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Blockade of store-operated calcium entry alleviates ethanol-induced hepatotoxicity via inhibiting apoptosis

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ABSTRACT

Extracellular Ca^{2+} influx has been suggested to play a role in ethanol-induced hepatocyte apoptosis and necrosis. Previous studies indicated that store-operated Ca^{2+} entry (SOCE) was involved in liver injury induced by ethanol in HepG2 cells. However, the mechanisms underlying liver injury caused by SOCE remain unclear. We aimed to investigate the effects and mechanism of SOCE inhibition on liver injury induced by ethanol in BRL cells and Sprague–Dawley rats. Our data demonstrated that ethanol (0–400 mM) dose-dependently increased hepatocyte injury and 100 mM ethanol significantly upregulated the mRNA and protein expression of SOC for at least 72 h in BRL cells. Blockade of SOCE by pharmacological inhibitors and sh-RNA knockdown of STIM1 and Orai1 attenuated intracellular Ca^{2+} overload, restored the mitochondrial membrane potential (MMP), decreased cytochrome C release and inhibited ethanol-induced apoptosis. STIM1 and Orai1 expression was greater in ethanol-treated than control rats, and the SOCE inhibitor corosolic acid ameliorated the histopathological findings and alanine transaminase and aspartate transaminase activity as well as decreased cytochrome C release and inhibited alcohol-induced cell apoptosis. These findings suggest that SOCE blockade could alleviate alcohol-induced hepatotoxicity via inhibiting apoptosis. SOCE might be a useful therapeutic target in alcoholic liver diseases.

Introduction

Alcoholic liver disease (ALD), ranging from alcoholic fatty liver, alcoholic hepatitis, and alcoholic fibrosis to alcoholic cirrhosis (Rong et al., 2012), is the most common liver disease and accounts for 40% of deaths from cirrhosis or 28% of all deaths from liver disease (Kim et al., 2002). ALD has a complex and incompletely known pathogenesis. The mechanisms include direct hepatotoxicity, production of reactive oxygen species (ROS) induced by ethanol and its metabolites, tissue hypoxia, endoplasmic reticulum (ER) stress, iron overload, cell apoptosis and immunological mechanisms, which play a key role in the development and progression of alcoholic liver injury (Gramenzi et al., 2006). However, some of the pathogenesis of ALD still remains obscure.

Ca^{2+}, as a ubiquitous second messenger, is involved in a plethora of cellular functions, including energy metabolism and cell death (Henke et al., 2012). Store-operated Ca^{2+} channels (SOCs), mediated by the sensor stromal interactive molecules 1 (STIM1) and Ca^{2+} release-activated Ca^{2+} channel (CRAC, also called Orai1), has a tight relationship with Ca^{2+} homeostasis by sensing ER Ca^{2+} level and mediating extracellular Ca^{2+} entry to increase cytosolic Ca^{2+} level and refill Ca^{2+} stores (Soboloff et al., 2012). We previously demonstrated that chronic ethanol significantly increased extracellular calcium influx by upregulating the expression of these SOC protein molecules in HepG2 cells (Cui et al., 2013; Liu et al., 2012). However, although HepG2 is a widely used model to research in vitro toxicity in the liver because it retains many phase I and II enzymes expressed in human hepatocytes, HepG2 cells still cannot fully represent primary human hepatocytes. Furthermore, the role of SOCs in liver injury induced by ethanol has been poorly investigated.

In this study, we aimed to investigate the role of SOCs on liver injury induced by ethanol application and the effectiveness of SOC inhibitors in alleviating alcohol-induced hepatotoxicity in rat liver cells (BRL) and Sprague–Dawley rat livers, which may provide an experimental basis for finding a new target for clinical treatment of ALD.

