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Original article

Diallyl trisulfide attenuates ethanol-induced hepatic steatosis by inhibiting oxidative stress and apoptosis

Lian-Yun Chen[a,b], Qin Chen[a,b], Yi-Feng Cheng[a,b], Huan-Huan Jin[a,b], De-Song Kong[a,b], Feng Zhang[a,b], Li Wu[a,b], Jiang-juan Shao[a,b], Shi-Zhong Zheng[a,b,c,*]

[a] Departement of Pharmacy, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China
[b] National First-Class Key Discipline for Traditional Chinese Medicine of Nanjing University of Chinese Medicine, Nanjing 210023, China
[c] Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Material Medical, Nanjing University of Chinese Medicine, Nanjing 210023, China

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ABSTRACT

Inhibiting the major characteristics of alcoholic fatty liver (AFL) such as lipid accumulation, oxidative stress and apoptosis is a promising strategy of treating AFL. Diallyl trisulfide (DATS) is the major constituent isolated from garlic, which shows promise in the treatment of chronic liver disease. However, the effects of DATS on ethanol-induced liver injury and the related mechanisms remain unclear. The aim of this study was to evaluate the potential protective effects of DATS on AFL and the potential mechanisms. A single intragastric dose of ethanol was given to rats in vivo, while ethanol-stimulated LO2 cells were used as an in vitro model. Our results demonstrated that DATS prevented ethanol-induced injury, as indicated by the reduced activities of aspartate transaminase (AST) and alanine aminotransferase (ALT) in the serum and culture medium, and inhibition of cell apoptosis. Furthermore, DATS reduced hepatic steatosis by up-regulating the expression of peroxisome proliferator-activated receptor-alpha (PPAR-α) and down-regulating the expression of sterolregulatory element binding protein 1c(SREBP-1c). In addition, DATS alleviated ethanol-induced oxidative stress by enhancing non-enzymatic antioxidant and enzymatic antioxidants contents and by reducing the levels of reactive oxygen species (ROS) and malondialdehyde (MDA). These data collectively revealed that DATS protected ethanol-induced liver injury by inhibiting lipid accumulation and oxidative stress.

1. Introduction

In recently years, alcoholic fatty liver (AFL) has become one of the global public health problems, and its prevalence was increasing with epidemics of diabetes, obesity, and metabolic syndrome [1]. AFL is a clinicohistopathological entity characterized by lipid accumulation in the liver after excess alcohol intake [2]. With continued alcohol consumption and abuse over years, steatosis can progress to steatohepatitis, which has the potential to induce fibrosis, cirrhosis, and even hepatocellular carcinoma [3,4] Therefore, it is important to understand the potential molecular mechanisms that underlie fat accumulation and discover efficacious pharmacological agents to treat steatosis as early as possible.

Available evidence suggests that reactive oxygen species (ROS)-mediated oxidative stress critically participates in AFL progression and acts as mediators of molecular and cellular events implicated in AFL [5]. Excess production of ROS can inhibit cell proliferation and subsequent promote hepatocyte apoptosis after alcohol consumption[5]. Moreover, ROS also worsens lipid metabolism disturbances via controlling the genes of transcription involved in steatosis, such as peroxisome proliferator-activated receptor-alpha (PPAR-α), sterolregulatory element binding protein 1c (SREBP-1c) and adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) [6,7].

Functional foods with their nutritional value are attractive for management of chronic diseases. Garlic and their active ingredients are well-known medicines and have been used for a long time as disease-preventing food and folk medicine for reducing the risk of cardiovascular disease, aging, cancer and diabetes, stimulating the immune system and protecting against infection throughout history [8,9]. Diallyl trisulfide (DATS), a major lipid-soluble organosulfur component derived from garlic, is responsible for the pharmacological effects of garlic [10,11]. Modern pharmacological studies have claimed that DATS has the anti-inflammation, anti-oxidation and anti-tumor properties [6,12]. A recent

* Corresponding author at: Department of Clinical Pharmacy, College of Pharmacy, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Nanjing, Jiangsu 210023, China. Fax: +86 25 86798188.
E-mail address: nytws@163.com (S.-Z. Zheng).

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report from our laboratory also confirmed that DATS showed powerful hepatoprotective effects against oxidative damage caused by several hepatotoxins, such as carbon tetrachloride [12,13]. Hence, in view of its potential benefits, the aim of our present study was to examine whether DATS treatment exerted any protective influence on the ethanol-induced fatty liver in vivo and in vitro, and to elucidate further the detailed mechanisms underlying.

