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Intestinal alkaline phosphatase administration in newborns decreases systemic inflammatory cytokine expression in a neonatal necrotizing enterocolitis rat model

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Abstract

Background—Supplementation of intestinal alkaline phosphatase (IAP), an endogenous protein expressed in the intestines, decreases the severity of necrotizing enterocolitis (NEC)—associated intestinal injury and permeability. We hypothesized that IAP administration is protective in a dose-dependent manner of the inflammatory response in a neonatal rat model.

Materials and methods—Pre- and full-term newborn Sprague–Dawley rat pups were sacrificed on day of life 3. Control pups were vaginally delivered and dam fed. Preterm pups were delivered via cesarean section and exposed to intermittent hypoxia and formula feeds containing lipopolysaccharide (NEC) with and without IAP. Three different standardized doses were administered to a group of pups treated with 40, 4, and 0.4 U/kg of bovine IAP (NEC + IAP40, IAP4, or IAP0.4 U). Reverse transcription-real-time polymerase chain reaction (RT-PCR) for inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)- α on liver and lung tissues and serum cytokine analysis for interleukin (IL)-1 β , IL-6, IL-10, and TNF- α were performed. Data were analyzed by Kruskal–Wallis and Mann–Whitney tests, expressed as mean standard error of the mean and $P < 0.05$ considered significant.

Results—Levels of cytokines IL-1 β , IL-6, and TNF- α increased significantly in NEC *versus* control, returning to control levels with increasing doses of supplemental enteral IAP. Hepatic and pulmonary TNF- α and iNOS messenger ribonucleic acid expressions increased in NEC, and the remaining elevated despite IAP supplementation.

Conclusions—Proinflammatory cytokine expression is increased systemically with intestinal NEC injury. Administration of IAP significantly reduces systemic proinflammatory cytokine

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expression in a dose-dependent manner. Early supplemental enteral IAP may reduce NEC-related injury and be useful for reducing effects caused by a proinflammatory cascade.

Keywords

Necrotizing enterocolitis; Intestinal alkaline phosphatase; Systemic inflammation; Cytokine

1. Introduction

Necrotizing enterocolitis (NEC) is a multifactorial neonatal gastrointestinal disease occurring in 0.3–2.4 per 1000 live births and is one of the most common causes of neonatal 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 it has developed.

Severe intestinal damage is often accompanied by multiple organ dysfunction resulting in hepatic, renal, and/or pulmonary failure [6,7]. A large amount of research attributes the gut as a major source of cytokine production and resulting remote organ injury after hemorrhagic shock and/or ischemia or reperfusion injury [8–13]. Similar mechanisms have also been implicated in the development of NEC [14–16]. Previous work has shown that the liver undergoes inflammatory and morphologic alterations in NEC [17–22] and that the lung is susceptible as well to lipopolysaccharide (LPS)-induced injury [23–28]. Clinical studies have demonstrated that elevated cytokine levels exist in infants with NEC and multiple organ dysfunction [6], as well as correlating with poor growth and cognitive development [29].

Intestinal alkaline phosphatase (IAP), a member of the family of alkaline phosphatases, is an endogenous brush border enzyme expressed in the intestines. IAP has been shown to detoxify LPS by dephosphorylating the lipid-A moiety, thereby preventing it from binding to the toll-like receptor-4 (TLR-4). Previous studies have demonstrated that monophosphoryl LPS is unable to induce an equivalent reaction to that produced by intact LPS [30,31]. In the absence of IAP, intestinal inflammation is exacerbated, and replacement or supplementation of IAP prevents intestinal inflammation and endotoxemia [17,32–34]. Our previous work has demonstrated that supplemental enteral IAP reduced the severity of histologic NEC-associated intestinal injury [35]. We have also demonstrated that supplemental enteral IAP prevented NEC-associated loss of gut barrier function (in press). Based on our previous results, we hypothesized that supplemental enteral IAP would mitigate NEC-induced systemic end organ inflammation.

