Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry xx (2015) xxx–xxx

Journal of Nutritional Biochemistry

Glutamine supplementation attenuates ethanol-induced disruption of apical junctional complexes in colonic epithelium and ameliorates gut barrier dysfunction and fatty liver in mice

Kamaljit K. Chaudhry[a], Pradeep K. Shukla[a], Hina Mir[a], Bhargavi Manda[a], Ruchika Gangwar[a], Nikki Yadav[a], Megan McMullen[b], Laura E. Nagy[b], RadhaKrishna Rao[a,*]

[a]Department of Physiology, University of Tennessee Health Science Center, Memphis, TN
[b]Cleveland Clinic Foundation, Cleveland, OH

Received 6 April 2015; received in revised form 23 July 2015; accepted 8 August 2015

Abstract

Previous *in vitro* studies showed that glutamine (Gln) prevents acetaldehyde-induced disruption of tight junctions and adherens junctions in Caco-2 cell monolayers and human colonic mucosa. In the present study, we evaluated the effect of Gln supplementation on ethanol-induced gut barrier dysfunction and liver injury in mice *in vivo*. Ethanol feeding caused a significant increase in inulin permeability in distal colon. Elevated permeability was associated with a redistribution of tight junction and adherens junction proteins and depletion of detergent-insoluble fractions of these proteins, suggesting that ethanol disrupts apical junctional complexes in colonic epithelium and increases paracellular permeability. Ethanol-induced increase in colonic mucosal permeability and disruption of junctional complexes were most severe in mice fed Gln-free diet. Gln supplementation attenuated ethanol-induced mucosal permeability and disruption of tight junctions and adherens junctions in a dose-dependent manner, indicating the potential role of Gln in nutritional intervention to alcoholic tissue injury. Gln supplementation dose-dependently elevated reduced-protein thiols in colon without affecting the level of oxidized-protein thiols. Ethanol feeding depleted reduced protein thiols and elevated oxidized protein thiols. Ethanol-induced protein thiol oxidation was most severe in mice fed with Gln-free diet and absent in mice fed with Gln-supplemented diet, suggesting that antioxidant effect is one of the likely mechanisms involved in Gln-mediated amelioration of ethanol-induced gut barrier dysfunction. Ethanol feeding elevated plasma transaminase and liver triglyceride, which was accompanied by histopathologic lesions in the liver; ethanol-induced liver damage was attenuated by Gln supplementation. These results indicate that Gln supplementation ameliorates alcohol-induced gut and liver injury.

Keywords: Alcohol; Tight junction; Adherens junction; Occludin; Oxidative stress; Claudin; Cadherin

1. Introduction

Epithelial tight junctions form a selective barrier for the diffusion of solutes and macromolecules [1]. In the gastrointestinal tract, epithelial tight junctions prevent the diffusion of toxins, allergens and pathogens from the intestinal lumen into the mucosa and systemic circulation. Disruption of tight junctions and diffusion of bacterial toxins play an important role in the pathogenesis of many gastrointestinal diseases. Recent studies indicated that colonic epithelial barrier dysfunction impacts the systemic organs, beyond the gastrointestinal tract, due to impact of endotoxemia on different organ systems. One of such conditions is alcoholic liver disease (ALD), which is associated with the intestinal mucosal barrier dysfunction and endotoxemia [2,3]. Endotoxemia was detected not only in alcoholics with the symptoms of liver disease [3–5] but also in the experimental models of ALD [2,3,6–8]. Endotoxin-mediated Kupffer cell activation and subsequent inflammatory reactions are involved in the pathogenesis of ALD [9]. Therefore, alcohol-induced gut barrier dysfunction is a crucial event in the initiation and progression of ALD.

Tight junctions are multiprotein complexes consisting of transmembrane proteins such as occludin, claudins, tricellulin and junctional adhesion molecules [10]. The intracellular domains of transmembrane proteins bind to intracellular adapter proteins such as ZO1, ZO2 and ZO3 [11]. These protein complexes interact with other tight junction-specific proteins such as cingulin, AF6, 7H6 and catenins [12–14]. These protein–protein interactions are essential for the assembly and maintenance of tight junctions. Adherens junctions, the junctional complexes that lie beneath the tight junctions, are also multiprotein complexes composed of E-cadherin (the transmembrane

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; ECL, enhanced chemiluminescent; EF, ethanol fed; EGF, epidermal growth factor; ERK, extracellular receptor activated kinase; FITC, fluorescein isothiocyanate; Gln, glutamine; GF, Gln free; GN, Gln at regular dietary dose; GS, Gln supplemented; HRP, horseradish peroxidase; PF, pair fed; PMSF, phenylmethanesulfonyl fluoride; ZO-1, zona occludens-1

