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Occludin deficiency promotes ethanol-induced disruption of colonic epithelial junctions, gut barrier dysfunction and liver damage in mice

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ABSTRACT

Background: Disruption of epithelial tight junctions (TJ), gut barrier dysfunction and endotoxemia play crucial role in the pathogenesis of alcoholic tissue injury. Occludin, a transmembrane protein of TJ, is depleted in colon by alcohol. However, it is unknown whether occludin depletion influences alcoholic gut and liver injury.
Methods: Wild type (WT) and occludin deficient ($Ocln^{-/-}$) mice were fed 1–6% ethanol in Lieber–DeCarli diet. Gut permeability was measured by vascular-to-luminal flux of FITC-inulin. Junctional integrity was analyzed by confocal microscopy. Liver injury was assessed by plasma transaminase, histopathology and triglyceride analyses. The effect of occludin depletion on acetaldehyde-induced TJ disruption was confirmed in Caco-2 cell monolayers.
Results: Ethanol feeding significantly reduced body weight gain in $Ocln^{-/-}$ mice. Ethanol increased inulin permeability in colon of both WT and $Ocln^{-/-}$ mice, but the effect was 4-fold higher in $Ocln^{-/-}$ mice. The gross morphology of colonic mucosa was unaltered, but ethanol disrupted the actin cytoskeleton, induced redistribution of occludin, ZO-1, E-cadherin and β-catenin from the junctions and elevated TLR4, which was more severe in $Ocln^{-/-}$ mice. Occludin knockdown significantly enhanced acetaldehyde-induced TJ disruption and barrier dysfunction in Caco-2 cell monolayers. Ethanol significantly increased liver weight and plasma transaminase activity in $Ocln^{-/-}$ mice, but not in WT mice. Histological analysis indicated more severe lesions and fat deposition in the liver of ethanol-fed $Ocln^{-/-}$ mice. Ethanol-induced elevation of liver triglyceride was also higher in $Ocln^{-/-}$ mice.
Conclusion: This study indicates that occludin deficiency increases susceptibility to ethanol-induced colonic mucosal barrier dysfunction and liver damage in mice.

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1. Introduction

Epithelial cells lining the gut lumen provide the first line of defense by maintaining an intricate balance between absorption of nutrients and prevention of harmful substances from entering into internal organs. Epithelial tight junctions (TJ) partly impart this sieving capacity and maintain gut mucosal homeostasis. Dysregulation of gut homeostasis due to stress, infection, altered gut flora or immune response may lead to change or damage in TJ and *vice versa*. Such conditions are involved in the pathogenesis of various gastrointestinal and other pathologies, such as inflammatory bowel disease, fatty liver disease, viral or bacterial infection, type-1 diabetes and allergies among many others. Alcoholic liver disease is associated with disruption of TJ, increased gut permeability and endotoxemia [1,2]. Although the disruption of and barrier dysfunction in Caco-2 cell monolayers by ethanol or its metabolic product, acetaldehyde, has been addressed by us and others [3–6], the mechanistic aspect of intestinal mucosal barrier dysfunction and increased gut permeability in mice and rats by ethanol feeding has not been defined yet [1,2]. It is essential to understand the mechanisms involved in alcohol-mediated TJ disruption and barrier dysfunction in order to understand the pathogenesis of alcoholic diseases and design of novel therapeutics.

Occludin is one of the transmembrane proteins of TJ. A single occludin gene exhibits alternative splicing resulting in four splice variants [7] which are under tight regulation at the post transcriptional level [8]. The C-terminal domain of occludin is highly conserved, the phosphorylation status of which is important in TJ assembly and disassembly in different epithelia under varying physiologic/pathophysiologic conditions [9–13]. Occludin down regulation in keratinocytes decreases cell–cell adhesion, Ca^{2+} homeostasis and reduces susceptibility of these cells to

Abbreviations: ALT, alanine aminotransferase; Cldn, claudin; ECL, enhanced chemiluminescent; EF, ethanol fed; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LPS, lipopolysaccharide; $Ocln^{-/-}$, occludin gene knockout; PF, pair fed; shRNA, short hairpin RNA; TER, transepithelial electrical resistance; WT, wild type; ZO-1, zonula occludens-1.

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apoptosis [14]. Interference with differential occludin expression during follicular development suppresses follicular growth in primates [15]. Another study using cell lines of different origin shows critical role of occludin in regulating polarized migration during wound healing [16]. Occludin is required for recruiting aPKC-Par3/PA-TJ complex to the leading edge and is crucial for activation of PI3K and lamellopodia formation during cell migration. Proteasomal degradation of occludin has been associated with the pathophysiology of irritable bowel syndrome [17]. However, surprisingly, occludin knockout mice ($Ocln^{-/-}$ mice) showed no apparent anomaly in the intestinal epithelial TJ [18]. These mice were not challenged, and therefore, there is no information present whether occludin deficient mice are susceptible or resistant to challenges relevant to physiologic and pathophysiologic conditions.

Ethanol and acetaldehyde treatment induces redistribution of occludin from the intercellular junctions of intestinal epithelium *in vivo* and *in vitro* leading to disruption of TJ and barrier dysfunction [3,5,19,20]. Experimental studies indicate that ethanol feeding depletes occludin in colonic epithelium [21–23]. However, the significance of occludin depletion *per se* in alcoholic barrier dysfunction is unclear. We hypothesized that initial depletion of occludin would sensitize intestinal epithelium to ethanol-induced increase in gut permeability and liver damage. Therefore, we evaluated the susceptibility of $Ocln^{-/-}$ mice to ethanol-induced gut barrier dysfunction as well as liver damage. The effect on the intestinal epithelium was confirmed in Caco-2 cell monolayers, a cell culture model of the intestinal epithelium.