2. Materials and methods

2.1. Animal model

The Medical College of Wisconsin Institutional Animal Care and Use Committee approved all animal experiments. An *in vivo* NEC model was used [35–39]. Control litters of full-term Sprague–Dawley rat pups (Harlan Laboratories, Madison, WI) were spontaneously delivered and dam fed. Experimental pups were delivered 1 d prematurely by cesarean section and divided into four experimental groups. All the experimental pups were maintained in an

incubator at 37°C and gavage fed formula with LPS, via an orogastric tube every 4 h. Pups also underwent three hypoxic episodes each day consisting of 5% oxygen for 10 min, Praxair Biosperix (Lacona, NY) chamber, at room temperature. Three of the four groups of NEC pups additionally received bovine IAP generously donated by Am-Pharma (Bunnik, Netherlands). Pups received enterally 0.4, 4, or 40 U of IAP in formula once daily. All the pups were sacrificed on day of life 3. In the absence of LPS, IAP was administered enterally to avoid interaction of LPS with IAP before administration. Liver and lung sections were placed in 300 µL of RNA-later (Qiagen, Valencia, CA) for ribonucleic acid (RNA) storage.

2.2. Serum cytokine assay

Blood was allowed to clot on ice for 10 min and then centrifuged (5000 rpm) for 10 min at 4°C. The serum was then transferred to a clean polypropylene tube. To completely remove platelets and precipitates, the samples were centrifuged again at 10,000 rpm for 10 min at 4°C. Serum was collected and diluted 1:2 in sample diluent. Sera were assayed for cytokine levels (interleukin [IL]-1 β , IL-6, IL-10, and tumor necrosis factor [TNF]- α) using a magnetic bead-based immunoassay kit (Bio-Rad, Hercules, CA). Cytokine assays were performed as described by the manufacturer's protocol. The limit of detection of this assay is 1 pg/mL, intra-assay coefficient of variation is <10%, interassay coefficient of variation is <15%, accuracy (percentage recovery) is 80%–120%, and cross-reactivity is <1%. Each reaction in the kit was performed in duplicate, with the experiment repeated four times.

2.3. Rat RNA preparation and reverse transcription and real-time polymerase chain reaction

Total RNA was isolated from lung and liver tissues stored in RNAlater using the RNeasy-Plus MiniKit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. Samples were incubated with RNase-free deoxyribonuclease (20 U per reaction) for 15 min at 37°C to eliminate deoxyribonucleic acid (DNA) contamination. RNA purity and concentration were determined using a NanoDrop (SPEC-TRAmas PLUS; Molecular Devices, Sunnyvale, CA). RNA integrity was determined by the Experian RNA StdSens Analysis Kit (Bio-Rad).

Real-time polymerase chain reaction (PCR) assays were performed to quantify messenger RNA (mRNA) levels of iNOS and TNF- α . Complementary DNA was synthesized from equal amounts (2 µg) of deoxyribonuclease-treated total using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol. Reverse transcription-real-time PCR was performed using ABI Prism 7900HT software (Applied Biosystems, Carlsbad, CA) together with SYBR Green RT-PCR Master Mix (Stratagene, Santa Clara, CA), as specified by the manufacturer's protocols. Primers and probes were designed using National Institute of Health or National Center for Biotechnology Information program. PCR amplification for 95°C $\times$ 3 min, 40 cycles of 95°C $\times$ 20 s, 60°C $\times$ 30 s, and 72°C $\times$ 30 s was used. GAPDH was used as an internal gene control. All gene amplification reactions were performed in triplicate. The primers had the following sequences: iNOS, sense 5'-GGCTGGAAGCCCCGCTATGG-3'; antisense primer, 5'-GGCAGGCAGCGCATACCACT-3', TNF- α a proprietary mix (SABiosciences, Frederick, MD), GAPDH, sense 5'-GGCAAATTCAACGGCACAGT-3'; antisense primer, 5'-

AGATGGTGATGGGCTTCCC-3'. mRNA levels are expressed as relative to control and are presented as mean $\pm$ standard error of the mean [40].

2.4. Statistical analysis

For serum cytokine assays, data analysis was performed using Bio-Plex manager software (version 6; Bio-Rad). A five-parameter logistic regression model was used when more than six standards were within 70%–130% of the expected values.

Using SPSS software (version 19; SPSS Inc, Chicago, IL), data were analyzed using nonparametric tests because the data have outliers and were skewed. Comparisons were made between the control and the NEC groups as well as each of these groups *versus* the IAP treatment groups. Means and standard errors are reported for normally distributed data, and groups were compared using a *t*-test. Kruskal–Wallis test was used for all groups. Medians and interquartile 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.