Materials and methods

Reagents. 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), lanthanides La^{3+} and 2-aminoethyldiphenyl borate (2-APB) were from Sigma-Aldrich Co. (St. Louis, MO, USA). GSK7579A and 2,6-difluoro-N-{5-[4-methyl-1-(5-methyl-thiazol-2-yl)-1,2,5,6-tetrahydro-pyridin-3-yl]-pyrazin-2-yl}-benzamide (RO2959) were from Glixx Laboratories (Southborough, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, antibiotics (penicillin, streptomycin) and trypsin–EDTA solution were from

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Gibco (Invitrogen, Grand Island, NY, USA). Hanks' Balanced Salt Solution (HBSS, pH 7.4) was from Gibco (Invitrogen Corp.) and consisted of 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$–$6H_2O$, 0.407 mM $MgSO_4$–$7H_2O$, 137.93 mM NaCl, 5.33 mM KCl, 4.17 mM $NaHCO_3$, 0.441 mM KH_2PO_4, 0.34 mM Na_2HPO_4, and 5.56 mM D-glucose. Fluo-3/acetoxymethyl ester (Fluo-3/AM) and pluronic F-127 were from Biotium (Hayward, CA, USA). Rhodamin 123 was from Sigma. All antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Cell culture and treatments. The rat liver cell line BRL was purchased from the Chinese Academy of Science, and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator (37 °C, 5% CO_2). BRL cells grown to 80–90% confluence in complete growth medium were treated with ethanol at different concentrations (0, 25, 50, 100, 200, 400 mM) or times (0, 24, 48 and 72 h). For inhibition experiments, corosolic acid (50 μM), La^{3+} (1 μM), 2-APB (50 μM), GSK-7975A (3.4 μM) and RO2959 (200 nM) were added with 100 mM ethanol for 24 h. To minimize ethanol evaporation during prolonged incubation, plates were sealed with parafilm and the medium containing ethanol was changed once every 24 h. To exclude the effect of sealing alone on cell viability, control plates were also sealed with parafilm. Cell lysates were prepared for western blotting analysis.

Assessment of cell viability. Viability of BRL cells was determined by standard MTT assay. Briefly, MTT solution (20 μl, 5 mg/ml) was added into each well for incubation at 37 °C for 4 h. After 4 h, the solution was removed by aspiration, the insoluble formazan crystals were dissolved in 150 μl/well DMSO, and absorbance at 490 nm was measured by use of a Varioskan Flash spectral scanning multimode reader (Thermo Electron, Vantaa, Finland). The spectrophotometer was calibrated to zero absorbance with culture medium without cells. The percentage cell survival was determined by comparing the average absorbance of the treated cells to that of untreated cells. All doses were tested in triplicate and the experiment was repeated at least 3 times.

Measurement of cytoplasmic free Ca^{2+} concentration. The free cytosolic Ca^{2+} concentration was determined with the fluorescent calcium indicator Fura 3-acetoxymethy ester (Fura3-AM) by flow cytometry. Equal volumes of Fluo-3/AM and pluronic F127 were added into HBSS just before incubation as the Fluo-3 loading solution (final concentration of Fluo-3/AM and pluronic F127 5 μM and 0.1%, respectively). After BRL cells were treated with ethanol (100 mM), ethanol plus corosolic acid, La^{3+}, 2-APB, GSK-7975A and RO2959 for 24 h, they were loaded with Fura3/AM for 45 min in the dark at 37 °C in a humidified incubator, washed 3 times in PBS, detached with trypsin, resuspended in HBSS, and directly introduced into a FACSCalibur flow cytometer to detect green fluorescence with FL1. Data for 10,000 fluorescent events were obtained by use of Cell Quest software and analyzed with FCS Express 4.0 (De Novo Software, Los Angeles, CA, USA) and expressed as mean fluorescence intensity of 10,000 cells.

