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Role of defective methylation reactions in ethanol-induced dysregulation of intestinal barrier integrity

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ABSTRACT

Alcoholic liver disease (ALD) is a major healthcare challenge worldwide. Emerging evidence reveals that ethanol administration disrupts the intestinal epithelial tight junction (TJ) complex; this defect allows for the paracellular translocation of gut-derived pathogenic molecules to reach the liver to cause inflammation and progressive liver injury. We have previously demonstrated a causative role of impairments in liver transmethylation reactions in the pathogenesis of ALD. We have further shown that treatment with betaine, a methylation agent that normalizes liver methylation potential, can attenuate ethanol-induced liver injury. Herein, we explored whether alterations in methylation reactions play a causative role in disrupting intestinal mucosal barrier function by employing an intestinal epithelial cell line. Monolayers of Caco-2 cells were exposed to ethanol or a-pan methylation reaction inhibitor, tubercidin, in the presence and absence of betaine. The structural and functional integrity of intestinal epithelial barrier was then examined. We observed that exposure to either ethanol or tubercidin disrupted TJ integrity and function by decreasing the localization of TJ protein occludin-1 to the intracellular junctions, reducing transepithelial electrical resistance and increasing dextran influx. All these detrimental effects of ethanol and tubercidin were attenuated by co-treatment with betaine. We further show that the mechanism of betaine protection was through BHMT-mediated catalysis. Collectively, our data suggest a novel mechanism for alcohol-induced gut leakiness and identifies the importance of normal methylation reactions in maintaining TJ integrity. We also propose betaine as a potential therapeutic option for leaky gut in alcohol-consuming patients who are at the risk of developing ALD.

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1. Introduction

Alcoholic liver disease (ALD) is one of the leading causes of liver diseases and a major cause of morbidity and mortality in the United States and worldwide [1]. The gut–liver interactions have emerged as a critical component in the progression of alcohol-induced liver disease both in patients and in experimental models [2–4]. Increasing evidence demonstrates that the elevated portal endotoxin level is an important etiological factor in the pathogenesis of ALD [5–8]. Gut-derived endotoxins normally penetrate the gut epithelium in only trace amounts due to tightly regulated intestinal barrier defenses. Disruption of this barrier has been suggested to be a leading cause of alcohol-induced endotoxemia [9–12]. The barrier is provided by paracellular apical junction complexes, including tight junctions (TJs) and adherens junctions, located at the apical end of epithelial cells. TJs are complex assemblies of transmembrane proteins (occludin, claudins, junctional adhesion molecules), scaffolding proteins (zona occludens family, including ZO-1) and signaling proteins [13–15]. Recent studies have revealed that ethanol and its metabolite, acetaldehyde, dysregulate intestinal TJ proteins, thereby disrupting the structural integrity of the mucosal barrier [11,16]. This defect results in increased permeability of pathogenic molecules like endotoxins that translocate through the epithelium to other

Abbreviations: 4-MP, 4-methylpyrazole; ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; BHMT, betaine-homocysteine methyltransferase; CYP 2E1, cytochrome P 450 2E1; DMG, dimethylglycine; SAH, S-adenosylhomocysteine; TEER, transepithelial electric resistance; TJ, tight junctions.

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extraintestinal sites including the liver. However, the molecular mechanisms of TJ disruption are not fully understood.

We have previously shown that ethanol consumption alters hepatic methionine metabolism that impairs several essential methylation reactions resulting in the generation of many hallmark features of alcoholic liver injury [17–23]. In particular, the ethanol-induced elevations in S-adenosylhomocysteine (SAH) levels are especially detrimental as it can alter the cellular methylation potential by inhibiting numerous methyltransferases [17–19,23,24]. We have further shown that treatment with a safe and effective methylating agent, betaine, can prevent the increases in hepatocellular SAH levels via betaine-homocysteine methyltransferase (BHMT)-mediated catalysis and thereby attenuates many indices of alcoholic liver injury [17–22].

This study was undertaken to examine whether such impairment in essential methylation reaction(s) in the intestinal tract could also be responsible for the alcohol-induced dysregulation of the TJ integrity and barrier function. We also were interested in examining the effect of betaine on these parameters. To test our hypothesis, we used human colon adenocarcinoma derived cell line Caco-2 cells. These cells spontaneously differentiate into a monolayer of cells that morphologically resemble normal small intestinal enterocytes with a defined apical brush border and a highly organized TJ network [25]. This cell model has been extensively used for studying structural barrier integrity by conducting microscopic and Western blot analysis of TJ proteins and determining the functional barrier integrity using transepithelial electrical resistance (TEER) and paracellular solute flux measurements [16,26–29].

In this study, we describe the effect of ethanol and tubercidin (an agent that specifically elevates intracellular SAH levels) in disrupting TJ integrity and barrier function. We further examined whether betaine treatment could prevent both the ethanol and tubercidin-induced defects to preserve TJ integrity and barrier function. Finally, we also investigated whether mechanistically, betaine exerted its protective effects via BHMT-mediated catalysis that utilizes betaine to prevent the increase in SAH.