* Corresponding author at: Department of Physiology, University of Tennessee Health Science Center, 894 Union Avenue, Nash 426, Memphis, TN 38163, USA. Tel.: +1-901-448-3235; fax: +1-901-448-7126.
E-mail address: rrao2@uthsc.edu (R. Rao).

http://dx.doi.org/10.1016/j.jnutbio.2015.08.012
0955-2863/

protein) and catenins (the adapter proteins) [15]. Adherens junctions are not diffusion barriers for macromolecules, but they indirectly regulate the integrity of tight junctions and, therefore, the barrier function [16]. Both tight junctions and adherens junctions interact with numerous signaling proteins and are regulated by the activity of intracellular signaling cascades. Tight junction and adherens junction protein complexes interact with the actin cytoskeleton [17,18], which is essential for the assembly and maintenance of tight junctions and adherens junctions.

Factors that protect tight junctions and adherens junctions against injurious agents are of importance in developing therapeutics for the prevention and treatment of various gastrointestinal diseases. Growth factors and nutritional components are known to help maintain the gastrointestinal mucosal homeostasis and preserve the epithelial barrier function [19]. Glutamine (Gln) is one such nutritional component that plays an important role in preservation of intestinal mucosal homeostasis [20]. Gln is the most abundant amino acid in blood stream accounting for 30–35% of plasma amino acid nitrogen and is classified as a nonessential amino acid as it is readily synthesized in muscle, liver, brain and stomach tissue [20]. However, it has been relabeled as a conditionally essential amino acid, as the body depends on dietary Gln under pathophysiologic conditions. Gln is an essential source of fuel for the gastrointestinal tract [21]. Therefore, gastrointestinal tract is the first organ that responds to depletion of plasma Gln. Gln supplementation was found to be beneficial in the treatment of burn injury, malnutrition, radiation injury and critically ill patients [22,23]. Evidence indicates that Gln plays a critical role in maintenance of the intestinal epithelial barrier function [24–26]. Depletion of Gln by Gln synthase increases paracellular permeability in Caco-2 cell monolayers [27], and Gln treatment protects tight junctions and preserves barrier function from acetaldehyde in Caco-2 cell monolayers and human colonic mucosa *in vitro*[28,29].

In the present study, we evaluated the influence of dietary Gln on ethanol-induced intestinal mucosal barrier dysfunction and liver injury. Results of this study provide evidence to the beneficial effect of dietary Gln supplementation in alcoholic gut and liver injury.

2. Methods and materials

2.1. Chemicals

Maltose dextrin was purchased from Bioserv (Flemington, NJ, USA). Regular Lieber DeCarli ethanol diet (Dyet # 710260) and Gln-free diet (Dyet # 717781) were purchased from Dyets Inc. (Bethlehem, PA, USA). EnzyChrom Alanine transaminase (EALT-100) and EnzyChrom Aspartate transaminase (EAST-100) assay kits were purchased from BioAssay systems (Hayward, CA, USA). Triglyceride assay kit was purchased from Pointe Scientific Inc., (Canton, MI, USA). Hoechst 33342 dye and BODIPY FL-*N*-(2-aminoethyl) maleimide were purchased from Life technologies (Grand Island, NY, USA). N-ethylmaleimide (NEM) and tris(2-carboxyethyl)phosphine were from Sigma Aldrich (St Louis, MO, USA). All other chemicals were purchased from either Sigma Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Tustin, CA, USA).

2.2. Antibodies

Anti-ZO-1, antioccludin and anti-claudin-3 antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Anti-E-Cadherin and anti-β-catenin antibodies were purchased from BD Biosciences (Billerica, MA, USA). Horseradish peroxidase (HRP)-conjugated antimouse IgG, HRP-conjugated antirabbit IgG and anti-β-actin antibodies were obtained from Sigma Aldrich (St. Louis, MO, USA). AlexaFlour-488-conjugated antimouse IgG and Cy3-conjugated antirabbit IgG were purchased from Molecular Probes (Eugene, OR, USA).

2.3. Animals and diets

Female C57BL/6 mice (12–14 weeks, Harlan Laboratories, Houston, TX, USA) were used for all experiments. All animal experiments were performed according to the protocols approved by UTHSC Institutional Animal Care and Use Committee. Animals were housed in institutional animal care facility with 12-h light and dark cycles. All mice had free access to regular laboratory chow and water until the start of experiments.

