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Intestinal alkaline phosphatase prevents the systemic inflammatory response associated with necrotizing enterocolitis

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Abstract

Background—Necrotizing enterocolitis (NEC) is the most common surgical emergency in neonates, with an incidence of 0.5–2.4 cases per 1000 live births and a mortality rate between 10% and 50%. Neonates affected by NEC develop a septic injury that is associated with increased risk of neurological impairment due to intraventricular bleeding and chronic lung disease. Intestinal alkaline phosphatase (IAP) is an endogenous protein that has been shown to inactivate the endotoxin lipopolysaccharide (LPS), and has recently been used successfully as an adjunct to treat sepsis in adult patients. We tested the hypothesis that systemic, exogenous IAP will mitigate the inflammatory response as measured by serum levels of proinflammatory cytokines in a rat model of NEC.

Methods—Newborn Sprague-Dawley rats were divided into groups. Control pups were dam fed. NEC was induced by feeding formula containing LPS and exposure to intermittent hypoxia. NEC pups were given intraperitoneal injections of 4 or 40 glycine units (U) of IAP or placebo twice daily. Intestine and serum was collected for cytokine analysis as well as measurement of alkaline phosphatase activity.

Results—Systemic IAP administration significantly increased serum alkaline phosphatase activity in a dose- and time-dependent fashion. The proinflammatory cytokines tumor necrosis factor α , interleukin 6, and interleukin 1 β were significantly increased in NEC rats *versus* controls on days 2 and 3. Importantly, treatment with 40 U systemic IAP decreased these proinflammatory cytokines back to near-control levels.

Conclusions—Systemic IAP administration appears effective in mitigating the systemic inflammatory response associated with NEC, and may prove to be a valuable adjunctive treatment for NEC.

Keywords

Necrotizing enterocolitis; Intestinal alkaline phosphatase; Lipopolysaccharide

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1. Introduction

Necrotizing enterocolitis (NEC) is the most common surgical emergency in the neonatal intensive care unit, with an incidence of 0.5–2.4 cases per 1000 live births [1–4]. With improvements in care for premature and very low birth weight infants (<1500 g), the overall survival for these preterm newborns has increased past the immediate postnatal period. As a consequence, the number of neonates with the highest risk of NEC development has increased. The incidence of NEC in these very low birth weight populations reaches 7%–10% of live births and has not changed over the past 2 decades [5]. Most concerning is that NEC is associated with a mortality rate between 10% and 50% as well as a significantly increased risk for short bowel syndrome, intracranial hemorrhage, impairment of neurodevelopment, and respiratory distress syndrome in its survivors [4,6–8]. Neonates affected by NEC also have hospital stays and associated costs that are 60 d and \$186,000 greater than neonates without NEC. It is estimated that this translates into \$5 billion annually for the initial treatment of NEC in the United States [9]. Despite the devastation that NEC causes, and the research dollars dedicated to studying it, our understanding of its pathophysiology, prevention, and treatments is incomplete [3,6].

Intestinal alkaline phosphatase (IAP) is an enzyme produced by apical enterocytes and secreted into the luminal brush border of the intestine in response to enteral feeding [10,11]. The IAP enzyme is localized to the upper intestine *in vivo* and has its greatest activity in the duodenum. IAP is thought to be involved in gut mucosal defense, mainly through the detoxification of lipopolysaccharide (LPS). It does so by dephosphorylation of the lipid A moiety of LPS [10–13]. It has also been shown to affect bicarbonate secretion and adenosine metabolism, which may also have beneficial protective effects [14,15]. We previously reported a protective role of supplemental enteral IAP in the setting of experimental NEC. Interestingly, recent research has demonstrated in both animal models and human patients with sepsis that systemic IAP could mitigate the septic response and end-organ injury [12,13,16–18].

Systemic inflammation and sepsis are common in NEC and contribute significantly to its morbidity and mortality. We hypothesize that systemic IAP may also mitigate the inflammatory response in an NEC model.

