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Intestinal alkaline phosphatase to treat necrotizing enterocolitis

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Abstract

Background—Intestinal alkaline phosphatase (IAP) activity is decreased in necrotizing enterocolitis (NEC), and IAP supplementation prevents NEC development. It is not known if IAP given after NEC onset can reverse the course of the disease. We hypothesized that enteral IAP given after NEC induction would not reverse intestinal injury.

Materials and methods—NEC was induced in Sprague–Dawley pups by delivery preterm followed by formula feedings with lipopolysaccharide (LPS) and hypoxia exposure and continued up to 4 d. IAP was added to feeds on day 2 until being sacrificed on day 4. NEC severity was scored based on hematoxylin and eosin-stained terminal ileum sections, and AP activity was measured using a colorimetric assay. IAP and interleukin-6 expression were measured using real time polymerase chain reaction.

Results—NEC pups' alkaline phosphatase (AP) activity was decreased to 0.18 U/mg compared with controls of 0.57 U/mg ($P < 0.01$). Discontinuation of LPS and hypoxia after 2 d increased AP activity to 0.36 U/mg ($P < 0.01$). IAP supplementation in matched groups did not impact total AP activity or expression. Discontinuing LPS and hypoxia after NEC onset improved intestinal injury scores to 1.14 compared with continued stressors, score 2.25 ($P < 0.01$). IAP supplementation decreased interleukin-6 expression two-fold ($P < 0.05$), though did not reverse NEC intestinal damage ($P = 0.5$).

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Conclusions—This is the first work to demonstrate that removing the source of NEC improves intestinal damage and increases AP activity. When used as a rescue treatment, IAP decreased intestinal inflammation though did not impact injury making it likely that IAP is best used preventatively to those neonates at risk.

Keywords

Intestinal alkaline phosphatase; Necrotizing enterocolitis

1. Introduction

Necrotizing enterocolitis (NEC) is a common gastrointestinal inflammatory disease of the neonate associated with significant morbidity and mortality. Those newborns that survive NEC may have long-term medical conditions such as short bowel syndrome and developmental delays [1–4]. NEC is associated with known risk factors such as prematurity, low birth weight, formula feeding, bacterial colonization, and ischemia. Unfortunately, there exists no definitive treatment for NEC, and care is largely supportive for these high-risk premature newborns. A better understanding of the pathophysiology is needed to identify treatments to prevent NEC.

Previous work has shown the preterm intestine to be more sensitive to early enteral stressors [5]. Our laboratory has been interested in the potential for exogenous intestinal alkaline phosphatase (IAP) as a treatment to protect this preterm intestine. IAP is an endogenous protein expressed by the intestinal epithelium, with fractions bound to the brush border or secreted in the intestinal lumen. Our laboratory has shown IAP activity to be decreased in preterm rat pups compared with that in full-term rat pups [6]. Additionally, we have shown daily enteral IAP supplementation beginning on day 0 shows promise to be a treatment to prevent the development of NEC. Rat pups fed IAP and exposed to stressors that induce the development of NEC had significantly decreased intestinal and systemic inflammatory response, intestinal damage, and maintained gut barrier function [5,7–10].

The spectrum of mechanism(s) by which IAP may protect the intestine remains unknown, and it is a common area of research. One of the most elucidated mechanisms, by which IAP is thought to work, is by dephosphorylating lipopolysaccharide (LPS) thereby rendering it inactive and unable to induce a robust inflammatory response by its interaction with toll-like receptor-4 [11,12]. Though other possible roles in controlling inflammation include dephosphorylating other proinflammatory molecules such as uridine diphosphate, flagellin, and CpG DNA [13,14]. IAP has also been shown to decrease bacterial translocation in rats, and we have previously demonstrated IAP to decrease toll-like receptor-4 expression, which has been shown to be crucial to NEC development [15–17].

An important clinical concern is whether the use of supplemental enteral IAP should be given prophylactically to all at-risk preterm newborns or if it could be used as treatment for preterm newborns who develop NEC. However, we currently do not know if it is possible for enteral IAP to reverse NEC development once it has begun. Previous work in adult mouse models has shown IAP administration in the setting of chronic colitis is able to reverse gut inflammatory markers. Additionally, IAP administration was found to lead to

reepithelialization and lessen gut injury scores [18]. Based on these data, we hypothesized that use of IAP after the development of NEC would decrease the severity of NEC. To test this, we exposed preterm newborn rats to NEC stressors for 2 d to induced NEC and then gave supplemental enteral IAP. We examined the effect of the supplemental IAP with and without continued exposure to the NEC stressors for two additional days.