3. Results

3.1. Supplemental enteral IAP decreased systemic proinflammatory cytokine production

In control pups, low levels of TNF- α were found in the serum (Fig. 1A). After induction of NEC, there was a four-fold increase in the level of serum TNF- α (control 1082 *versus* NEC 4417 pg/mL, *P* ≤ 0.001). However, NEC animals fed with IAP had a dose-dependent decrease in the level of serum TNF, with the group that received 40 U of IAP having over a three-fold reduction compared with the NEC pups (NEC 4417 *versus* IAP40 U 1298 pg/mL, *P* ≤ 0.01).

IL-6 levels were low in control pups (Fig. 1B). After NEC induction, there was a 26-fold increase in the IL-6 level in the NEC group (control 379 *versus* NEC 9782 pg/mL, *P* ≤ 0.001). The level of IL-6 trended down with increasing doses of IAP administered. The group that received 40 U of IAP had a fourfold decrease in the IL-6 level (NEC 9782 *versus* IAP40 U 2216 pg/mL, *P* ≤ 0.01).

Baseline IL-1 β level was measured in control pups (Fig. 1C), and it underwent a four-fold increase in the NEC-stressed group (control 3431 *versus* NEC 13,945 pg/mL, *P* ≤ 0.001). Similarly to IL-6, IL-1 β level trended down with increasing doses of IAP administration and decreased significantly with a three-fold change in the IAP40 U/kg groups (NEC 13,945 *versus* IAP40 U 5173 pg/mL, *P* ≤ 0.01).

IL-10 levels were detected at baseline (Fig. 1D) and increased three-fold with NEC stress (control 9505 *versus* NEC 28,528 pg/mL, *P* ≤ 0.001). However, the levels of IL-10 did not decrease to control levels, although they trended down with increasing IAP administration.

3.2. Enteral IAP did not reduce NEC-related hepatic and pulmonary inflammation

We sought to determine if the protective effects of IAP on reducing intestinal injury and permeability would translate into decreased liver and lung inflammation. Sections of liver

and lung from each group were used to analyze mRNA expression of proinflammatory iNOS (Figs. 2A and 3A) and TNF- α (Figs. 2B and 3B). Compared with the control group, the hepatic and pulmonary tissues of the NEC group had increased relative mRNA expression of both iNOS (lung control 1 *versus* NEC 2, $P = 0.05$ and liver control 1 *versus* NEC 39.8, $P = 0.001$) and TNF- α (lung control 1 *versus* NEC 4.6, $P = 0.001$ and liver control 1 *versus* NEC 11, $P = 0.005$). Although there appears to be a decreasing trend in the levels of both iNOS and TNF- α , these differences were not statistically significant.

4. Discussion

Using a rodent NEC model, we have demonstrated that supplemental enteral IAP decreases systemic inflammatory mediators in a dose-dependent fashion. With NEC stress, there was an associated increase in hepatic and pulmonary inflammation that was not ameliorated by enteral IAP.

Administration of enteral IAP decreased systemic inflammatory cytokines. These results are consistent with our early findings that supplemental enteral IAP effectively reduced histologic evidence of neonatal rat NEC intestinal injury and permeability. Cytokines are produced by various cell types, such as monocytes, neutrophils, and endothelial cells, and act as potent regulatory molecules of the immune system in infants with NEC [41,42]. In our rat pup model, we saw the levels of TNF- α , IL-1 β , and IL-6 elevate with NEC stress and decrease with increasing doses of enteral IAP, without diminishing IL-10. Elevated levels of IL-6 in the fetal blood significantly increase neonatal morbidity and outcomes in NEC [43]. In addition, during the septic response, enterocytes increase secreted IL-6 [44]. IL-1 β participates in the inflammatory cascade by increasing neutrophils at sites of injury [45] and plays a role in protecting intraluminal gut integrity during times of stress [46]. Interestingly, levels of IL-6 and IL-1 β appear to be correlative values that rise together with increasing inflammation (data not shown). TNF- α is increased in NEC and is a potent proinflammatory cytokine capable of increasing other cytokines in the inflammatory cascade [20]. IL-10 is a counter-regulatory or anti-inflammatory cytokine that normally serves to downregulate the inflammatory response. In severe NEC, the concentration of IL-10 is significantly increased [47]. It appears in recent research that IL-10 is protective against NEC development [48] and results in spontaneous chronic colitis in IL-10 knockout mice [49]. Our results support its counter-regulatory role, as it is observed increasing with NEC stress and then trending down with IAP administration (although not a statistically significant difference). The results of our experiments suggest that supplemental enteral IAP may inhibit the systemic inflammatory response that occurs in NEC and therefore prevent the occurrence of septic shock.

