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Alteration in intestine tight junction protein phosphorylation and apoptosis is associated with increase in IL-18 levels following alcohol intoxication and burn injury

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ABSTRACT

Intestinal mucosal barrier is the first line of defense against bacteria and their products originating from the intestinal lumen. We have shown a role for IL-18 in impaired gut barrier function following acute alcohol (EtOH) intoxication combined with burn injury. To further delineate the mechanism, this study examined whether IL-18 alters intestine tight junction proteins or induces mucosal apoptosis under these conditions. To accomplish this, rats were gavaged with EtOH (3.2 g/kg) prior to ~12.5% total body surface area burn or sham injury. One day after injury, EtOH combined with burn injury resulted in a significant decrease in total occludin protein and its phosphorylation in small intestine compared to either EtOH or burn injury alone. There was no change in claudin-1 protein content but its phosphorylation on tyrosine was decreased following EtOH and burn injury. This was accompanied with an increase in mucosal apoptosis ($p<0.05$). The treatment of rats with anti-IL-18 antibody at the time of burn injury prevented intestine apoptosis and normalized tight junction proteins following EtOH and burn injury. Altogether, these findings suggest that IL-18 modulates tight junction proteins and cause apoptosis leading to impaired intestinal mucosal integrity following EtOH intoxication combined with burn injury.

1. Introduction

The intestine is the second largest immunological organ in the body. It has a large surface area with multiple functions. One of the primary functions of intestine is to absorb nutrition. Another major task for the gut is to maintain a local barrier which prevents the translocation of bacteria and endotoxin contained within the intestinal lumen to the extra-intestinal sites. The intestinal barrier is mainly formed by a layer of epithelial cells joined together by tight junction (TJ). TJ is a complex of membrane-bound proteins (e.g., occludin and claudins) and their adaptor and scaffolding proteins (e.g., junctional adhesion molecule, ZO-1, ZO-2 and ZO-3 [1,2]. These proteins form a structure at the boundary of two adjacent cells working as a barrier within the epithelial cell space [2]. The TJ proteins are the rate-limiting step in the paracellular pathway and form a selectively permeable barrier to the solutes, fluid and other nutrition elements as well as the bacterial movement across the intestinal mucosa [3]. Therefore, an intact intestinal epithelial barrier plays a critical role in maintaining the normal physiological function and protecting from gut-derived pathogens. In addition to the physical epithelial barrier, the secretion of immunoglobulin A (IgA) is another important defense factor on mucosal surfaces [4,5]. IgA is secreted by mucosal plasma cells residing under epithelial cells. Following release, IgA is coated on intestinal epithelium to prevent adherence of bacteria to mucosal surface. IgA can also neutralize toxin, regulate the microbial environment of intestine, and prevent local inflammation [4,5].

Several lines of evidence indicate that intestine barrier is impaired following major trauma; burn injury as well as alcohol/ethanol (EtOH) exposure [6–9]. Nearly, one million burn injuries are reported every year within the United States and almost half of these injuries are reported to occur under the influence of EtOH [10–14]. Studies have also indicated that the intoxicated patients require frequent intubations, experience delayed wound healing and longer hospital stay. The intoxicated patients were found to be more susceptible to infection and had significantly higher mortality rate compared to burn patients who were not intoxicated at the time of injury. In addition, intoxicated patients died of smaller burns [11–15]. Similar findings were obtained in experimental models of EtOH and burn injury [7,14,16,17]. We have shown that a single dose of EtOH or a

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minor burn injury alone was not able to produce severe adverse effects in the intestine; however, when the EtOH and minor burn injury were combined, they caused intestinal tissue damage, leakiness, and a significant increase in bacterial translocation [18–21]. This was accompanied with an increase in intestinal IL-18 levels [18–21]. IL-18, a proinflammatory cytokine, belongs to IL-1 cytokine superfamily. It is synthesized as a precursor protein (pro-IL-18) which in the presence of IL-1β-converting enzyme (ICE, or caspase-1) matures into 18-kDa active protein [22–26]. It is produced by macrophages, dendritic cells, neutrophils, and epithelial cells. IL-18, like IL-12 was discovered initially to be a factor that drives T cell towards Th1 cells as an IFN-γ-inducing factor [22–26]. However, later studies have indicated that IL-18 induces tissue damage in inflammatory bowel disease, arthritis and sepsis [22,23,25–28]. In our previous studies we have shown that IL-18 plays a role in increased gut leakiness following EtOH and burn injury. We also showed that IL-18 plays a key role in increased neutrophil recruitment to the intestine and the lung following EtOH intoxication and burn injury [18–21]. However, the mechanism by which IL-18 causes gut leakiness following EtOH and burn injury remains largely unknown. This study examined whether IL-18 alters intestine tight junction proteins or induces mucosal apoptosis as changes in any of these parameters may cause gut leakiness following EtOH intoxication and burn injury.

