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Enteral intestinal alkaline phosphatase administration in newborns decreases iNOS expression in a neonatal necrotizing enterocolitis rat model

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Abstract

Purpose—To determine if intestinal alkaline phosphatase (IAP) decreases intestinal injury resulting from experimentally induced necrotizing enterocolitis (NEC). We hypothesized that IAP administration prevents the initial development of NEC related intestinal inflammation.

Methods—Pre- and full-term newborn Sprague–Dawley rat pups were sacrificed on day 1 of life. Preterm pups were exposed to intermittent hypoxia and formula containing LPS to induce NEC. Select NEC pups were given 40, 4 or 0.4 units/kg of bovine IAP (NEC+IAP40u, IAP4u or IAP0.4u) enterally, once daily. Ileal sections were evaluated by real-time PCR (qRT-PCR) for IAP, iNOS, IL-1 β , IL-6, and TNF- α mRNA and immunofluorescence for 3-nitrotyrosine (3-NT).

Results—Experimentally induced NEC decreased IAP mRNA expression by 66% ($p = 0.001$). IAP supplementation increased IAP mRNA expression to control. Supplemental enteral IAP decreased nitrosative stress as measured by iNOS mRNA expression and 3-NT staining in the NEC stressed pups ($p = 0.01$), as well as decreased intestinal TNF- α mRNA expression. In addition, IAP decreased LSP translocation into the serum in the treated pups.

Conclusions—We conclude that enterally administered IAP prevents NEC-related intestinal injury and inflammation. Enteral IAP may prove a useful strategy in the prevention of NEC in preterm neonates.

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Keywords

Prematurity; Formula; Intestinal alkaline phosphatase; Inflammation; Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the most common life-threatening gastrointestinal surgical emergency encountered in premature infants [1]. Multiple risk factors have been implicated in the pathogenesis of NEC, including prematurity, formula feeding, bacterial colonization and dysregulated inflammatory pathways, yet the exact etiology remains undefined.

Prior to widespread histologic evidence of NEC, inflammation is present in the immature intestine [2]. One of the potential reasons NEC may develop in neonates is that immature intestinal cells mount a more robust and prolonged inflammatory response than adult intestinal cells [3]. Previous studies by Ford et al. [4] suggested that acute NEC in neonates is associated with increased inducible nitric oxide synthase (iNOS) expression and nitrosative. Furthermore nitrosative stress induced by increased iNOS expression is well known to increase enterocytes apoptosis and impair normal restorative processes [5].

Increased productions of pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6, have all been linked to NEC. Indeed, severity of inflammatory cytokine production has been suggested as a biomarker of intestinal injury that has been linked to clinical outcomes in NEC. Sharma et al. [6] demonstrated that elevated circulating endotoxin in those with NEC was associated with higher levels of serum cytokines, which predisposed the infant to multiple organ failure and subsequent mortality. While Lodha et al. [7] showed that the inflammatory profile and magnitude of production were associated with increased long-term neurologic injury in infants with NEC. Exactly how pro-inflammatory cytokine production changes in the early stages of NEC remain unclear.

Intestinal alkaline phosphatase (IAP) is an endogenous enzyme expressed in the brush border that is hypothesized to play an important role in the pathogenesis of NEC. Premature neonatal rat pups with experimentally induced NEC have diminished levels of IAP activity compared with healthy controls. Interestingly, IAP supplementation was recently shown to reduce the severity of intestinal injury [8].

While these studies show that IAP protects against experimental NEC, no studies were performed evaluating the mechanism by which this occurred at the molecular level. In the present study we show the effect of IAP in experimentally induced NEC with respect to IAP, iNOS and cytokine mRNA expression as well as serum LPS activity.

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. Four experimental groups of NEC stressed pups were delivered 1-day prematurely via cesarean

section and gavage-fed formula with LPS every 4h. Pups were exposed to three periods of hypoxia (5% oxygen for 10min) (Praxair Biosperix, Lacona, NY)[8,9]. Three of the four groups of NEC pups received added enteral 0.4, 4 or 40units/kg bovine IAP (generously donated by Am-Pharma, Netherlands) in formula once daily. All pups were euthanized on day 1. IAP was administered between feeds to prevent interaction and inactivation of LPS in the formula with IAP administration.

Pups were injected with IP ketamine and xylazine mixture (100 mg/kg, 10 mg/kg) and then euthanized by creating a pneumothorax via a thoracotomy. Ileal sections were placed in 300 µl of *RNAlater* (Qiagen, Valencia, CA) or fixed in formalin (Richard-Allan Scientific, Kalamazoo, MI) prior to immunofluorescent studies.