2.4. Study protocol

In the first study, adult female (12–14 weeks age) mice were fed with Lieber-DeCarli liquid diet (#710260), with or without ethanol (0% for 2 days, 1% for 2 days, 2% for 2 days, 4% for 1 week, 5% for 1 week and 6% for 1 week), and with (GN) or without (GS) L-Gln (8.4 g/L) supplementation for 4 weeks. The diet without ethanol was supplemented with isocaloric maltose dextrin. In the second set of the studies, animals were fed Gln-free diet (Dyet # 717782), a modified Lieber-DeCarli ethanol diet, with no Gln (GF) or with L-Gln, 8.4 g/L (GN) or 16.8 g/L (GS). Corresponding control groups received isocaloric maltose dextrin in place of ethanol. Diet intake was recorded daily, and body weights were recorded twice a week. The diet intake of mice fed with ethanol-containing diet was 10–15% lower than that in mice fed with control diets. Therefore, control mice were pair fed to maintain constant diet and caloric intakes in all groups. At the end of experiment, gut permeability was measured as described below. Intestinal segments and liver were collected for further analyses.

2.5. Gut permeability in vivo

Mucosal barrier dysfunction was evaluated by measuring gut permeability to FITC-inulin (6 kDa). On the last day of experiment, mice were intravenously injected with FITC-inulin (50-mg/ml solution; 2-μl/g body weight) via tail vein. One hour after injection, blood samples were collected by cardiac puncture under isoflurane anesthesia for plasma preparation. Luminal contents from intestinal segments were flushed with 0.9% saline. Fluorescence in plasma and luminal flushing was measured using fluorescence plate reader. Fluorescence values in the luminal flushing were normalized to fluorescence values in corresponding plasma samples and calculated as percent of amount injected.

2.6. Immunofluorescence microscopy

Cryo-sections of intestine (10-μm thickness) were fixed in acetone methanol mixture (1:1) at −20 °C for 2 min and rehydrated in phosphate-buffered saline (PBS). Sections were permeabilized with 0.2% Triton X-100 in PBS for 15 min and blocked in 4% nonfat milk in TBST (20-mM Tris, pH 7.2 and 150-mM NaCl). It was then incubated for 1 h with primary antibodies (mouse monoclonal antioccludin and rabbit polyclonal anti-ZO-1 antibodies or mouse monoclonal E-cadherin and rabbit polyclonal anti-β-catenin antibodies), followed by incubation for 1 h with secondary antibodies (AlexaFluor-488-conjugated antimouse IgG and Cy3-conjugated antirabbit IgG antibodies from Molecular Probes, Eugene, OR, USA) containing Hoechst 33342. The fluorescence was examined by using a confocal microscope (Zeiss 710), and images from x to y sections (1 μm) were collected using Zen software. Images were stacked using the Image J software (NIH) and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

2.7. Preparation of detergent-insoluble fraction

Actin-rich detergent-insoluble fraction was prepared as described previously [30,31]. Mucosal scrapping from distal colon and ileum were incubated on ice for 15 min with lysis buffer-CS (Tris buffer containing 1% Triton-X100, 2-μg/ml leupeptin, 10-μg/ml aprotinin, 10-μg/ml bestatin, 10-μg/ml pepstatin-A, 10-μl/ml of protease inhibitor cocktail, 1-mM sodium vanadate and 1-mM PMSF). Briefly, mucosal lysates were centrifuged at 15,600×*g* for 4 min at 4 °C to sediment the high-density actin-rich detergent-insoluble fraction. Supernatant was used a detergent soluble fraction. The pellet was suspended in 100 μl of preheated lysis buffer-D (20-mM Tris buffer, pH 7.2, containing 10 μl/ml of protease inhibitor cocktail, 10-mM sodium fluoride, 1-mM sodium vanadate and 1-mM PMSF) and sonicated to homogenize the actin cytoskeleton and heated at 100 °C. Protein content was measured by BCA method (Pierce Biotechnology, Rockford, IL, USA). Triton-insoluble and soluble fractions were mixed with equal volume of Laemmli's sample buffer (2× concentrated), heated at 100 °C for 5 min, and 25–40-μg protein sample was used for immunoblot analysis.

2.8. Immunoblot analysis

Triton soluble and insoluble fractions were separated by SDS-polyacrylamide gel (7%) electrophoresis and transferred to PVDF membranes as described before [30,32,33]. Membranes were immunoblotted for different proteins using specific antibodies for different tight junction and adherens junction proteins with β-actin as housekeeping protein in combination with HRP-conjugated antimouse IgG or antirabbit IgG secondary antibodies. The blots were developed using ECL chemiluminescence method (Pierce) and quantitated by densitometry using Image J software. The density for each band was normalized to density of corresponding actin band.

2.9. Protein thiol assay

Protein thiols in colonic sections was assessed as described before [34]. Reduced protein thiols were evaluated by staining cryosections colon with BODIPY FL-*N*-(2-aminoethyl) maleimide (Flm) and confocal microscopy at excitation and emission wavelengths, 490 nm and 534 nm, respectively. For oxidized protein thiols, the reduced protein thiol was first alkylated with NEM followed by reduction of oxidized protein thiols with tris(2-carboxyethyl)phosphine prior to staining with Flm. Control staining is