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Pomegranate prevents binge alcohol-induced gut leakiness and hepatic inflammation by suppressing oxidative and nitrative stress

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ABSTRACT

Alcoholic liver disease (ALD) is a major chronic liver disease worldwide and can range from simple steatosis, inflammation to fibrosis/cirrhosis possibly through leaky gut and systemic endotoxemia. We investigated whether pomegranate (POM) protects against binge alcohol-induced gut leakiness, endotoxemia, and inflammatory liver damage. After POM pretreatment for 10 days, rats were exposed to 3 oral doses of binge alcohol (5 g/kg/dose) or dextrose (as control) at 12-h intervals. Binge alcohol exposure induced leaky gut with significantly elevated plasma endotoxin and inflammatory fatty liver by increasing the levels of oxidative and nitrative stress marker proteins such as ethanol-inducible CYP2E1, inducible nitric oxide synthase, and nitrated proteins in the small intestine and liver. POM pretreatment significantly reduced the alcohol-induced gut barrier dysfunction, plasma endotoxin and inflammatory liver disease by inhibiting the elevated oxidative and nitrative stress marker proteins. POM pretreatment significantly restored the levels of intestinal tight junction (TJ) proteins such as ZO-1, occludin, claudin-1, and claundin-3 markedly diminished after alcohol-exposure. In addition, the levels of gut adherent junction (AJ) proteins (e.g., β-catenin and E-cadherin) and desmosome plakoglobin along with associated protein α-tubulin were clearly decreased in binge alcohol-exposed rats but restored to basal levels in POM-pretreated rats. Immunoprecipitation followed by immunoblot analyses revealed that intestinal claudin-1 protein was nitrated and ubiquitinated in alcohol-exposed rats, whereas these modifications were significantly blocked by POM pretreatment. These results showed for the first time that POM can prevent alcohol-induced gut leakiness and inflammatory liver injury by suppressing oxidative and nitrative stress.

1. Introduction

Chronic and/or binge alcohol (ethanol) consumption is known to cause alcoholic liver disease (ALD), which is one of the leading causes of hepatic diseases and liver-related death worldwide [1–3]. The clinical spectrum of ALD includes alcoholic fatty liver (steatosis), steatohepatitis (inflammation), fibrosis/cirrhosis, and increased risk of hepatocellular carcinoma [1,3]. Alcohol can directly affect the physiological functions of different liver cells including hepatocytes, Kupffer cells and stellate cells. In addition, alcohol can indirectly stimulate the liver injury by changing gut microbiome accompanied with the impaired gut barrier function, leading to increased leaky gut and translocation of intestinal bacteria with elevated plasma levels of bacterial endotoxin (e.g., lipopolysaccharide, LPS) [4–6]. Many recent reports showed that circulating LPS can reach the liver via portal vein and interact with hepatic toll-like receptor-4 (TLR4) to initiate the inflammatory cascades for the accelerated development of ALD [4–6]. Alcohol-induced gut leakiness is critically important in the progression to more severe ALD such as fibrosis/cirrhosis since the elevated levels of endotoxin are correlated with the development of liver cirrhosis [7].

Under the influence of ethanol, the expression and the levels of many genes and proteins are altered through transcriptional and post-transcriptional mechanisms, including epigenetics [8]. The ethanol-inducible cytochrome P450-2E1 (CYP2E1) is one of the well-characterized proteins that are induced and/or activated by chronic and/or binge alcohol intake [9]. Under relative low amounts of alcohol, hepatic and

Abbreviations: POM, pomegranate; ALD, alcoholic liver disease; TJ, tight junction; AJ, adherent junction; CYP2E1, ethanol-inducible cytochrome P450-2E1; iNOS, inducible nitric oxide synthase; nitroxidative stress, oxidative and nitrative stress; KI mice, knock-in mice; KO mice, knock-out mice; UA, urolithin A; EA, Ellagic acid; CMZ, chlormethiazole; BAC, blood alcohol concentration; ER, endoplasmic reticulum; TLR4, toll-like receptor-4; PERK, protein kinase-like endoplasmic reticulum kinase; eIF2α, eukaryotic translation initiation factor-2α; PDI, protein disulfide isomerase; PTM, post-translational modification; ROS, reactive oxygen species; TEER, trans-epithelial electrical resistance; FITC-D4, FITC-labeled 4-kDa dextran; IP, immunoprecipitation; JNK, c-Jun *N*-terminal protein kinase; LPS, lipopolysaccharide

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gastric alcohol dehydrogenases usually metabolize alcohol. However, after long-term alcohol drinking or under high blood alcohol concentration (BAC), CYP2E1 becomes involved in the ethanol metabolism (K_m about ~ 10 mM ethanol), resulting in the production of acetaldehyde and reactive oxygen species (ROS), which can negatively affect many cellular components and can cause cell death [10]. Death of parenchymal cells can activate liver-resident inflammatory cells such as Kupffer cells or infiltrated neutrophils that can significantly promote inflammatory ALD and severe disease [2,9,11]. Indeed, the specific involvement of CYP2E1 in ALD has been demonstrated by utilizing CYP2E1 overexpressed transgenic (Tg), knock-out (KO), and knock-in (KI) mice [2,12,13]. Recently, our group reported that CYP2E1 is also involved in n-6 fatty acid containing western-style high-fat diet induced non-alcoholic steatohepatitis [14] and aging-related liver and kidney damage [15,16] through increasing the oxidative and nitrative (nitroxidative) stress. Furthermore, both intestinal and hepatic CYP2E1 induced (i.e., via protein stabilization) by chronic alcohol drinking [17] or binge alcohol exposure seems critically important in promoting alcohol-mediated increased nitroxidative stress, gut leakage, and endotoxemia, contributing to apoptosis of hepatocytes and steatohepatitis [18,19].

