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Intestinal alkaline phosphatase administration in newborns is protective of gut barrier function in a neonatal necrotizing enterocolitis rat model

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Abstract

Background: Previously, we have shown that supplementation of intestinal alkaline phosphatase (IAP) decreased severity of necrotizing enterocolitis (NEC)–associated intestinal injury. We hypothesized that IAP administration is protective of intestinal epithelial barrier function in a dose-dependent manner.

Methods: Control rat pups were vaginally delivered and breast-fed. Premature rats were divided into 4 groups: formula fed with lipopolysaccharide and hypoxia (NEC) or additional daily bovine IAP 40, 4, or 0.4 U/kg (NEC + IAP 40 U, IAP 4U, or IAP 0.4 U).

Results: Necrotizing enterocolitis is associated with decreased IAP protein expression and activity. Supplemental IAP increases IAP activity in intestinal homogenates and decreased NEC injury score in a dose-dependent manner. Intestinal injury as measured by fluorescein isothiocyanate–dextran flux from ileal loops showed increased permeability vs control, but supplemental IAP reversed this. Tight junction proteins claudin-1, claudin-3, occludin, and zonula occludin 1 were elevated in the NEC and IAP-treated groups with differences in expression patterns. No differences in messenger RNA levels were observed on postinjury day 3. Intestinal alkaline phosphatase administration decreases intestinal NEC injury in a dose-dependent manner.

Conclusion: Early enteral supplemental IAP may reduce NEC-related injury and may be useful for preserving the intestinal epithelial barrier function.

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Necrotizing enterocolitis (NEC) is a multifactorial neonatal gastrointestinal disease occurring in 0.3 to 2.4 per 1000 live births and is one of the most common causes of neonatal and infant death in premature newborns [1-5]. Despite recognition of risk factors including prematurity, ischemia, formula feeding, and bacterial colonization of the intestine, little can be done to mitigate the disease progression once developed.

Normal intestinal function depends on establishment and maintenance of distinct epithelial lined compartments [6,7]. Tight junction (TJ) proteins interact between adjacent cells to hold epithelial cells together. There are multiple families of TJ proteins, including the transmembrane proteins occludin, claudin, and junctional adhesion proteins. In addition, zonula occludin proteins (ZO-1) function as adaptors between the transmembrane proteins and the cell cytoskeleton [8-10]. In the neonatal intestine, occludin and claudins are crucial for creating and maintaining a functional epithelial intestinal barrier [11]. Failure of the gut barrier function is known to cause systemic inflammation from translocation of bacterial products such as lipopolysaccharide (LPS), progressing to a clinical picture of sepsis and multiple organ dysfunction [12,13]. Thus, preservation of the intestinal barrier implies prevention of the systemic inflammatory response because of translocation.

Inflammatory diseases of the intestine such as NEC alter TJs and lead to increased barrier permeability [14]. In preterm infants, several common stressors associated with prematurity, such as intermittent hypoxia, infections, and treatment with indomethacin and glucocorticoids, may compromise TJs and thus epithelial integrity [15]. Altered expression and localization of claudin and occludin proteins in NEC is associated with increased intestinal permeability [11,16].

Intestinal alkaline phosphatase (IAP), a member of the family of alkaline phosphatases, is an endogenous brush border enzyme expressed in the intestines. Intestinal alkaline phosphatase has been shown to detoxify LPS by dephosphorylating the lipid A moiety, thereby preventing it from binding the TLR4 receptor. It has been shown that in the absence of IAP, intestinal inflammation is exacerbated and that replacement or supplementation prevents intestinal inflammation and endotoxemia [17-20]. Our previous work has demonstrated that enteral supplemental IAP reduced the severity of histologic NEC-associated injury [21]. We hypothesized that the protective effect of IAP may be a result of preservation of the gut barrier function and that supplementation of IAP in formula will prevent intestinal injury in a dose-dependent fashion.

The aim of this study was to investigate in a neonatal rat model of NEC whether supplemental, enteral IAP led to increased IAP activity, decreased NEC-related intestinal injury, prevented the increased gut permeability associated with NEC, and effected the expression of TJ proteins. In addition, we sought to determine if these effects of IAP were dose dependent.

1. Materials and methods

1.1. Animal model

Animal experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Control litters of full-term Sprague-Dawley rat pups (Harlan Laboratories, Madison, WI) were spontaneously delivered and dam fed. To induce NEC, a modified in vivo model was performed [22-24]. Preterm pups were delivered 1 day early by cesarean delivery and split into 4 experimental groups. All NEC pups were maintained in an incubator at 37°C and gavage fed formula with LPS 2 mg/kg LPS per feed via an orogastric tube (1 mg LPS in 1.0 mL PBS, *Escherichia coli*; Sigma-Aldrich, St. Louis, MO) [21]. Of the 4 NEC groups, 3 additionally received bovine IAP generously donated by AM-Pharma, Netherlands. The 3 groups received 0.4, 4, or 40 U/kg of IAP mixed with formula once daily. Intestinal alkaline phosphatase was administered enterally in formula in the absence of LPS to avoid IAP from inhibiting LPS ex vivo.

On day of life (DOL) 4, all surviving pups were euthanized using intraperitoneal injections of a ketamine and xylazine mixture (100 mg/kg, 10 mg/kg) followed by creation of a pneumothorax. Intestines were dissected from the mesentery, were placed in 1 mL MOPS buffer [25], homogenized while on ice, and centrifuged, and supernatant was removed for IAP activity assay. Alternately, ileal sections were placed in sucrose-based potassium phosphate buffer for Western blot. A short segment of terminal ileum was fixed in formalin (Richard-Allan Scientific, Kalamazoo, MI). Hematoxylin and eosin staining was performed on 4-μm-thick sections. Tissues were graded using a 0- to 4-point scale previously described [23,24,26].

1.2. Western blot

Protein was quantified from each homogenate using the bicinchoninic acid reagent per the manufacturer's protocol (Pierce, Rockford, IL). As described [21] with the following modifications, equal volumes of 5× Laemmli sample buffer (Bio-Rad, Hercules, CA) and 20% βME (Sigma-Aldrich) were added to each protein sample. Buffer was used to bring the total volume to 15-μL. Primary antibodies used were rabbit, anti–Z0-1 (1:250; Invitrogen), antioccludin (1:250; Invitrogen), anti–claudin-1 (1:170; Invitrogen) and anti–claudin-3 (1:500; Abcam), and mouse monoclonal anti–β-actin antibody to control for loading (1:7500; Santa Cruz). Secondary antibodies (goat antirabbit IgG HRP [1:500] and goat antimouse IgG HRP [1:75000; Santa Cruz]) were incubated for 1 hour at room temperature. Bands were detected using SuperSignal West Pico or West Dura Chemiluminescent Substrate according to the manufacturer's instructions (Thermo Scientific, Rockford, IL). Western blot band intensities were quantified using UN-