Transfection. BRL cells were cultured in DMEM supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 at 37 °C in 60-mm flasks. When cells reached 90% confluence, they were transfected with the plasmid U6/GFP/Neo-short hairpin RNA (sh-RNA) (Shanghai GenePharma) targeting STIM1 (p.STIM1.sh-RNA; 8.0 μg with 20 μl Lipofectamine 2000 [Invitrogen, Shanghai] in each flask), and plasmid U6/GFP/Neo-sh-RNA (Shanghai GenePharma Corp.) targeting Orai1 (p.Orai1.sh-RNA; 8.0 μg with 20 μl Lipofectamine 2000 in each flask). The non-transfected and negative control sh-RNA-transfected cells were used as controls. The sh-RNA sequences were for STIM1, 5′-GGAAGACCTCAATTACCAT-3′; Orai1, 5′-GCAACGTCCACAACCTCAACT-3′; negative control, 5′-GTTCTCCGAACG TGTCACGT-3′. The medium was changed after 6 h. The efficiency of transfection was observed by fluorescence microscopy (Olympus IX-70, Olympus, Prague). The efficiency of gene silencing was confirmed by assaying STIM1 and Orai1 protein levels. After 24-h transfection, cells were treated with 100 mM ethanol for 24 h. Then cells were collected.

Apoptosis analysis by annexin-V FITC/PI staining. Cell apoptosis was evaluated by use of an Annexin V-FITC/PI kit. Briefly, after treatment, attached cells were collected, washed with PBS twice. An amount of 400 μl binding buffer, 5 μl Annexin-V FITC and 5 μl PI was successively added to the cell suspension. After 15 min of incubation in dark, the cells were subjected to flow cytometry. (Becton Dickinson, San Jose, CA, USA). At least three independent experiments were carried out.

Cell cycle analysis by PI staining. Cell cycle analysis was evaluated by PI staining. Briefly, about 12×10^5 BRL cells were seeded in a 6 well plate, allowed to attach for 12 h, then cells were collected by the trypsin method, washed with PBS, and fixed overnight at 4 °C in 70% ethanol. Fixed cells were washed with cold PBS twice, 20 μl RNase A was added and incubated in 37 °C for 30 min to hydrolyze RNA, then cells were stained with PI in the dark for another 30 min, and the cell cycle was evaluated by flow cytometry. The experiments were repeated at least three times independently.

Rat model of ALD. This study was approved by the institutional review board of Qilu Hospital. We obtained 40 male Sprague–Dawley rats (6 weeks old) from the Experimental Animal Center of Shandong University. After 1-week acclimatization, rats were randomly divided into 4 groups ($n = 10$ each). Each group was fed standard food with olive oil.

Group I (control), normal control group: no intragastrical or intravenous treatment.

Group II (ethanol-treated group), intragastric ethanol infusion: 60% alcohol (Red Star Er Guo Tou Jiu, Beijing) diluted with water was given intragastrically in increasing doses: 4.5, 6.5 and 9 g/kg/day for 1–4, 5–8, and 9–12 weeks, respectively. The daily dose of alcohol was administered in 2 divided doses, 12 h apart.

Group III (therapeutic group 1), daily oral dose of 4 ml 20% corosolic acid followed 10 min later by an oral administration of ethanol given by the same schedule as for group II. Administration of corosolic acid started at 8 weeks after ethanol and continued simultaneously with ethanol for the 4-week treatment period.

Group IV (therapeutic group 2), concomitant treatment with both corosolic acid and ethanol as in group III but corosolic acid intake started 4 weeks after the initiation of ethanol and continued simultaneously with ethanol for the 8-week treatment period.

Rats were weighed at the beginning and end of the experiment, blood samples were obtained from left ventricles, and rats were killed by intraperitoneal injection of sodium pentobarbital to the right lower abdomen. Liver tissue was obtained for histopathological sections, and other portions were kept in liquid nitrogen for western blot analysis. Animal welfare and experimental procedures were in accordance with the care and use of laboratory animals (National Research Council, Washington, DC, USA).

Histology. Liver lobes from controls and ethanol-treated groups were trimmed from the porta hepatis; liver tissue was harvested from the right hepatic lobe and fixed with 4% paraformaldehyde solution. Paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E). Hepatic tissue sections were observed under an optical microscope (Olympus BX51, Olympus, Tokyo).

Biochemistry. Damage of BRL cells was evaluated by alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) leakage in culture supernatant collected from treated cells by use of an Automatic Biochemistry Analyzer (Hitachi 7170A, Tokyo, Japan). Rat blood was allowed to stand for 10 min before being centrifuged at 2000 rpm