2. Methods

2.1. Reagents and antibodies

DATS (purity >80%) was purchased from Cheng Du Micxy Chemical Co., Ltd. (Chengdu, China) and was dissolved in olive oil for in vivo experiments. Tiopronin was obtained from Yifeng Pharmacy Chain Co., Ltd. (Nanjing, China). DATS (purity >97%) was purchased from Shenzhen Minn Bolin Biotechnology Co., Ltd. (Shenzhen, China). It was dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for in vitro experiments. The vehicle (DMSO) was used as a control throughout the experiments at a final concentration of 0.02% (w/v). The following primary antibodies were used in this study: PPAR-α and SREBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bcl-2, Bax, pro-caspase-9, cleaved-caspase-9, pro-caspase-8, cleaved-caspase-8, pro-caspase-3, cleaved-caspase-3, full-length PARP-1, cleaved-PARP-1, and β-actin (Cell Signaling Technology, Danvers, MA, USA).

2.2. Animals and experimental design

Adult male Sprague–Dawley rats (180–220 g body weight) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All these rats were maintained at approximately $22 \pm 2\,^{\circ}C$ and $55 \pm 5\%$ relative humidity, with a 12-h light: 12-h dark cycle, and had free access to laboratory chow and tap water. The experimental was approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China). All animals were cared for in accordance with the National Institutes of Health (USA) guidelines. After adaptive feed for a week, sixty rats were randomly divided into six groups with 10 rats in each group. Group 1 was the vehicle control in which rats were not given ethanol or DATS but received equal volume solvent. Group 2 was AFL model group in which rats were gavaged with ethanol (56%, v/v, 10 mL/kg) without DATS. Groups 3, 4, and 5 were treatment groups in which rats were gavaged with ethanol and orally given DATS (purity >80%) at 25, 50, and 100 mg/kg, respectively. Group 6 was the positive control in which rats were orally administrated with ethanol and tiopronin at150 mg/kg, respectively. Rats in Groups 2–6 were orally given ethanol every day for 8 weeks. DATS and tiopronin were dissolved in olive oil and given once daily by gavage during weeks 5–8. At the end of the experiment, rats were sacrificed after being anesthetized by intraperitoneal injection (i.p.) pentobarbital (50 mg/kg). Blood was collected, and livers were isolated and weighted quickly and stored at $-80\,^{\circ}C$ until analysis.

2.3. Cell culture and treatments

Human normal liver cell LO2 was obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin. The incubator kept the condition of 95% air and 5% CO_2 humidified atmosphere at 37 °C. The cells were exposed to 100 mM ethanol in the absence/presence of DATS (purity >97%) (1, 2.5, 5 μM) for 24 h, and were harvested in accordance with the applied technique. The doses of ethanol and DATS were chosen by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) cell proliferation assay.

2.4. Determination of blood biochemistry

Twenty-four hours after the last oral administration, whole blood was collected. Sera were collected from blood after centrifugation at $3000 \times g$ for 20 min and stored at $-80\,^{\circ}C$ for further analyses. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in serum (2 mL/sample) were detected, strictly adhering to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All absorbance values were determined using a SPECTRA max™ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Experiments were carried out in three independent experiments.

2.5. Determination of hepatic biochemistry

Levels of TG, TC, glutathione (GSH) and malondialdehyde (MDA), and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) in the liver homogenate were determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Small pieces of the liver tissue collected from the rats were homogenized and sonicated in 0.5 mL of ice-cold buffer and centrifuged. The supernatant was collected and measured using the reagents in the kits following the protocols from the manufacturer. Experiments were performed in triplicate.

2.6. Histopathology and immunohistochemistry

Liver samples were removed and fixed by immersion in 4% phosphate-buffered paraformaldehyde, and then conventionally processed, embedded in paraffin, and sectioned to a thickness of 5 μm. Briefly, the sections were deparaffinized and rehydrated by employing standard techniques, hematoxylin-eosin (H&E) staining was performed, and typical imaging of liver sections were examined by light microscopy. Immunohistochemistry was performed using antibodies against CD45. All histopathological analyses were undertaken by an experienced histopathologist in a blinded manner.

2.7. Oil Red O Staining

Analysis of intracellular lipid droplets was determined using Oil Red O staining kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the culture medium was discarded and the cells were washed three times in phosphate buffer solution, as well as 10-μm-thick frozen sections, then fixed with 4% paraformaldehyde for 30 min and stained with 5% Oil Red O solution for 30 min. They were then washed with 60% isopropanol for 30 s and then rinsed with ddH_2O for 30 s, counterstained with hematoxylin for 3 min, rinsed with ddH_2O for 5 min, fixed with neutral balata, and then observed under an upright microscope (Leica, Germany).

2.8. Western blot analysis

Liver samples and LO2 cells were lysed in radio-immunoprecipitation assay buffer (RIPA buffer) containing protease inhibitors