1.2. Immunofluorescent staining

Paraffin-embedded tissues were deparaffinized, immersed in xylene three times and hydrated with graded ethanol [9]. For antigen unmasking, slides were heated in 10mM sodium citrate buffer (pH 6.0) for 15min prior to treatment with 0.3% hydrogen peroxide for 30 min. Specimens were treated with 5% BSA in Tris-buffered saline with 0.05% vol/vol Tween-20 for 30min at room temperature followed by overnight incubation with Rabbit Anti-3-Nitrotyrosine (Sigma, St. Louis, MO) at 4 °C, followed with Cy2-anti-Rabbit antibody (Jackson ImmunoResearch Laboratories, PA, USA). Nuclei were labeled with DAPI (100ng/ml) (Invitrogen). Images were acquired by Leica SP2 confocal microscopy.

1.3. Serum lipopolysaccharide

Plasma endotoxin was assessed using a spectrophotometric method provided in an LAL assay, Pierce LAL Chromogenic Endotoxin Quantitation Kit (Pierce Biotechnology, Rockford, IL). Endotoxin catalyzes the activation of a proenzyme in LAL, and the rate of activation is determined by the concentration of endotoxin present. Endotoxin in a sample is compared with a standard curve of endotoxin.

1.4. RNA extraction and RT-PCR

Real-time PCR (RT-PCR) assays were performed to evaluate IL-1 β , IL-6, TNF- α , IAP and iNOS with GAPDH used as an internal gene control. Total ileal RNA was isolated with Qiagen RNeasyMini Kit (Qiagen Inc, Valencia, CA). RNA concentration and purity were quantified by the NanoDrop (SPECTRAMAS PLUS; Molecular Devices, Sunnyvale, CA). Complementary DNA (cDNA) was synthesized from equal amounts (2µg) of DNase-treated RNA using the iScript cDNA Synthesis kit (BioRad, Hercules, CA). RT-PCR was performed using ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA) with SYBR Green RT-PCR Master Mix (Stratagene, Santa Clara, CA). Primers and probes were designed using NIH/NCBI program RefSeq Accession No. iNOS (NM_012611.3), IAP (NM_022665.3), GAPDH (NM_017008.3), IL-1b (NM_031512.2), IL-6 (NM_012589.1), TNF- α (NM_012675.3). Gene-amplification reactions were performed in triplicate. RNA expression was compared using the Ct method.

1.5. Statistical analysis

Using SPSS software (ver. 19; SPSS Inc., Chicago, IL), data were analyzed using non-parametric tests since the data have outliers and were skewed. Comparisons were made between the control and NEC groups as well as each of these groups versus the IAP treatment groups. Means and standard errors (SE) are reported for normally distributed data and groups were compared using the Student's *t* test. Medians and inter-quartile ranges were reported for skewed data and groups were compared using a Mann–Whitney test. Differences were considered statistically significant if the *p*-value was ≤ 0.05 . All tests were conducted as two-tailed tests.

2. Results

2.1. Administration of IAP during NEC preserves endogenous IAP expression

Expression of IAP in response to stress was measured in the ileum using RT-PCR in control and NEC pups on day 1. The Ct value for control pups was set to 1. IAP mRNA expression in the ileum of the pups was significantly lower in NEC stressed pups vs. control ($p < 0.05$, Fig. 1). In stressed pups fed exogenous 4 or 40 units/kg IAP, IAP mRNA expression was significantly increased compared with untreated pups ($p < 0.05$) to nearly control.

2.2. Enteral IAP decreases ileal expression of iNOS and 3-nitrotyrosine (3-NT) staining

In order to demonstrate that IAP supplementation inhibits stress-induced nitric oxide production and subsequent reactive nitrogen stress in the ileum, iNOS expression was measured with quantitative PCR and 3-NT was evaluated with fluorescent immunohistochemistry. When Ct values for control pups was set to 1, expression of iNOS mRNA increased 40-fold with NEC stress vs. control on day 1 ($p < 0.05$) (Fig. 2A). All enteral dose regimens of IAP significantly reduced iNOS expression relative to the NEC pups. Importantly, IAP supplementation decreased 3-NT generation in NEC pups to control levels in a dose-dependent manner (Fig. 2B).

2.3. IAP decreases intestinal TNF- α expression in NEC

We also sought to determine if supplemental, enteral IAP could inhibit the production of inflammatory cytokines by the intestine. We chose to measure TNF- α , IL-1 β and IL-6 by RT-PCR, all pro-inflammatory cytokines that have been linked to NEC. Experimentally induced NEC increased TNF- α mRNA levels compared with control pups (3.5 vs. 1.0, $p < 0.001$, Fig. 3). IAP supplementation dose-dependently decreased TNF- α mRNA expression across all treatment groups (0.5 to 0.8 vs. 3.5, $p < 0.05$). No significant differences were found in IL-1 β or IL-6 expression in control, NEC or NEC pups fed IAP (data not shown).