2. Materials and methods

2.1. Animal model

All animal protocols were approved by the Institutional Animal Care and Use Committee and followed a well-described neonatal rat model of NEC [19–21]. Litters of full-term Sprague-Dawley rat pups (Harlan Laboratories, Madison, WI) were split into experimental groups after birth. Control pups remained with the mother, were dam fed, and were kept at normoxia and normothermia. To induce NEC, the remaining groups of experimental pups were housed separately from the mother in an Air-Shields T-100 infant transport incubator (Soma Technologies, Bloomfield, CT) set at 37°C. They were exposed to hypoxia by placing them in a room-temperature Praxair Biosperix (Lacona, NY) chamber, and subjected to 5%

oxygen for 10 min, three times daily. Additionally, they were gavage-fed transorally with 0.2 mL formula three times per day using a 24-gauge blunt-tipped angiocatheter (Instech Solomon, Plymouth Meeting, PA). Neonatal rats were fed a base formula of Similac infant formula (4 g; Abbott Laboratories, Columbus, OH) in Esbilac canine milk replacement (20 mL; PetAg Inc, Hampshire, IL). The formula was supplemented with LPS (1 mg in 1.0 mL phosphate-buffered saline [PBS], *Escherichia coli*; Sigma-Aldrich, St Louis, MO), resulting in administration of 2 mg/kg LPS per feed.

2.2. Intraperitoneal treatment of NEC animal model

The IAP groups received two 100- μ L intraperitoneal (IP) injections into the RLQ using a 27.5-gauge needle. The injections consisted of either 4 or 40 glycine units of bovine IAP suspended in PBS 1 h prior to the first and last feeding each day (7:00 AM and 3:00 PM). After injection, light pressure was applied to the injection site for 60 s with sterile gauze to ensure that the injected fluid remained in the peritoneal cavity. The NEC and control groups received two 100- μ L injections of PBS with no IAP at these same time points. The pups were sacrificed on days 1, 2, and 3 of life using IP injections of a ketamine and xylazine mixture (100 mg/kg, 10 mg/kg). Thoracotomies were performed and whole blood was collected via cardiac puncture. Whole blood samples were centrifuged for 10 min at 5000 RPM and 4°C. The serum was stored at -80°C for later use to measure serum cytokines. Next, laparotomies were performed and the entire intestinal tract from the distal esophagus to distal rectum was removed. Enteric contents were gently evacuated from the lumen and the intestinal tissue was placed in fresh Hanks balanced salt solution (HBSS; Sigma-Aldrich, St Louis, MO) on ice. A short segment of terminal ileum from the pups was harvested for histology grading of NEC. Tissues were fixed in zinc formalin (Richard-Allan Scientific, Kalamazoo, MI) processed, and subsequently embedded in paraffin. Hematoxylin-eosin staining was performed on 4- μ m-thick sections. Tissues were graded using a 4-point scale previously described.²² The entire intestinal mesentery was then carefully dissected from the bowel and discarded. The remaining intestinal tissue was placed in fresh HBSS on ice and subsequently used for alkaline phosphatase (AP) activity analysis.

2.3. Protein quantification

Intestines from adult rats and neonatal rat pups were placed in fresh HBSS (500 mL) in a 2-mL microfuge tube (Axygen Scientific, Union City, CA). The tissue was cut into small pieces using alcohol-treated fine scissors and homogenized while on ice (three bursts, 10 s each burst) using a hand-held Polytron PT 1200 E homogenizer (Kinematica, Inc, Bohemia, NY). Homogenates were lysed with MOPS buffer (500 mL) containing protease and phosphatase inhibitors (Sigma-Aldrich). The tubes were centrifuged at 14,000 rpm for 10 min at 4°C to pellet cell debris. The supernatant was removed and saved for protein quantification, AP activity assay, and Western blot analysis. Protein content was quantified using the bicinchoninic acid reagent per the manufacturer's protocol (Pierce Chemical Company, Rockford, IL).

