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Treatment with baicalein attenuates methionine–choline deficient diet-induced non-alcoholic steatohepatitis in rats

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ABSTRACT

Baicalein, a naturally occurring flavone, has been proved as a promising chemopreventive compound for many chronic human diseases. The aim of this work was to investigate whether treatment with baicalein prevented nonalcoholic steatohepatitis (NASH) induced by methionine–choline-deficient (MCD) diet. Rats were divided into four experimental groups and fed for 8 weeks as follows: (1) control rats; (2) control rats treated with baicalein (intraperitoneal injection of 10 mg/kg); (3) MCD-diet-fed rats; (4) MCD-diet-fed rats treated with baicalein. Treatment with baicalein prevented MCD-diet-induced NASH, as evidenced by reduced histological scores, apoptosis, activities of ALT and AST, and hepatic fat accumulation in rats. Treatment with baicalein abated MCD-diet-induced oxidative stress through enhancing Nrf2/HO-1 pathway and activities of SOD and catalase in livers. Treatment with baicalein preserved hepatic mitochondrial function in MCD-diet fed rats. Treatment with baicalein reduced hepatic NO formation through suppressing MCD-diet-induced iNOS activation, and suppressed MCD-diet-induced inflammation through suppressing NFκB activation and reducing IL-6 and TNFα expressions in livers. Treatment of MCD-diet fed rats with baicalein had a beneficial modulation on expression profiles of fatty acid metabolism genes in livers. The results support the investigation of baicalein as a therapeutic candidate for NASH induced by MCD diet in rats.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as hepatic fat accumulation exceeding 5–10% of liver weight, in the absence of excess alcohol consumption or any other liver disease or other causes of steatosis, such as certain toxins and drugs (Vanni et al., 2010; Zivkovic et al., 2007), and its prevalence is increasing with epidemics of obesity, diabetes, and metabolic syndrome. NAFLD includes a spectrum of pathological hepatic changes such as steatosis, steatohepatitis, advanced fibrosis, and cirrhosis. As a progressive stage of NAFLD, nonalcoholic steatohepatitis (NASH) is characterized by steatosis along with ballooning degeneration and inflammation. Despite its clinical significance, treatment options for NASH are limited.

The dried roots of *Scutellaria baicalensis* (*S. baicalensis*) Georgi (common name: Huangqin in China) have been widely employed for many centuries in traditional Chinese herbal medicine. Baicalein (5, 6, 7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), one of the major flavonoids contained in the dried roots, is known for its potential therapeutic effects, such as cardioprotective (Huang et al., 2005), anticancer (Donald et al., 2012) and neuroprotective properties (Li et al., 2012). In the recent years, accumulating evidence suggested an important protective effect of baicalein in liver. In vitro, baicalein suppressed LPS-induced liver endothelial cell activation and inhibited hepatic stellate cell migration (Chen et al., 2013), and attenuated tert-butyl hydroperoxide-induced hepatic toxicity in rat hepatocytes (Hwang et al., 2005). In vivo, baicalein alleviated carbon tetrachloride induced acute and chronic liver injury in rodents (Huang et al., 2012; Sun et al., 2010), and attenuated acute liver failure induced by D-galactosamine/LPS in mice (Wu et al., 2010). Recently, it was found that treatment with baicalein attenuated obesity, dyslipidemia, fatty

Abbreviations: MCD, methionine–choline-deficient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MDA, malondialdehyde; PCO, protein carbonyl; SOD, superoxide dismutase; Nrf2, Nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ATP, adenosine triphosphate; iNOS, inducible nitric oxide synthase; NOx, NO_2^- + NO_3^-; PPARγ, peroxisome proliferator-activated receptor γ; TLR-4, Toll-like receptor 4; TLR-2, Toll-like receptor 2; PPARα, peroxisome proliferator-activated receptor α; SREBP-1c, sterol regulatory element binding protein 1c; Fas, fatty acid synthase; CD36, cluster of differentiation 36; L-FABP, liver fatty acid binding protein

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liver, diabetes and insulin resistance induced by high-fat diet in mice (Pu et al., 2012).

Genetic models of rodent obesity or the feeding of high-fat diets induce hepatic steatosis without progression to steatohepatitis. In contrast, feeding a high-sucrose diet deficient in methionine and choline (MCD diet) causes hepatic steatosis, hepatocyte injury, inflammation, and ultimately fibrosis, a spectrum of changes that mimic the hepatic pathology of NASH (Vetelainen et al., 2007). In this study, we investigated the effect of treatment with baicalein on MCD-diet-induced NASH in rats.

2. Materials and methods

2.1. Materials, animals and study design

Unless otherwise specified, regents, antibodies and drugs were purchased from Sigma-Aldrich (St Louis, MO, USA).

Male Sprague-Dawley (SD) rats aged 10 weeks were purchased from the Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). All the rats used in this work received humane care in compliance with institutional animal care guidelines, and were approved by the Local Institutional Committee. All the surgical and experimental procedures were in accordance with institutional animal care guidelines. All the rats were entrained at controlled temperature (22–25 °C), 12-h light and 12-h dark cycles (light, 08:00–20:00 h; darkness, 20:00–08:00 h), and free access to tap water.