Lowering inflammatory cytokines is beneficial to outcomes in NEC. One of the important mechanisms responsible for intestinal inflammation in NEC is the enterocytes. In preterm newborns, the immature enterocytes mount an unbalanced proinflammatory response after activation by bacterial ligands [50–52]. Compared with mature enterocytes, immature enterocytes more rapidly degrade the Nuclear Factor-Kappa Beta (NF- κ B) inhibitor Inhibitory factor kappa beta (I κ B α) and increase NF- κ B–DNA binding and transcriptional activity, resulting in the transcription of inflammatory mediators [52]. Therefore, given that

the mechanism of action of IAP is likely by inhibiting LPS action on TLR-4 and subsequent NF- κ B activation, the decrease we observed in proinflammatory cytokines may result from preventing the initiation of intracellular signaling cascades in the enterocytes. More experimentation is required to determine if supplemental enteral IAP affects the immature enterocytes' predisposition to increased NF- κ B activation and whether this specifically leads to decreased production of inflammatory mediators.

The absence of mitigating effects on hepatic and pulmonary inflammation after enteral IAP administration suggests that these organs are not responsible for the observed reduction in serum proinflammatory cytokines. However, although the mechanism of IAP enterally may be to dephosphorylate LPS and prevent the activation of the TLR-4 receptor and downstream NF- κ B activation, the mechanism of pulmonary and hepatic injury may be through an alternate pathway. Inflammation initiated by the enterocytes and circulating lymphocytes is also a source of increased serum cytokines associated with NEC. Further investigations are planned to measure the effects of supplemental enteral IAP on lymphocyte activation and recruitment within the intestine.

We have previously shown that IAP activity decreases in NEC and returns to near control levels with supplemental IAP administration. In all groups of NEC-stressed pups, we found statistically increased hepatic and pulmonary iNOS and TNF- α expressions compared with the control pups. Enteral administration of IAP does not affect the pulmonary or hepatic system during times of systemic inflammation caused by NEC. This may result from inadequate stress to the liver and lung. In the course of our experiments, we did not observe significant injury on liver or lung histology (data not shown). Alternatively, an explanation for the lack of effect on the hepatic and pulmonary system may be that enteral IAP may not be absorbed systemically. This description is supported by investigations demonstrating that IAP is expressed and secreted by enterocytes, localizes to the brush border, and is secreted into the intestinal lumen [53]. Although alkaline phosphatase is found in the serum, the majority consists of the tissue nonspecific alkaline phosphatase isoform produced by kidney, liver, and bone. IAP administered via intraperitoneal injection is taken up rapidly from the circulation by the asialoglycoprotein receptor on hepatocytes [17,54,55]. Therefore, enteral IAP administration may not be entering the circulation in a quantity sufficient to ameliorate lung and liver inflammation. Thus, the dramatic reduction of serum cytokines is likely because of the reduced intestinal injury and permeability, which resulted in decreased lymphocyte activation and migration to the intestine.

Our results raise interesting questions concerning enteral IAP administration in the NEC disease process. In the future, we will evaluate terminal ileal inflammation with IAP administration and examine NF- κ B, iNOS, and recruitment of active gut lymphocytes. Additionally, exploration of histologic correlation between cytokine expression and pulmonary and hepatic mRNA expression patterns may yield further information. Severity of histologic lung and liver injury requires refinement as well. It may be prudent to evaluate serum IAP activity after enteral IAP administration, as it appears that given our lung and liver results, minimal IAP entered the systemic circulation. Although liver function alterations with LPS administration have been demonstrated in the adult rat [17] and weaned piglet liver, there is evidence that serum transferase levels initially fall in response to LPS

challenge despite a rise in hepatic NF- κ B and evidence of hepatic injury [56]. In our study, we were limited by the quantity of serum sample obtained from our rat pups. Finally, the route of IAP administration may necessitate alteration once pulmonary and hepatic treatments are desired.

