction

Alcohol abuse and chronic alcohol consumption remain as a global public health problem. Excessive alcohol consumption is one of the critical causative factors leading to alcoholic liver disease (ALD) [1]. ALD is characterized by a wide spectrum of liver damage ranging from steatosis and steatohepatitis to fibrosis and cirrhosis [2]. Alcoholic liver fibrosis (ALF) is regarded as a turning point in ALD because it can lead to cirrhosis [3]. Accumulating evidence suggests that liver fibrosis is reversible and that recovery from cirrhosis may be possible [4], but the molecular determinants of fibrosis regression in animals and humans need to be more comprehensively defined.

The development of liver fibrosis in alcoholics has been linked to the oxidation of ethanol to the highly reactive compound acetaldehyde, which is the principal metabolite of alcohol and known to stimulate the production of several extracellular matrix (ECM) components, including type I collagen, by activating the hepatic stellate cell (HSC) [5,6]. HSC activation represents a critical event in alcohol-induced fibrosis because these cells become the primary source of ECM in liver upon injury [7]. As response to acetaldehyde-induced stimulation, HSCs change from quiescent vitamin A-storing cells to activated myofibroblast-like cells, which proliferate and become fibrogenic [8].

Coffee is one of the most frequently consumed beverages worldwide [9]. Epidemiological studies have shown that coffee intake attenuates the progression of chronic liver disease, including alcoholic liver cirrhosis [10–12], although there are controversies on whether these beneficial effects are related to caffeine or other specific components in this popular beverage. In recent years, limited experimental data further suggest that caffeine intake exert beneficial effects in experimental liver fibrosis

Abbreviations: α-SMA, smooth muscle α-actin; A2AR, adenosine A2A receptor; ALD, alcoholic liver disease; ALF, alcoholic liver fibrosis; ALT, alanine aminotransferase; AST, aspartate transaminase; cAMP, cyclic adenosine monophosphate; C IV, type IV collagen; CREB, cyclic adenosine monophosphate response element bonding protein; CYP2E1, cytochrome P450 enzymes; DMEM, Dulbecco's modified eagle medium; ECM, extracellular matrix; FCS, fetal calf serum; HA, hyaluronic acid; HE, hematoxylin-eosin; HSC, hepatic stellate cell; LN, laminin; PIIINP, N-terminal procollagen type III; PKA, protein kinase A; TBS, tris-buffered saline.

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[13–16]. Previous work from our laboratory has also indicated that caffeine could alleviate chronic alcohol-induced liver injury via inhibition of oxidative stress and inflammation [17]. However, to date, few studies have investigated the effects of caffeine directly on the HSCs and ALF.

Caffeine, a widely consumed psychoactive substance and an active ingredient in coffee [18], acts as a nonselective antagonist of adenosine A2A receptor (A2AR) [19]. During the past few years, a growing body of evidence indicates that A2AR is expressed on rat and human HSCs, and its activation plays a crucial role in the pathogenesis of hepatic fibrosis [20–22]. A2AR was originally identified by virtue of its ability to elevate intracellular levels of cAMP via receptor interaction with the heterotrimeric (alpha beta gamma) stimulatory G protein (Gs) and subsequent activation of adenylyl cyclase [23]. Using an in vitro cell culture model of acetaldehyde-induced HSC-T6 cells, we have previously demonstrated that caffeine inhibits the activation of acetaldehyde-induced HSC-T6 cells via A2AR mediated cAMP signal pathway [24]. However, the molecular mechanisms involved in the regulation of the cAMP-dependent pathway by which caffeine exerts beneficial effects in a rat model of ALF remain largely unclear.

In the present study, the rat model of ALF was induced by intragastric infusion of increasing concentration of ethanol 2 times per day for 12 weeks, and the primary HSCs were isolated from Sprague Dawley (SD) rats by in situ perfusion and density gradient centrifugation. Furthermore, we evaluated the protective effects of caffeine on rat model of ALF and assessed whether caffeine regulate the activation of HSCs via cAMP/PKA/CREB signaling pathway.

