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Diallyl trisulfide protects against ethanol-induced oxidative stress and apoptosis via a hydrogen sulfide-mediated mechanism

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ABSTRACT

Garlic is one natural source of organic sulfur containing compounds and has shown promise in the treatment of chronic liver disease. Dietary garlic consumption is inversely correlated with the progression of alcoholic fatty liver (AFL), although the exact underlying mechanisms are not clear. Our previous studies also have shown that diallyl trisulfide (DATS), the primary organosulfur compound from *Allium sativum* L, displayed anti-lipid deposition and antioxidant properties in AFL. The aim of the present study was to clarify the underlying mechanisms. In the present study, we used the intragastric infusion model of alcohol administration and human normal liver cell line LO2 cultured with suitable ethanol to mimic the pathological condition of AFL. We showed that accumulation of intracellular reactive oxygen species (ROS) was lowered significantly by the administration of DATS, but antioxidant capacity was increased by DATS. Additionally, DATS inhibited hepatocyte apoptosis via down-regulating Bax expression and up-regulating Bcl-2 expression, and attenuated alcohol-induced caspase-dependent apoptosis. More importantly, using iodoacetamide (IAM) to block hydrogen sulfide (H_2S) production from DATS, we noted that IAM abolished all the above effects of DATS in ethanol-treated LO2 cells. Lastly, we found DATS could increase the expressions of cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS), the major H_2S-producing enzymes. These results demonstrate that DATS protect against alcohol-induced fatty liver via a H_2S-mediated mechanism. Therefore, targeting H_2S may play a therapeutic role for AFL.

1. Introduction

The liver is not only the vital organ responsible for metabolizing alcohol, but also the main target organ for alcoholic toxicity [1,2]. Excess alcohol consumption becomes the major etiologic factor in the development of alcoholic fatty liver (AFL), which is characterized by accumulation of large lipid droplets and mitochondrial enlargement in hepatocytes [3]. Therefore, it is necessary to prevent AFL as early as possible, and it's likely to block or delay the development of steatosis to more severe forms, such as steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma [4,5]. Thus, it is important to study the pathogenesis of AFL, and discover efficacious pharmacological agents to treat steatosis.

Most evidences suggest that oxidative stress contributes to alcohol-induced liver injury [6]. Excess alcohol is oxidized to acetaldehyde through three metabolic pathways, and then to acetic acid, which leads to generation of reactive oxygen species (ROS) [7]. Accumulation of ROS reduces cellular antioxidant abilities, and enhances oxidative stress in liver [7]. In addition, the cytotoxic effects of alcohol metabolism and excess ROS induce death of hepatocytes, as evidenced by apoptosis in AFL [7,8]. Ingested alcohol contributes to oxidative stress-induced cell apoptosis, using the intragastric infusion model of alcohol administration and human normal liver cell line LO2 cultured with suitable ethanol [8–10]. We can mimic the change to investigate the potential mechanisms of AFL and evaluate the effects of novel therapeutic agents in cellular and rat models.

Diallyl trisulfide (DATS) is an active constituent isolated from the vegetable of garlic, a Chinese herbal medicine, which has been clinically used in the treatment of bacterial infections, diabetes and cardiovascular diseases for many years [11,12]. Investigation has demonstrated that DATS reduced alcohol-induced liver injury and fat deposition, which was associated with attenuation of hepatic oxidative stress [13]. However, the exact mechanisms of how DATS affects AFL are not fully understood. Interestingly, emerging evidence suggests that hydrogen sulfide (H_2S) as the third gasotransmitter, which is synthesized from L-

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cysteine by enzymes such as cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MST), along with cysteine aminotransferase (CAT), is thought to play an important role in diseases [14]. Moreover, drugs that are capable of producing safe levels H_2S or target H_2S signaling pathway in vivo may have potential biological and clinical significances for chronic liver diseases [15]. Considering the fact that DATS, which has the highest content accounting for 41.5% among the organosulfur compounds in garlic and been found to be the most effective compound, serves as an exogenous H_2S donor, and the liver has a high ability to produce and clear H_2S [16–18], we speculated that DATS attenuated oxidative stress induced-apoptosis through generation of H_2S in AFL. The current study was designed to validate the hypothesis.