2.4. Alkaline phosphatase activity assay

Because of the lack of commercially available IAP antibodies that react with rat, our experiments used tissue-nonspecific AP antibodies when analyzing intestinal tissue. AP

activity was measured using a colorimetric assay kit (BioVision, Mountain View, CA). Equal amounts of protein (0.5 µg) from the lysate of each intestinal tissue sample and equal volumes of serum (5 µL of a 1:5 dilution) were analyzed according to the manufacturer's protocol. Absorbance was read at 405 nm on a 96-well plate reader. Activity was calculated in U/mg for intestine and U/mL for serum.

2.5. Serum cytokine analysis

Plasma concentrations of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor α (TNF-α) were measured by using a Bio-Plex human cytokine assay kit (Bio-Rad Laboratories, Inc, Hercules, CA).

2.6. Statistical analysis

Statistical analysis was performed on all data using the paired Student *t*-test and analysis of variance with *post hoc* test where appropriate, with a *P* value 0.05 considered to be significant.

3. Results

3.1. Systemic IAP does not prevent intestinal injury

In contrast to our previous research that demonstrated enteral IAP was effective in preventing intestinal injury in our NEC model, systemic IAP did not have any apparent effect in preventing NEC-related intestinal injury. Both NEC and the two IAP-treated groups averaged a histologic NEC score greater than 2 (2.25, 2.06, and 2.09, respectively, Fig. 1). Similarly, in contrast to what we previously found with enteral supplementation of IAP, the pups treated with systemic IAP did not have an increase in intestinal AP activity, as shown in Figure 2.

3.2. Systemic IAP increased serum AP levels

To determine if IP administration of IAP was effectively entering the circulation, we measured AP activity of serum from healthy pups on days 1–3. As shown in Figure 3, treatment with IP IAP significantly increased serum AP activity in a dose-dependent fashion on each day.

3.3. NEC is associated with increased serum cytokine expression

To determine if increased serum levels of AP had any effect on the systemic inflammatory response, we then measured the serum levels of TNF-α, IL-6, IL-1β, and IL-10 in control and NEC pups. As shown in Figure 4A–C, we found that the proinflammatory cytokines (measured in pg/mL) TNF-α and IL-6 were significantly increased in NEC rats as compared with controls on days of life (DOL) 2 and 3 (TNF-α: 3-fold increase on DOL 2 and 2-fold on DOL 3; IL-6: 6-fold on DOL 2 and 5-fold on DOL 3) (*P* 0.05 for all). IL-1β levels were also increased in NEC rats compared with controls on DOL 3 (2-fold), *P* 0.05.

3.4. Systemic IAP decreased serum cytokine expression

Importantly, we found that treatment with 40 U of systemic IAP not only increased serum AP levels, but also decreased these proinflammatory cytokines (measured in pg/ml) back to near-control levels (TNF- α : 297 on DOL 2 and 993 on DOL 3; IL-6: 1380 on DOL 3; IL-1b: 2930 on DOL 3) ($P = 0.05$ for all). Although not statistically significant, there was a trend for 40 U of systemic IAP to decrease IL-10 levels in NEC (Fig. 4D).

4. Discussion

The results of our experiments demonstrate that increased serum AP levels as a consequence of systemic IAP administration were associated with mitigation of the systemic inflammatory response in our NEC model. Interestingly, this occurred without preventing intestinal injury in our NEC model or increasing the intestinal AP levels that we have previously demonstrated with enteral IAP [22–24]. Our results are novel for NEC, and raise the possibility that systemic IAP could be used to treat NEC-associated sepsis, similar to what was demonstrated to be effective in adult human clinical trials [25].