2. Materials and methods

2.1. Materials and reagents

Caffeine and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Ethanol was obtained from Shanghai Suyi Chemical Reagent (Shanghai, China). DMEM and FCS were purchased from GIBCO (CA, USA). Reagent kits for ALT and AST were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). HA, LN, PIIINP and CIV radioimmunoassay kits were obtained from Beijing Huaying Bioengineering Research Company (Beijing, China). TRIzol reagent was obtained from Invitrogen (CA, USA). PrimeScript™ RT Master Mix (Perfect Real Time) was obtained from TaKaRa (Dalian, China). Maxima SYBR Green qPCR Master and ECL-chemiluminescent kit were obtained from Thermo Scientific (CA, USA). ^{125}I-cAMP radio immunoassay kit was obtained from Shanghai University of Traditional Chinese Medicine (Shanghai, China). Antibody for procollagen I was obtained from Bioss Biological Engineering Co. (Beijing, China). Antibody for procollagen III, secondary antibodies for goat anti-rabbit immunoglobulin IgG horse radish peroxidase (HRP) and goat anti-mouse IgG HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). BCA protein assay kit and Antibodies for α-SMA and PKA were obtained from Boster Biological Engineering Co. (Wuhan, China). Antibody for CREB and phosphorylation-dependent antibody for CREB was obtained from Abcam (Cambridge, UK). All the other chemicals were of the commercially available highest grade.

2.2. Animals

Adult male Sprague–Dawley rats (specific pathogen free level, body weight of 180–220 g) were provided by the Experimental Animal Center of Anhui Medical University. All animals had free access to a standard laboratory chow diet and normal tap water. After one-week adaptive feeding, we randomly assigned those rats into six groups (20 rats/group) as follows: one untreated group (GI) and five groups (GII–GVI) treated with ethanol gavage twice a day and interval time was 10 h. The ethanol dose (g) = absolute ethanol volume (ml) × required ethanol concentration (%) × 0.8 (g/ml). From the first day to the third day, we adopted 5 g/kg ethanol (30%) for adaptive gavage. From the fourth day, the concentration of ethanol was increased by 5% and the dose was increased by 0.6 g/kg every 3 days till the ethanol concentration reached 50% and the dose reached 8 g/kg. Then we maintained the concentration and dose to gavage the rats until the experiment was completed. At the same time, the rats were treated with 5 mg/kg/day caffeine (GIII), 10 mg/kg/day caffeine (GIV), 20 mg/kg/day caffeine (GV), and 0.1 mg/kg/day colchicine (GVI) as positive control through gavage. The concentration of caffeine was referenced from the standard values of caffeinated beverages and daily intake range of adults.

After 8-week and 12-week treatment, quarter of the rats in each group were euthanatized to harvest liver tissues and collect blood samples. Tissue samples were fixed in 10% formaldehyde. The other half was prepared to isolate HSCs. The animal experimental protocol was approved by the University Animal Care and Use Committee.

2.3. Measurement of liver biochemical indicators

The collected blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to obtain the serum. The activity levels of ALT and AST were detected with commercial reagent kits according to the manuals.

2.4. Determination of serum levels of HA, LN, PIIINP and CIV

Serum levels of HA, LN, PIIINP and CIV were determined by radioimmunoassay using commercially available kits according to the manuals.

2.5. Histological and immunohistochemical analysis

Liver samples were taken from all the animals and fixed for 24 h with 10% formaldehyde in phosphate-buffered saline. Tissue pieces were washed with tap water, dehydrated in alcohol and embedded in paraffin. Then, 5-mm sections were placed on glass slides and covered with saline. Hematoxylin-eosin (HE) staining, Sirius red staining and immunohistochemical staining were performed on the slides.

HE staining was performed to assess inflammation and steatosis. To detect ECM, the tissue slides were incubated for 5 min in the hematoxylin solution. After water flushing and adding 0.5% ammonium hydroxide for 30-second treatment, the tissue slides were put in 0.5% eosin for 2-minute dyeing.

Sirius red staining allows the lasting staining of collagen. The observation of polarizing microscope indicates that it can enhance collagen fiber refraction. The tissue slides were put in 0.1% saturated picrate Sirius scarlet dye solution and dyeing time was more than 1 h.

Immunohistochemical staining was performed with the mouse monoclonal α-SMA antibody and indirect immunoperoxidase protocol according to the manuals of the LSAB2-kit. Immunohistochemical staining was performed with monoclonal anti-a-SMA antibody (dilution ratio of 1:500).

2.6. Isolation and cultivation of HSCs

Primary HSC was isolated from livers of rats mentioned above. The rats were anesthetized with chloral hydrate. The in-situ liver perfusion and digestion were performed with Pronase E, collagenase IV and DNase, and the obtained liver cell suspension was purified through density gradient centrifugation with 18% Nycodenz [25]. The cells were resuspended in DMEM. At this point, fairly pure HSC preparation with a high rate of viability, as estimated by Trypan blue exclusion test, was obtained. Primary cells of 5–7 days were used and the purity was more than 95%. Cells grew in standard tissue culture plastic flasks in DMEM with 10% fetal calf serum (FCS).