2. Materials and methods

2.1. Reagents and antibodies

The following compounds were used in this study: DATS (purity >97%; Shenzhen Minn Bolin Biotechnology Co., Ltd., Shenzhen, China, iodoacetamide (IAM; Nanjing Dingguo Changsheng Biotechnology Co., Ltd., Nanjing, China), they were dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for in vitro experiments and kept at −20 °C if necessary. The vehicle (DMSO) was used as a control throughout the experiments and the final concentration of DMSO never exceeded 0.02% (w/v), which had no cytotoxicity in this study (data not shown). DATS (purity >80%) was purchased from Cheng Du Micxy Chemical Co., Ltd. (Chengdu, China) and was dissolved in olive oil for in vivo experiments. The following primary antibodies were used in this study: catalase(CAT), superoxide dismutase(SOD) and glutathione reductase (GR) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); CSE and CBS (Epitomics, San Francisco, 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 procedures

Male Sprague-Dawley rats (180–220 g body weight) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All these rats grew in the specific pathogen free clean room with the condition of 21 to 25 °C as well as a 12-h dark/light 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, fifty rats were randomly divided into five groups with 10 rats in each group. Group1 was the vehicle control in which rats were not given ethanol or DATS but received equal volume solvent. Group 2 was ALD 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 orally given ethanol and DATS at 25, 50, and 100 mg/kg, respectively. Rats in groups 2–5 were orally given ethanol every day for 8 weeks. DATS was 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). Livers were isolated and weighted quickly and stored at −80°C until analysis.

2.3. Cell culture

Human LO2 hepatocyte cells were purchased 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 cells were grown in humidified atmosphere of 95% air and 5% CO2 humidified atmosphere at 37 °C. The cells were exposed to 100 mM ethanol in the absence/presence of DATS or IAM for 24 h, and were harvested in accordance with the applied technique. LO2 hepatocytes were then treated with DATS at 1, 2.5, and 5 μM and/or IAM at 50 μM, respectively. Cell morphology was assessed under an inverted microscope (Leica, Germany).

2.4. Analyses of apoptosis

LO2 cells were seeded into 6-well plates and cultured in DMEM with 10% FBS for 12 h. LO2 hepatocytes were then treated with various reagents at the indicated concentrations for 24 h. Morphological changes characteristic of apoptosis were detected by Hoechst staining kits and TUNEL staining kits (Beyotime Institute of Biotechnology, Haimen, China) according to the protocol. The cells were observed and photographed using an inverted fluorescence microscope (Nikon, Tokyo, Japan). In certain experiments, cells were treated as indicated, collected, and stained with Annexin V-FITC apoptosis assay kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Apoptotic cells were detected by flow cytometry, and the extent of apoptosis was calculated with FlowJo software (FACSCalibur; BD, Franklin Lakes, NJ, USA). Moreover, liver tissue samples embedded in paraffin were prepared, and used for TUNEL assay. Results were from triplicate experiments.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of H_2S in liver tissues and LO2 were determined with an ELISA kit (Beijing equations biological Co., Ltd., Beijing, China) according to the protocol. Results were from triplicate experiments.

2.6. Detection of reactive oxygen species (ROS)

The content of intracellular ROS was detected by DCFH-DA staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The intensity of cellular fluorescence intensity was determined by an MRC 1024 laser confocal microscope (Bio-Rad Laboratories). Results were from triplicate experiments.

2.7. Measurement of malondialdehyde (MDA), glutathione (GSH) and SOD

Levels of intracellular MDA, GSH and SOD were determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). LO2 cells were seeded into 6-well plates and cultured in DMEM with 10% FBS for 12 h. LO2 were then treated with various reagents at the indicated concentrations for 24 h, and were lysated with RIPA buffer. The lysates were collected and deproteinated using the reagents in the kits. The levels of MDA, GSH and SOD were determined according to the instructions. Experiments were performed in triplicate.

2.8. Western blot analyses

LO2 cells were treated with various reagents at indicated concentrations for 24 h. Liver samples and LO2 cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease inhibitors and/or phosphatase inhibitors. The protein levels were detected using a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL). Proteins (50 μg/well) were subjected to sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a poly(vinylidene fluoride) membrane (Millipore, Burlington, MA, USA).The membrane were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). The blots were incubated overnight with the corresponding primary antibodies in TBST at 4 °C and subsequently by horseradish peroxidaseconjugated secondary