2. Materials and methods

2.1. Chemicals

Maltose dextrin, feeding tubes and holders were purchased from Bioserv (Flemington, NJ). Lieber DeCarli liquid diet (Dyet #717780) was purchased from Dyets Inc. (Bethlehem, PA). EnzyChrom Alanine transaminase (EALT-100) assay kit was purchased from BioAssay systems (Hayward, CA). Triglyceride reagent kit set was purchased from Pointe Scientific Inc., (Canton, MI). Hoechst 33342 dye was from Life technologies (Grand Island, NY). All other chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Tustin, CA).

2.2. Antibodies

Anti-ZO-1 (rabbit polyclonal), anti-occludin (mouse monoclonal), anti-claudin-2 (mouse monoclonal) and anti-claudin-3 (rabbit polyclonal) antibodies were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-toll like receptor-4 (TLR4) antibody was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-E-cadherin (mouse monoclonal) and anti-β-catenin (rabbit polyclonal) antibodies were purchased from BD Biosciences (Billerica, MA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG and anti-β-actin (mouse monoclonal) antibodies were obtained from Sigma-Aldrich. AlexaFlour-488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR).

2.3. Animals

All animal experiments were performed according to the protocol approved by the University of Tennessee Health Science Center (UTHSC) Institutional Animal Care and Use Committee (IACUC). $Ocln^{-/-}$ mice (mixed background), generated as described before [24], were bred and the progeny genotyped to obtain wild type and $Ocln^{-/-}$ mice. Animals were housed in institutional animal care facility with 12-h light and dark cycles, and were fed regular laboratory chow until the start of experiments. Both male and female mice were used for this study. Although female mice were slightly more sensitive to ethanol effects, we had to use mice of both gender, due to limited availability of $Ocln^{-/-}$ mice. Due to infertility of male $Ocln^{-/-}$ mice heterozygote male and female mice were used for breeders. The total numbers of mice used in 3 independent experiments were 12 (6 males + 6 females), 15 (7 males + 8 females), 15 (7 males + 8 females), and 11 (5 males + 6 females) for Wild type-Pair fed, Wild type-Ethanol fed, $Ocln^{-/-}$ Pair fed and $Ocln^{-/-}$ Ethanol fed groups, respectively. All mice had free access to standard rodent diet and water before the study.

2.4. Ethanol feeding

Wild type and $Ocln^{-/-}$ mice (10–14 wk. old) were fed Lieber–DeCarli liquid diet containing ethanol (1% 2 days, 2% 2 days, 4% 1 week, 5% 1 week and 6% 1 week). Control mice were pair fed with isocaloric maltodextrin. To ensure similar diet intake all groups were pair fed. Diet intake and body weight were recorded. In all experiments animals were maintained in pairs to facilitate body temperature maintenance. The ethanol-feeding model used in this study is the same as the one previously used by others [21] and us [25,26]. Ethanol concentration at 6% corresponds to 32% dietary calorie, which is in the range of ethanol-derived calorie consumption by alcoholics.

2.5. Gut permeability in vivo

At the end of 4 weeks of ethanol feeding, mice were intravenously injected with FITC-inulin (50 mg/ml solution; 2 μl/g body weight) *via* tail vein using a restrainer. One hour after injection, blood samples were collected by cardiac puncture under isoflurane anesthesia to prepare plasma. Mice were euthanized by cervical dislocation under isoflurane anesthesia. Luminal contents of intestinal segments were flushed with 0.9% saline. Fluorescence in plasma and luminal flushing was measured using a fluorescence plate reader. Fluorescence values in the luminal flushing were normalized to fluorescence values in corresponding plasma samples.

2.6. Cell culture and transfection

Caco-2 cells (ATCC, Rockville, MD) were passaged under standard cell culture conditions as described before [27]. The shRNA specific for human occludin and control RNA were purchased from Origene (Rockville, MD; Cat#TL311039). Cells grown in 6-well plates for 24 h showing ~75% confluence were transfected using serum-free Opti-MEM®, 1.0 μg shRNA constructs and 3.15 μl of Lipofectamine® LTX with plus reagent as described previously [28]. Cells were seeded onto transwell inserts (6.5 mm diameter). Experiments were performed on day 3 after seeding. Knockdown was confirmed by immunoblot analysis as described below.

2.7. Acetaldehyde treatment

Acetaldehyde (200 μM) treatment was performed as previously described [3]. Cells were pre-incubated in PBS containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, bovine serum albumin and glucose for one hour. Then cells were exposed to vapor-phase acetaldehyde to achieve 200 μM concentration in sealed culture plates (25).

2.8. Analysis of barrier function TJ integrity

Barrier function was evaluated by measuring transepithelial electrical resistance (TER) and unidirectional flux of FITC-inulin.

2.8.1. Transepithelial electrical resistance (TER)

TER was measured as described previously (25) by using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). Basal TER of supporting semi-permeable membrane of transwells was subtracted from all values (80–100 Ohms/cm^2). The baseline TER on day-3 post