Animals were randomly divided into four groups and treated as follows (n=32–34 in each group): (1) control group; (2) control group treated with baicalein (Sigma, 98% purity); (3) MCD group; (4) MCD group treated with baicalein. MCD-diet fed rats (MCD group) were fed a methionine and choline deficient diet. Control rats consumed the same diet but sufficient in DL-methionine and choline bitartrate (Serviddio et al., 2008, Lee et al., 2007) (Composition of diet was shown in supplementary data, Table 1S). The baicalein was initially dissolved in dimethylsulfoxide (DMSO) and then diluted in PBS (pH 7.4) (de Carvalho et al., 2011), and the final concentration of DMSO in all groups was no more than 0.1%. The same volume of DMSO was used as control PBS. The rats received intraperitoneal injections of baicalein (10 mg/kg) or control PBS daily for 8 weeks.

2.2. Steatohepatitis assessment

Paraffin-embedded liver sections were stained with hematoxylin-eosin for evaluation of steatosis, lobular inflammation and ballooning degeneration. Steatosis (0–4): 0 ≤ 5%; 1=5–25%; 2=25–50%; 3=50–75%; 4=75–100%. Ballooning (0–3): 0 =absent; 1 =mild (focal involving fewer than three hepatocytes); 2 =moderate (focal involving more than three hepatocytes or multifocal); 3 =severe (multifocal with more than two foci of three or more hepatocytes). Inflammation (0–4): 0 =absent; 1 =minimal (zero to one focus per 20 × field); 2 =mild (two foci); 3 =moderate (three foci); 4 =severe (four or more foci). Slides were blindly evaluated and scored for steatosis and inflammation. The final result was calculated as the arithmetic sum of all individual scores. Fibrosis was minimal in all samples after staining with Masson-trichrome and was therefore not scored.

2.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis

Apoptotic activity of the livers was assessed according to a TUNEL method with a commercial kit (Roche Applied Science, Shanghai, China). TUNEL-positive cells were counted in five randomly selected 10 × microscopic fields per liver.

2.4. Biochemical analysis

Glucose levels of serum were determined spectrophotometrically using the Trinder assay (Sigma, St Louis, MO, USA).

Cholesterol levels in serum and livers were determined by using a Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, CA, USA). Triacylglycerols levels in serum and livers were determined by using a MaxDiscovery™ Triglycerides Enzymatic Assay Kit (Bioo, Texas, USA). The results were corrected for their protein content.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) content in serum were determined according to the enzymatic kinetic method by using an automatic biochemical analyzer (Olympus UA2700, Tokyo, Japan).

2.5. Measurement of malondialdehyde (MDA) and protein carbonyl in livers

Hepatic homogenates were used for the determination of MDA levels by using a kit (Cayman, Ann Arbor, USA). The results were corrected for their protein content.

Protein carbonyl (PCO) content was estimated using the 2, 4-dinitrophenylhydrazine (DNPH)-based procedure. The absorbance of each sample was measured at 320 nm and the tissue protein carbonyl content was calculated based on the molar extinction coefficient of DNPH ($2.2 \times 10^4\ cm^{-1}\ M^{-1}$) (Reznick and Packer, 1994). The results were corrected for their protein content.

2.6. Total reactive oxygen species and $OONO^-$ production in livers

Hepatic total reactive oxygen species and $OONO^-$ production were detected as the method described by Elks et al. (2009). Briefly, samples were incubated at 37 °C with 6.6 μl of CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine, 200 μM) for 30 min for reactive oxygen species measurement; 30 μl of CPH (1-hydroxy-3-carboxypyrrolidine, 500 μM) for 30 min for $OONO^-$ measurement.

2.7. Measurement of SOD activity and catalase activity in livers

Hepatic SOD activity was determined with an SOD-525™ reagent kit (OXIS International, Foster, CA, USA) as previously reported (Umemoto et al., 2004). The final results were corrected for their protein content.

Catalase activity was measured by using a Catalase Activity Assay Kit (Cell Biolabs, San Diego, CA, USA). The final results were corrected for protein content.

2.8. Western blotting

Equal amount of protein preparations (5 μg/μl) was run on SDS-polyacrylamide gels, electrotransferred to polyvinylidine difluoride membranes, and blotted with a primary antibody against Nrf2, HO-1, NF-κB p65, iNOS (Abcam, Cambridge, UK) overnight at 4 °C using slow rocking. Then, they were blotted with responding HRP-conjugated secondary antibody and HRP-conjugated monoclonal antibody against β-actin. Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, Beyotime Institute of Biotechnology, Shanghai, China), and results were expressed as the ratio of the density of specific bands to the corresponding β-actin.

2.9. Measurement of mitochondrial ATP production and reactive oxygen species formation

Mitochondria were isolated by differential centrifugation of hepatic homogenates as the method described previously (Mariappan