Pomegranate (POM, *Punica granatum*) is one of the edible fruits and mainly grown in middle east, India, southern Asia and Mediterranean Europe, as well as in warm climate areas of the world including Americas [20]. It is a rich source of polyphenolic compounds like anthocyanidins (delphinidin, cyanidin and pelargonidin) and hydrolysable tannins (such as punicalagin, pedunculagin, punicalin, gallagic, ellagic acid esters of glucose) [21] against a variety of diseases including cancer [22], cardiovascular disorders [23], diabetes [24], Alzheimer's disease [25], aging [26], and AIDS [27]. Recently, it was shown to prevent development of non-alcoholic fatty liver disease in rats [28] and ethanol-induced toxicity in HepG2 human hepatoma cells [29]. Acute and sub-chronic toxicity studies with rodents revealed that oral administration of its extracts for up to ~ 4330 mg/kg/day is considered safe with no observable adverse effects [30–32].

Based on our recent results on alcohol-induced gut leakiness [18,19], we hypothesized that POM, with various antioxidants and anti-inflammatory ingredients, can also prevent alcohol-mediated oxidative stress critically important in promoting gut leakiness and inflammatory liver injury. In this present study, we have evaluated the beneficial effects of POM on alcohol-induced gut leakiness and inflammatory liver injury in rats and investigated the protective mechanisms in rats as well as cultured hepatocytes and T84 colonic cells.

2. Material and methods

2.1. Materials

All chemicals, urolithin A (UA) and ellagic acid (EA) used in this study were from Sigma Chemical (St. Louis, MO, USA). POM extracts (Bulk Supplements; 40% EA) were obtained from Amazon. T84 human colonic cell line (CCL-248) and AML12 normal mouse liver cells (CRL-2254) were purchased from the ATCC (American Type Culture Cells, Manassas, VA). Other materials not described here were the highest grades available and/or the same, as recently described [19,33,34].

2.2. Animal treatments

All animal experimental procedures were carried out by following the National Institutes of Health (NIH) guidelines for small animal experiments and approved by the NIAAA Institutional Animal Care and Use Committee. All rats were maintained under controlled lighting (12-h light/dark cycle) with food and water provided ad libitum. Age-matched 7-weeks old female Fischer 344 wild-type (WT) rats were orally administered a daily dose of 600 mg POM/kg, based on the safety and effective dosages of POM extracts [30–32,35,36]. Control rats were orally administrated a vehicle (water). After POM pretreatment for 10 days, some rats (n ≥ 4/group) were exposed to 3 oral doses of binge alcohol (5 g/kg/dose) or dextrose (as control) at 12-h intervals and euthanized 1-h after the last ethanol dose.

2.3. Culture of liver cells and colon cells

The AML12 liver cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, 0.1 μM dexamethasone, and insulin-transferrin-selenium (Invitrogen). Cells were grown at 37 °C under 5% CO_2. AML12 cells were treated with 100 mM ethanol in the absence or presence of the selective inhibitor of UA (15 μM), EA (100 μM), or chlormethiazole (CMZ, 15 μM) for 24 h. T84 colon cells were grown in a humidified incubator under 95% air and 5% CO_2 at 37 °C in Ham's F-12 medium, supplemented with 10% FBS, 1% antibiotic-antimycotic solution. Confluent monolayers appeared 6–14 day after plating. T84 monolayer cells were exposed to 40 mM ethanol in the absence or presence of the selective inhibitor of UA (15 μM), EA (100 μM), or CMZ (15 μM) for 8 h prior to harvest for subsequent analyses.

2.4. Histological analysis and serum ALT measurement

Rats were briefly sedated to carbon dioxide gas followed by decapitation and immediate collection of trunk blood, small intestine, and liver from each rat. In this study, part of the largest liver lobe or small intestine from each rat exposed to POM pretreatment or control with or without binge ethanol exposure was fixed in neutral formalin. Paraffin-embedded blocks of formalin-fixed individual liver or small intestine sections were cut at 4 μm, stained with hematoxylin/eosin (H/E) by American Histolabs, Inc. (Gaithersburg, MD). To further support fat accumulation, frozen liver samples embedded in optimal cutting temperature compound were cut (10 μm) and stained with Oil Red O by American HistoLabs, Inc. The plasma ALT level in each rat was determined by using the standard end-point colorimetric assay kit (TECO Diagnostics, Anaheim, CA), as described [34].

2.5. Endotoxin assay

Plasma endotoxin levels were determined using the commercially available endpoint LAL Chromogenic Endotoxin Quantitation Kit with a concentration range of 0.015–1.2 EU/mL (Thermo Fisher Scientific, Waltham, MA), as described [19].

2.6. Triglyceride determination in liver

The amounts of hepatic triglyceride (TG) were assessed by using a commercially available kit (Thermo Fisher Scientific) [19,34].

2.7. Determination of the levels of hydrogen peroxide, nitrate/nitrite, lipid peroxidation and superoxide dismutase (SOD) activity in liver extracts

The levels of hepatic hydrogen peroxide, nitrate/nitrite, lipid peroxidation and superoxide dismutase (SOD) activity were assessed by using commercially available kits, respectively (Cayman; Abcam; Oxford Biomedical Research; Cayman).

2.8. Determination of plasma ROS levels

The amounts of plasma ROS were determined by visualization with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Thermo Fisher Scientific). Following incubation with DCFH-DA at 37 °C for 20 min, the DCFH-DA fluorescence was then determined by the method, as recently described [33].