Though its causes are still largely unknown, the consensus is that the development of NEC is multifactorial, with a combination of genetic predisposition, intestinal immaturity, formula feeding, bowel ischemia, and abnormal colonization of the immature gut leading to the uncontrolled inflammatory response that is seen in NEC [3,4,6]. The only consistent risk factors for the development of NEC are prematurity and enteral feeding, suggesting that development of NEC is related to immaturity of the alimentary tract. A potential strategy to prevent NEC is to prevent the interaction between LPS and its receptor Toll-like receptor 4 [26,27]. IAP is an enzyme produced by apical enterocytes and secreted into the luminal brush border of the intestine in response to enteral feeding. IAP is thought to be involved in gut mucosal defense, mainly through the detoxification of LPS [10]. It does so by dephosphorylation of the lipid A moiety of LPS [10–12,16]. It has also been shown to affect bicarbonate secretion and adenosine metabolism, which may also have beneficial preventative effects [14,15]. The IAP enzyme is localized to the upper intestine *in vivo* and has its greatest activity in the duodenum [28]. We previously reported a protective role of supplemental IAP in the setting of experimental NEC [22–24]. Prior research focused on sepsis and endotoxemia in a variety of animal models and humans suggest similar findings [11–13,16–18]. IAP expression is known to increase in postembryonic states, but its presence at earlier points of gestational age is largely unknown [29,30]. Goldberg *et al.* have shown that intestinal epithelial cells that express high levels of IAP have markedly decreased LPS-induced inflammation. They also found that IAP expression is lost following periods of starvation, suggesting that enteral feeding is paramount to its production and secretion [10]. Chen *et al.* have shown that IAP knockout mice are more susceptible to LPS-mediated intestinal disease [30]. IAP has been shown to have protective effects in human disease as well. Enterally administered IAP was effective in reducing intestinal inflammation and symptoms in patients with Crohn disease and ulcerative colitis in a recent prospective, randomized clinical trial in Europe. In addition to IAP's effect of neutralizing LPS, it has recently been shown to be important to maintaining intestinal homeostasis. Specifically, and important to NEC, the absence of IAP was associated with colonization of the intestine with

increasing amounts and varieties of pathologic bacteria [31]. Lastly, IAP has also been shown to attenuate the LPS-mediated inflammatory response in animal models of sepsis [17]. Therefore, it is reasonable to hypothesize that a relative deficiency of IAP in preterm newborns may predispose them to NEC, and that replacement of IAP may protect the susceptible infant from development of NEC. We also hypothesized that IAP would mitigate the systemic inflammatory response and sepsis associated with NEC as measured by proinflammatory cytokine levels.

We demonstrated that although systemic IAP had no effect on preventing NEC-related intestinal injury, it was effective in significantly decreasing the expression of serum inflammatory cytokine expression as a marker of the systemic inflammatory response associated with NEC. Specifically, we found that IAP administration led to a significant decrease in serum TNF- α , IL-1, and IL-6. All are proinflammatory cytokines linked to NEC-induced sepsis. Although we did observe a decrease in serum IL-10 following IAP administration, this did not reach statistical significance. IL-10 is believed to be a counter-regulatory cytokine and the upregulation of IL-10 in NEC has been suggested to ameliorate mucosal inflammation and injury in NEC [32].