In conclusion, our results demonstrate that supplemental enteral IAP decreases systemic cytokine inflammation but does not ameliorate pulmonary or hepatic organ inflammation. Thus, enteral IAP may be useful as a preventative strategy in newborns at risk of NEC.

References

1. Christensen RD, Gordon PV, Besner GE. Can we cut the incidence of necrotizing enterocolitis in half—today? *Fetal Pediatr Pathol.* 2010; 29:185. [PubMed: 20594142]
2. Sankaran K, Puckett B, Lee DS, et al. Variations in incidence of necrotizing enterocolitis in Canadian neonatal intensive care units. *J Pediatr Gastroenterol Nutr.* 2004; 39:366. [PubMed: 15448426]
3. Guillet R, Stoll BJ, Cotten CM, et al. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. *Pediatrics.* 2006; 117:e137. [PubMed: 16390920]
4. Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatr Perinat Epidemiol.* 2006; 20:498. [PubMed: 17052286]
5. Llanos AR, Moss ME, Pinzon MC, Dye T, Sinkin RA, Kendig JW. Epidemiology of neonatal necrotising enterocolitis: a population-based study. *Paediatr Perinat Epidemiol.* 2002; 16:342. [PubMed: 12445151]
6. Sharma R, Tepas JJ 3rd, Hudak ML, et al. Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis. *J Pediatr Surg.* 2007; 42:454. [PubMed: 17336180]
7. Proulx F, Joyal JS, Mariscalco MM, Leteurtre S, Leclerc F, Lacroix J. The pediatric multiple organ dysfunction syndrome. *Pediatr Crit Care Med.* 2009; 10:12. [PubMed: 19057438]
8. Zufferey C, Erhart D, Saurer L, Mueller C. Production of interferon-gamma by activated T-cell receptor-alphabeta CD8alphabeta intestinal intraepithelial lymphocytes is required and sufficient for disruption of the intestinal barrier integrity. *Immunology.* 2009; 128:351. [PubMed: 20067535]
9. Shenkar R, Coulson WF, Abraham E. Hemorrhage and resuscitation induce alterations in cytokine expression and the development of acute lung injury. *Am J Respir Cell Mol Biol.* 1994; 10:290. [PubMed: 8117448]
10. Shenkar R, Chang YH, Abraham E. Cytokine expression in Peyer's patches following hemorrhage and resuscitation. *Shock.* 1994; 1:25. [PubMed: 7538033]
11. Shenkar R, Abraham E. Effects of hemorrhage on cytokine gene transcription. *Lymphokine Cytokine Res.* 1993; 12:237. [PubMed: 8218596]
12. Grotz M, Regel G, Schratt HE, Seekamp A, Pape HC, Tscherne H. Intestinal cytokine liberation after intestinal ischemia in the rat—studies in the Ussing chamber system. *Z Gastroenterol.* 1996; 34:783. [PubMed: 9082659]
13. Deitch EA, Xu D, Franko L, Ayala A, Chaudry IH. Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. *Shock.* 1994; 1:141. [PubMed: 7749933]
14. Beeby PJ, Jeffery H. Risk factors for necrotising enterocolitis: the influence of gestational age. *Arch Dis Child.* 1992; 67:432. [PubMed: 1586186]
15. Stevenson JK, Graham CB, Oliver TK Jr, Goldenberg VE. Neonatal necrotizing enterocolitis. A report of twenty-one cases with fourteen survivors. *Am J Surg.* 1969; 118:260. [PubMed: 5798396]