2. Materials and methods

2.1. Animal model

The Medical College of Wisconsin Institutional Animal Care and Use Committee approved all animal protocols (AUA 92). Control Sprague–Dawley pups (Harlan Laboratories, Madison, WI) were spontaneously delivered vaginally, remained undisturbed with the maternal rat, and dam fed. Experimental pups received 2 d of NEC stressors. A previously used model was used to induce NEC [10]. Pups were delivered 1 d preterm via cesarean section. They were then housed in a temperature and humidity-controlled incubator for the duration of the experiments. (GQF Manufacturing, Savannah, GA). Pups were formula fed (FF) three times daily with 0128:B12 *Escherichia coli* LPS (Sigma, St. Louis, MO) 2 mg/kg added to the formula. Base formula was made of 4 g Similac infant formula (Abbott Laboratories, Columbus, OH) in 20 mL Esbilac canine milk replacement (PetAg Inc, Hampshire, IL). Additionally, pups were also exposed to hypoxia of 5% O₂ (Praxair Biospherix, Lacona, NY) after each feed. On day 2, pups were divided into four groups (Fig. 1). One group had continued NEC stressors of LPS and hypoxia (NEC); another had continued NEC stressors with 4 U/kg IAP supplemented to enteral feeds (NEC + IAP). Two other groups did not receive NEC stressors for the remainder of the experiments and were FF (NEC then FF) or had 4 U/kg IAP added to feedings three times per day (NEC then IAP).

To prove NEC was induced, control and NEC groups were sacrificed on day 2. All other groups were sacrificed on day 4. All pups were sacrificed using ketamine and xylazine followed by an open pneumothorax. While on ice, intestine was stripped of mesentery while bathed in phosphate-buffered saline. Adjacent terminal ileum sections were removed and saved for histology for NEC grading and in RNA later for real time polymerase chain reaction (RT-PCR). An additional section of ileum was snap frozen to be used for the AP activity assay.

2.2. AP activity assay

Ileum tissue was harvested and homogenized in assay buffer of 1 M diethanolamine-HCL, 1 mM MgCl₂, and 20 μM at pH 9.8. Homogenates were subsequently analyzed for alkaline phosphatase (AP) activity using a colorimetric assay, similar to those described previously [19]. AP is known to dephosphorylate para-nitrophenyl phosphate (pNPP) to para-nitrophenol producing a color change. Then absorbance is measured at 405 nM. Total protein loaded into reaction was standardized using a Bradford assay then added to the assay buffer mentioned previously. 0.5 mM pNPP was added to each sample, and absorbance was measured at 405 nM. Using a standard curve, μM of pNPP generated was determined and used to expressed units of AP activity.

2.3. Real time polymerase chain reaction

RT-PCR was performed to evaluate for mean relative messenger RNA (mRNA) levels of IAP and interleukin (IL)-6 (IAP: Forward-CTGGGCGTCCATCAATADCGCCA, Reverse-CCTCCTCCACTGGGATGACACCA; TNAP: Qiagen Primer Assay catalog number PPR52402 A; IL-6: CCTTCTGTGACTTAA CTCTCC) in the terminal ileum [20]. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for reference. All intestine samples obtained for RNA analysis using RT-PCR were stored in RNA later until the RNA was isolated using the Qiagen RNeasy mini kit (Valencia, CA) with on column DNase treatment. RNA concentration and purity were then determined using the NanoDrop spectrophotometer (SpectraMax Plus; Molecular Devices, Sunnyvale, CA). Complementary DNA was synthesized from equal amounts of RNA using the EasyScript cDNA Synthesis Kit (Lamda Biotech, St. Louis, MO). RT-PCR was performed using ABI Prism 7900HT software (Applied Biosystems, Grand Island, NY) together with iQ SYBR Green RT-PCR Supermix (Bio-Rad, Hercules, CA). All gene amplification reactions were performed in triplicate.

2.4. Intestinal injury grading

Terminal ileum sections were harvested and fixed in 10% formalin. Hematoxylin and eosin-stained sections were graded using a 4 points score as described previously [21].