There are many reports on the role of the inflammatory cascade in the pathogenesis and progression of NEC. Frost *et al.* reviewed the importance of proinflammatory cytokines and the development of NEC [33]. Levels of TNF- α , IL-6, and IL-1 β are all increased in both human and rat models of NEC. In addition, Lodha *et al.* found that increased levels of IL-6 at the time of NEC have been associated with worse growth and development outcomes when compared with NEC infants without increased cytokine levels [34]. In light of these findings, it seems paramount to find treatments that are effective in dampening the systemic inflammation associated with NEC as well as preventing the disease. Our data show that IP injections of IAP are effective in increasing serum activity of IAP. This increased activity is associated with a decrease in proinflammatory serum cytokines, namely TNF- α , IL-1 β , and IL-6. This finding is supported by the previous research of others. In animal and human studies of sepsis, both serum TNF- α and IL-6 levels have been shown to be markers of severity and outcomes, with decreased levels associated with improved survival [33,35–37]. IAP has been shown to effectively decrease the incidence and severity of peritonitis following intestinal perforation [35]. It was also effective at decreasing the levels of TNF- α and IL-6 in the peritoneal fluid following perforation [35]. Verweij *et al.* demonstrated that placental AP decreased mortality in a mouse model of sepsis [38]. Beumer *et al.* subsequently found that calf IAP prolonged survival and decreased TNF- α production in murine and porcine models of sepsis [17]. Su *et al.* used IAP in a sheep model of sepsis and found that IAP decreased levels of IL-6 and improved survival [39]. Finally, recently systemic IAP given to patients undergoing coronary artery bypass grafting appears to prevent the normal increase in serum TNF- α , IL-6, and IL-8 in patients on cardiopulmonary bypass [40].

This increase in serum AP activity did not, however, decrease the incidence of NEC as measured by histologic evidence of intestinal damage. This may be because of the mechanism by which IAP is effective in NEC prevention. One proposed mechanism is through the detoxification of LPS by dephosphorylation of its lipid-A moiety. In order for it

to prevent intestinal damage in this fashion, it must be present in the gut lumen to detoxify LPS prior to initiation of the inflammatory cascade. With IP administration it was not effectively transported to the gut lumen, as we did not see an increase in intestinal AP activity in those rats treated with IAP. Following intestinal damage, there is increased permeability of the intestinal epithelium and thus trans-location of luminal bacteria into the bloodstream, causing inflammation and sepsis. It is at this point where we feel that systemic administration of IAP exerts its effect of LPS detoxification by preventing further activation of the inflammatory cascade and systemic sepsis or, potentially, a secondary mechanism. Given the absence of a measurable difference in intestinal injury with systemic IAP, we would hypothesize that the decrease in systemic proinflammatory cytokines is through prevention of activation of circulating lymphocytes. Our study was not designed to determine this, but based on our results this does warrant further investigation.

We feel that our results support that IAP mitigates the systemic inflammatory response often seen in infants affected with NEC. In contrast to our previous research using enteral IAP, which may prevent NEC, these new data may support the use of systemic IAP in newborns who develop NEC as an adjunct to treat the septic systemic inflammatory response. More research needs to be done regarding the effect that IAP has on other end organs such as the liver and lung in order to assess if there is further clinical significance to the blunting of the inflammatory response.

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Fig. 1. Intestinal injury grading. Histologic injury grading of terminal ileum sections. A 4-point grading scale was used to characterize intestinal injury (0 = 1/4 normal histology, 1 = 1/4 mild submucosal/lamina propria separation, 2 = 1/4 moderate submucosal/lamina propria separation and/or edema, 3 = 1/4 severe edema, submucosal separation, and regional villous sloughing, 4 = 1/4 necrosis, loss of villi). Grading reported as mean score. Error bars represent SEM.

Fig. 2.

Intestinal AP activity in the ileum of rat pups was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. Data represent mean $\pm$ standard error.

* $P < 0.05$ versus control.

Fig. 3.

AP activity in the serum of rat pups was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. Data represent mean $\pm$ standard error. * $P < 0.05$ versus control.

Fig. 4.

(A) Serum levels of TNF- α (pg/mL) was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. (B) Serum levels of IL-6 (pg/mL) was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. (C) Serum levels of IL-1 β (pg/mL) was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. (D) Serum levels of IL-10 (pg/mL) was compared between control, NEC, and NEC pups given 4 or 40 U IAP over the first 3 DOL. Data represent mean $\pm$ standard error. * $P < 0.05$ versus control. (Color version of figure is available online.)