2. Materials and methods

2.1. Animals and reagents

Male Sprague–Dawley rats (250–275 g) were obtained from Charles River Laboratories (Wilmington, MA). Anti-occludin antibody and anti-claudin-1 antibody were obtained from Invitrogen (Carlsbad, CA). Anti-cleaved caspase-3 antibody, anti-phospho tyrosine antibody ($pTyr^{100}$) and anti-phospho threonine antibody (p-Thr-polyclonal) were obtained from Cell Signaling (Danvers, MA). Anti-rat IL-18 antibody was obtained from R&D Systems (Minneapolis, MN). Cell death detection ELISA kit was obtained from Roche Applied Science (Indianapolis, IN).

2.2. Rat model of acute EtOH intoxication and burn injury

As described previously [18,21], rats were randomly divided into four groups: sham vehicle, sham EtOH, burn vehicle and burn EtOH. In EtOH treated groups, blood EtOH levels in the range of 90–100 mg/dL were achieved by gavage feeding of 5 ml of 20% EtOH (~3.2 g/kg body weight) in water. In vehicle group, animals were gavaged with 5 ml of water. Four hours after gavage, all animals were anesthetized and transferred into a template fabricated to expose ~12.5% of the total body surface area. Animals were then immersed in a boiling water bath (95–97 °C) for 10–12 s. Sham-injured rats were subjected to identical anesthesia and immersed in lukewarm water (37° C) for 10–12 s. The animals were dried immediately and resuscitated intraperitoneally with 10 ml of physiological saline. After recovery from anesthesia, the animals were returned to their cages and allowed food and water *ad libitum*. In some experiments, rats were treated intraperitoneally with anti-IL-18 antibody (100 μg/kg BW) or same amount of isotype IgG (Santa Cruz Biotechnology, Santa Cruz, CA) immediately after burn injury. One day after injury, rats were sacrificed.

All the experiments were carried out in adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. These studies were initiated at the University of Alabama at Birmingham (UAB) and were approved by UAB and Loyola University Chicago Stritch School of Medicine Institutional Animal Care and Use Committees.

2.3. Preparation of mucosal homogenates

Leaving approximately the first 15 cm proximal segment of intestine, a 20 cm long small intestine piece was horizontally opened and cleaned. Mucosa was removed by scraping the intestine with a glass slide, suspended in 1 ml lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 0.5% Triton X-100, 10% glycerol and protease inhibitor cocktail (Sigma Chemical Co. St. Louis, MO), and sonicated twice for 10 s on ice [18,20]. Homogenates were cleared by centrifuging at 10,000 rpm for 15 min at 4° C, and supernatants were collected and stored at −70 °C until use. Protein levels in the homogenates were measured by BioRad assay kit (Hercules, CA).

2.4. Immunofluorescence localization of intestinal occludin and claudin-1 protein expression

About 1.0 cm long segment of last part of ileum was fixed in 10% formalin and sent to Histology Laboratory at Loyola University Medical Center where they were embedded in paraffin, and cut into ~5 μm thick sections. After dewaxing and rehydrating, the antigenic site retrieval of the sections was accomplished by boiling slides for 20 min in 0.01 M citric acid buffer (pH 6.0) [29]. Nonspecific binding sites were blocked with 5% goat serum for 2 h. The sections were incubated with rabbit anti-occludin or rabbit anti-claudin-1 antibodies for 2 h at room temperature. The sections were washed in PBS and incubated with goat anti-rabbit IgG conjugated with Alexa Fluor® 488 or Texas Red® (Invitrogen) for 1 h at room temperature. For nuclear staining, the sections were incubated with Hoechst (Invitrogen) for 2 min at room temperature. The sections were washed again, covered with cover slides by using gel mounting media (Fluoro-gel, Electron Microscopy sciences, Hatfield, PA) and stored in the dark at 4 °C. The distribution of occludin and claudin-1 were examined by using a Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss MicroImaging Inc. Thornwood, NY). For non specific staining controls, the sections were stained directly with secondary antibody (e.g. fluorescent labeled anti goat anti-rabbit IgG). We did not find any fluorescence signal in sections stained directly with secondary antibody.

2.5. Phosphorylation of mucosal occludin and claudin-1

To accomplish this, first occludin and claudin-1 proteins were immunoprecipitated from the mucosal homogenates using their respective antibodies as described previously in our study [30]. Briefly, equal amount of protein from mucosal homogenates was incubated with anti-occludin or anti-claudin-1 antibody for 1 h at 4 °C and then incubated with Protein G-Sepharose beads for another 2 h at 4 °C. The beads were washed five times with wash buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 0.5% Triton X-100 and centrifuged at 10,000 rpm for 3 min at 4° C. Bound proteins were analyzed by SDS-PAGE and transferred to Immobilon P membranes. Membranes were probed with anti-phospho-tyrosine antibody and were re-blotted with anti-phospho-threonine antibody after stripping [21,30,31]. Then membranes were stripped again and re-blotted with antibodies to occludin or claudin-1 to confirm the respective protein levels. Representative blots shown in the result section come from the same membrane which may have more samples in various groups.

2.6. Measurement of mucosal apoptosis and cleaved caspase-3 activity

The mucosal apoptosis was assessed by measuring cytoplasmic histone-associated DNA fragments (mono-and oligonucleosomes) using ELISA kit. The apoptosis in the intestinal mucosa was further confirmed by measuring caspase-3 activity by Western Blot analysis