2.5. Statistical analysis

Using Prism software (Prism, San Diego, CA) version 6, data were analyzed using a Student *t*-test because the data are normally distributed. The data are reported as the mean and standard error of the mean, and all tests were conducted as two-tailed tests. Differences were considered statistically significant if the *P* value was ≤ 0.05 .

3. Results

3.1. Intestinal injury

Shown in Figure 2, 2 d of NEC stressors resulted in intestinal injury scores of 1.50, which is significantly greater than 0.29 for controls ($P < 0.01$). NEC pups continued to have worse intestinal injury on day 4 with scores of 2.25 and 0.71, respectively ($P < 0.0001$). Day 4 NEC pups trended toward having worse intestinal injury compared with day 2, though this did not reach statistical significance ($P = 0.07$). Pups given supplemental enteral IAP after the development of NEC also had significantly increased grade of injury (2.00) compared with that of control ($P < 0.01$) and not significantly different than NEC ($P = 0.5$) by day 4 of life. Both the NEC then FF and NEC then IAP had significantly decreased severity of injury compared with NEC (NEC then FF = 1.14 and NEC then IAP = 1.30, $P < 0.01$) but were not different ($P = 0.55$) from each other and remained significantly increased from controls ($P = 0.02$).

3.2. IL-6 polymerase chain reaction

On day 4, groups with NEC stressors, NEC, and NEC + IAP had 3–4 fold higher levels of IL-6 expression compared with controls ($P < 0.05$). NEC and NEC + IAP groups did not

differ ($P = 0.28$; Fig. 3). In groups that had LPS and hypoxia discontinued after day 2, IL-6 expression was similar to controls (Fig. 4). NEC then FF mean was 2-fold high though was highly variable and was not significantly different than controls ($P = 0.29$). NEC then IAP was also similar to controls ($P = 0.26$).

3.3. IAP polymerase chain reaction

Shown in Figure 5, compared with controls, IAP expression was decreased approximately 5-fold in NEC on day 4 ($P < 0.01$). The addition of exogenous IAP in the NEC + IAP group did not restore IAP mRNA and remained less than control though this did not reach statistical significance ($P = 0.10$). NEC did not differ from NEC + IAP ($P = 0.12$). Removing LPS and hypoxia in the NEC then FF group reduced IAP expression to approximately half control levels, approaching statistical significance ($P = 0.06$) (Fig. 6). NEC then IAP also had slightly less than the IAP mRNA as controls ($P < 0.05$), and did not differ from NEC then FF ($P = 0.62$).

3.4. Alkaline phosphatase activity

As we have shown previously, AP activity is decreased in NEC. On day 2, control pups had total AP activity of 0.53 U/mg and NEC pups 0.32 U/mg ($P < 0.01$). At day 4, control pups had AP activity of 0.57 U/mg, NEC 0.18 U/mg, NEC + IAP 0.20, NEC then FF 0.36 U/mg, and NEC then IAP 0.33 U/kg. Control activity was higher than all other groups on day 4 ($P < 0.001$). Pups receiving continued NEC (NEC and NEC + IAP) had lower AP activity than both groups that had NEC stressors removed (NEC then FF and NEC then IAP) ($P < 0.01$). Additionally, day 4 NEC compared with NEC + IAP did not result in a measurable difference in AP activity ($P = 0.50$), as did NEC then FF compared with NEC then IAP ($P = 0.43$) (Fig. 7).

4. Discussion

Here we have shown that preterm delivery followed by 2 d of formula feedings with LPS and hypoxia stress is sufficient to induce significant intestinal injury consistent with NEC. This is different in that similar rat models have traditionally been carried out over 4 d. Consistent with this is previous work showing increased inducible nitric oxide synthase at just 1.5 h and IL-1 β and IL-6 at 24 h in this model [20]. The often unpredictable and rapid nature of NEC development makes it a disease that would be best suited for early intervention. This shorter model with histologically demonstrated intestinal damage will serve as a useful tool to investigate NEC and potential treatments early in its disease course. Not surprisingly, continued LPS and hypoxia until day 4 resulted in significantly worse intestinal injury and inflammation. Discontinuation of NEC stressors led to significantly decreased severity of intestinal injury and inflammation. However, supplemental enteral IAP was not able to counteract the structural damage or inflammation as measured by IL-6 expression in either pups that continued to be exposed to NEC stressor or those that did not.