2.4. Serum LPS decreased in response to enteral IAP administration

LPS is inactivated by IAP in the intestinal lumen. To determine if supplemental, enteral IAP could prevent translocation of LPS we measured LPS in the serum in day 3 control, NEC, and NEC pups fed IAP. The mean serum LPS concentrations were significantly higher in NEC vs. control pups (426 vs. 92 pg/ml, $p < 0.001$) (Fig. 4). At the lowest IAP dose used, no

change in serum LPS levels were observed. However at all higher doses, IAP effectively decreased serum LPS levels (91pg/ml for IAP 4 and 75pg/ml for IAP40, $p<0.05$).

3. Discussion

Preterm intestinal enterocytes have an immature stress response and inflammatory profile that is rapid and often poorly regulated [10]. Here we sought to exam the effect of IAP on mitigating intestinal inflammation at the early stages of experimentally induced NEC. Our data demonstrate that supplemental, enteral IAP was highly effective at reducing nitrosative stress, pro-inflammatory cytokines and LPS translocation in this NEC model. Importantly, we have shown that supplemental IAP helps to preserve baseline intestinal levels of IAP mRNA. These data suggest that IAP prevents the stages of intestinal necrosis that occurs late in NEC by decreasing intestinal inflammation early in its development.

Previous studies have demonstrated that iNOS up-regulation, NO generation and nitrosylation all play important roles in the pathogenesis of NEC. iNOS is induced by a variety of stimuli and cellular stressors including hypoxia, LPS, and pro-inflammatory cytokines. In this model, increased iNOS expression and 3-NT formation occurred early in NEC and IAP treatment significantly suppressed their expression. These data are consistent with previous work, demonstrating that iNOS co-localizes with apoptotic enterocytes at the tips of villi [4] The importance of such changes are that the marked increases in NO production inhibit intrinsic repair mechanisms [11,12].

Studies presented here show that NEC induces temporal changes in inflammatory cytokine production. While experimentally induced NEC has been shown to increase TNF- α and IL-6, enteral IAP treatment inhibited iNOS and TNF- α expression in the intestine. Interestingly, IL-6 and IL-1 β production was unaltered in either NEC or in NEC pups treated with supplemental IAP. Such observations may be the result of IAP's inability to alter IL-6 and IL-1 β production or may be related to shifts in the mechanisms that regulate production in early stages of NEC.

Importantly translocation of LPS is attenuated in pups treated with IAP. These data are consistent with our previous report demonstrating IAP inhibited the increased in intestinal permeability associated with NEC. One of the limitations of our studies is that we cannot conclude whether the reduction in serum LPS results from inactivation by IAP or whether this decrease actually resulted from an improvement in barrier function. Probably it is a function of both. Regardless, any reduction in biologically active LPS should play a key role in reducing systemic inflammatory responses associated with NEC.

Our results raise new questions concerning the use of enteral IAP administration in the treatment of NEC. What remains to be determined is whether enteral IAP can also decrease experimentally induced NEC in models that do not utilize LPS. Additional time courses could be used to further dissect the relationship between IAP administration and IAP expression in neonatal and preterm rat pups. Such studies would provide new information concerning the notion that preterm pups are predisposed to NEC as a consequence of decreased IAP expression.

In conclusion, our results demonstrate that supplemental enteral IAP decreases ileal inflammation and serum LPS levels, as well as reduces TNF- α expression in the stressed ileum. Such data support the notion that enteral IAP may be a useful strategy for prevention and for limiting NEC in at risk newborns.

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Fig. 1.

(A) Levels of mRNA levels for IAP day 1. The Ct for control pups is normalized to 1. Data are expressed as mean $\pm$ SEM, $n=7$ per group, $*p<0.05$.

Fig. 2.

(A) iNOS mRNA expression day 1. The Ct for control pups is normalized to 1. Data are expressed as mean $\pm$ SEM, $n=7$ per group, $*p<0.05$. (B) 3-Nitrotyrosine immunofluorescence staining on day 3. Nuclei are visualized with DAPI (blue) and 3-NT (green). Representative images of ileum from control (upper left), NEC stressed (upper right), treatment with 0.4units/kg IAP (lower left), treatment with 4units/kg IAP (lower center) and treatment with 40units/kg IAP (lower right) pups.

Fig. 3. TNF-α mRNA expression. The Ct for control pups is normalized to 1. Data are expressed as mean±SEM, $n=7$ per group, * $p<0.05$, ** $p<0.005$, *** $p<0.0001$.

Fig. 4. Serum LPS on day 3. Data are expressed as mean $\pm$ SEM, $n=7$ per group, * $p<0.05$, ** $p<0.005$, *** $p<0.0001$.