2. Materials and methods

2.1. Reagents

Cell culture reagents were purchased from Hyclone Laboratories (South Logan, UT). Anti Occludin-1 was purchased from Invitrogen/Life Technologies (Grand Island, NY). Anti-β actin, betaine, dimethylglycine (DMG), rhodamine dextran (70 kDa), 4-methylpyrazole (4-MP) and other specialized reagents were purchased from Sigma Chemical Co (St. Louis, MO). Anti-BHMT was from Aviva Systems Biology, Corp. (San Diego, CA). Anti-CYP2E1 was from Calbiochem/EMD Millipore (Billerica, MA). Anti-ADH was a gift from Dr. Michael Felder (University of South Carolina). CY3-conjucated goat anti rabbit was from Jackson Immuno Research (West Grove, PA). Hanks balanced salt solution (HBSS) and minimum essential medium (MEM) were purchased from Gibco/Life Technologies (Grand Island, NY).

2.2. Cell culture and treatment

Caco-2 cells from American Type Culture Collection were maintained at 37 °C in MEM supplemented with 10% (v/v) FBS, 100 units penicillin/mL and 100 μg streptomycin/mL in an atmosphere of 5% CO_2. Caco-2 cells from passages 5 to10 were used for the experiments. These cells were seeded onto 8 μM transwell polycarbonate membranes (6.5 mm insert, 24-well plate, 0.33 cm^2 growth surface) or six-well plates at the density of 4×10^4 cells per cm^2 for 21 days. Before treatments, the cells were washed and exposed to 50 mmol/L ethanol or varying concentration of tubercidin (0–10 μmol/L) in the presence or absence of 0–10 mmol/L betaine in serum-free MEM for 24 h as indicated in the text and figures. In some cases cell monolayers were also exposed to 5 mmol/L 4-MP or 10 mmol/L DMG to examine the specific role of ethanol metabolism and BHMT-mediated catalysis, respectively.

2.3. Microscope analysis

For microscopic analysis, the cells cultured on coverslips were treated as indicated and then washed and fixed in 4% paraformaldehyde. Cells were subsequently blocked in PBS containing 5% BSA for 1 h at RT followed by incubation with primary antibody (occludin-1 at 1:100 dilution) in PBS. The cells were then washed and incubated with goat anti-rabbit CY3 588, a secondary antibody, washed again and mounted on slides using Vectashield mounting medium with DAPI. All images were acquired with a Zeiss 510 Meta Confocal Laser Scanning Microscope.

2.4. Analysis of epithelial barrier function

The Caco-2 cell monolayer barrier function was assessed by measuring transepithelial electric resistance (TEER) and unilateral rhodamine dextran flux. TEER was recorded using Endohm EVOM epithelial tissue voltohmmeter (World Precision Instruments) on cell monolayers in 6.5 mm diameter transwell inserts before and after 24 h of treatment as described before [16,26–28]. Percent increase and decrease in TEER was calculated by subtracting the TEER value obtained after treatment from the TEER value obtained before treatment.

To measure paracellular permeability, the cell monolayers in the transwell inserts were washed after treatment and the medium replaced with HBSS. The cells were equibrated in this buffer for 30 min at 37 °C. The HBSS was replaced with 0.2 mL of 1 mg/mL rhodamine-dextran in HBSS in the apical compartment and 0.8 mL HBSS in the basal compartment and incubation continued at 37 °C for 1 h. After the incubation period, the apical-to-basolateral flux of the 70 kDa rhodamine-dextran was measured to determine paracellular leakage.

2.5. Preparation of detergent-insoluble fractions and Western blot

Cell monolayers were washed and lysed in buffer containing 50 mM Tris, 0.1% Triton-x 100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μg leupeptin, 10 μg pepstatin, aprotinin and bestatin. The lysate was centrifuged @ 15,600 × *g* for 4 min. The supernatant was used as detergent soluble fraction. The pellet, detergent-insoluble fraction, was sonicated in hot lysis buffer containing 20 mM Tris, 0.3% SDS, 10 mM sodium fluoride, 1 mM sodium orthovanadate and 10 μg/mL protease inhibitor cocktail. The proteins from detergent soluble and insoluble fractions were separated on 10% polyacrylamide gels and transferred on to nitrocellulose membrane. The membranes were incubated with different primary antibodies followed by incubation with specific secondary antibodies conjugated with infra-red dyes and developed using an Odyssey™ infrared imaging system (Licor, Inc.,Lutz, FL). The protein bands were quantified by densitometric analysis using Li-Cor software (Li-Cor bioscience).

2.6. Statistical analysis

Data are mean values ± SEM. We determined statistical significance by one way analysis of variance followed by Newman–Keuls post hoc analysis. A probability value (P) ≤0.05 was considered statistically significant.