These results differ from a similar experiment in an adult mouse model of colitis that found IAP was able to reverse intestinal damage and decrease inflammation when given after colitis had already been induced [18]. Given what we know about the mechanism of IAP to

dephosphorylate LPS, it is surprising to us that it did not significantly affect IL-6 levels when compared with matched groups. Possible reasons include the inability of the preterm immature gut to respond as well after an insult. This would be consistent with what we have previously shown the preterm gut to be much more sensitive to early insults compared with term controls [20]. Additionally, we only used 2 d of IAP supplementation compared with 7 d. Though given the often rapid progression of NEC, we felt it necessary to see a more rapid response to IAP so opted for a shorter duration. Nonetheless, it is unclear what would have happened if the experiment had been extended.

We have previously shown enteral IAP is able to maintain gut barrier function as measured by permeability assays and higher levels of tight junction proteins in the NEC model when IAP is given before NEC onset, and suspect this is due to its ability to protect the intestinal barrier from the inflammatory insult associated with LPS [8]. However, we are not aware of any mechanism of IAP that is involved in reforming tight junctions after intestinal damage such as that which we induced here. We suspect once the integrity of this barrier is compromised, there is a very robust local and systemic inflammatory response in which it is difficult for the preterm gut to recover and could contribute to our results. Perhaps combined enteral and intravenous or intraperitoneal supplementation of IAP would be appropriate in this instance, as we have previously shown the ability of intraperitoneal IAP to control systemic inflammation [9].

A similar pattern was seen with AP activity, where NEC pups had the lowest levels of activity, consistent with what we have previously shown [6]. Additionally, removing LPS and hypoxia in the NEC then FF resulted in some restoration of activity compared with NEC, though did not reach control levels. However, IAP supplementation in the NEC + IAP and NEC then IAP group did not increase AP activity. This is difficult to explain, though may be a limit of the activity assay. It would be prudent to know the relative contributions of endogenous versus exogenous AP activity to help answer this question; though at this time we are not able to do so. Additionally, pups were sacrificed and tissue harvested 24 h after the last dose of IAP. This is a limitation of the model in that we cannot give IAP in every feed. To prevent the dephosphorylation of LPS by IAP in the formula before administration, we gave the two in separate feeds. A feed with IAP is given once in the morning followed by those with LPS. The timing of feed could very well affect our ability to measure differences in activity. However, IAP expression as measured by mRNA showed a similar trend with controls being the highest on day 4, NEC the lowest, and NEC then FF showing a regain of IAP expression relative to NEC without reaching control levels. Exogenous IAP supplementation did not effect endogenous IAP expression.

It has been shown that higher serum levels of IAP may be predictive of NEC development in neonates [22]. This may seem in contradiction to our results showing lower IAP, measured in the intestinal tissue, in those with histologic injury consistent with NEC. The key difference is where it is measured. Quantifying IAP in the serum may likely be secondary to a luminal enzyme escaping into the systemic circulation after the gut barrier has been compromised. Nonetheless, lower IAP in intestinal tissue could serve as a marker of NEC severity when intestinal tissue is available.

It is important to examine these data in the context of previous work of our laboratory showing the promise of IAP in preventing NEC development when given shortly after birth in the NEC model [5,7,8,10]. It appears as though IAP has a significant role in protecting the intestine from insult. Given the sometimes rapid progression of NEC, and high rates of morbidity and mortality, it would have been easy to already advocate for a preventive approach compared with a wait-and-treat approach. This current work further supports the need to give IAP very early in life to all those identified as at risk.

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Fig 1.
Experimental groups.

Fig 2.
Average histologic grade of injury. * $P < 0.05$.

Fig 3. IL-6 mRNA expression of terminal ileum as measured by RT-PCR on day 4. *Statistically significant difference.

Fig 4.
IL-6 Expression mRNA expression of terminal ileum as measured by RT PCR.

Fig 5.
IAP Expression mRNA expression of terminal ileum as measured by RT PCR. * $P < 0.05$ vs control.

Fig 6.
IAP Expression mRNA expression of terminal ileum as measured by RT PCR. * $P < 0.05$ versus control.

Fig 7.

Terminal Ileal Alkaline Phosphatase Activity as measured by NNP assay. * $P < 0.05$.