16. Touloukian RJ, Posch JN, Spencer R. The pathogenesis of ischemic gastroenterocolitis of the neonate: selective gut mucosal ischemia in asphyxiated neonatal piglets. *J Pediatr Surg.* 1972; 7:194. [PubMed: 5023196]
17. Tuin A, Huizinga-Van der Vlag A, van Loenen-Weemaes AM, Meijer DK, Poelstra K. On the role and fate of LPS-dephosphorylating activity in the rat liver. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:G377. [PubMed: 16223948]
18. Khailova L, Dvorak K, Arganbright KM, Williams CS, Halpern MD, Dvorak B. Changes in hepatic cell junctions structure during experimental necrotizing enterocolitis: effect of EGF treatment. *Pediatr Res.* 2009; 66:140. [PubMed: 19390485]
19. Halpern MD, Holubec H, Saunders TA, et al. Bile acids induce ileal damage during experimental necrotizing enterocolitis. *Gastroenterology.* 2006; 130:359. [PubMed: 16472592]
20. Halpern MD, Holubec H, Dominguez JA, et al. Hepatic inflammatory mediators contribute to intestinal damage in necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol.* 2003; 284:G695. [PubMed: 12529262]
21. Andorsky DJ, Lund DP, Lillehei CW, et al. Nutritional and other postoperative management of neonates with short bowel syndrome correlates with clinical outcomes. *J Pediatr.* 2001; 139:27. [PubMed: 11445790]
22. Moss RL, Das JB, Raffensperger JG. Necrotizing enterocolitis and total parenteral nutrition-associated cholestasis. *Nutrition.* 1996; 12:340. [PubMed: 8875518]
23. Koyama I, Matsunaga T, Harada T, Kikuno A, Hokari S, Komoda T. Ambroxol reduces LPS toxicity mediated by induction of alkaline phosphatases in rat lung. *Clin Biochem.* 2004; 37:688. [PubMed: 15302612]
24. Kristof AS, Goldberg P, Laubach V, Hussain SN. Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am J Respir Crit Care Med.* 1998; 158:1883. [PubMed: 9847282]
25. Speyer CL, Neff TA, Warner RL, et al. Regulatory effects of iNOS on acute lung inflammatory responses in mice. *Am J Pathol.* 2003; 163:2319. [PubMed: 14633605]
26. Speyer CL, Steffes CP, Tyburski JG, Ram JL. Lipopolysaccharide induces relaxation in lung pericytes by an iNOS-independent mechanism. *Am J Physiol Lung Cell Mol Physiol.* 2000; 278:L880. [PubMed: 10781417]
27. Speyer CL, Ward PA. Role of endothelial chemokines and their receptors during inflammation. *J Invest Surg.* 2011; 24:18. [PubMed: 21275526]
28. Zeidler PC, Millecchia LM, Castranova V. Role of inducible nitric oxide synthase-derived nitric oxide in lipopolysaccharide plus interferon-gamma-induced pulmonary inflammation. *Toxicol Appl Pharmacol.* 2004; 195:45. [PubMed: 14962504]
29. Lodha A, Asztalos E, Moore AM. Cytokine levels in neonatal necrotizing enterocolitis and long-term growth and neurodevelopment. *Acta Paediatr.* 2010; 99:338. [PubMed: 19922507]
30. Henricson BE, Benjamin WR, Vogel SN. Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. *Infect Immun.* 1990; 58:2429. [PubMed: 1695201]
31. Madonna GS, Vogel SN. Early endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools. *J Immunol.* 1985; 135:3763. [PubMed: 3905964]
32. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther.* 2003; 307:737. [PubMed: 12970380]
33. Koyama I, Matsunaga T, Harada T, Hokari S, Komoda T. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clin Biochem.* 2002; 35:455. [PubMed: 12413606]
34. van Veen SQ, van Vliet AK, Wulferink M, Brands R, Boermeester MA, van Gulik TM. Bovine intestinal alkaline phosphatase attenuates the inflammatory response in secondary peritonitis in mice. *Infect Immun.* 2005; 73:4309. [PubMed: 15972524]
35. Whitehouse JS, Riggle KM, Purpi DP, et al. The protective role of intestinal alkaline phosphatase in necrotizing enterocolitis. *J Surg Res.* 2010; 163:79. [PubMed: 20599220]

36. Caplan MS, Hedlund E, Hill N, MacKendrick W. The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology*. 1994; 106:346. [PubMed: 8299901]
37. Jilling T, Lu J, Jackson M, Caplan MS. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. *Pediatr Res*. 2004; 55:622. [PubMed: 14764921]
38. Nadler EP, Dickinson E, Knisely A, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *J Surg Res*. 2000; 92:71. [PubMed: 10864485]
39. Liedel JL, Guo Y, Yu Y, et al. Mother's milk induced Hsp70 expression preserves intestinal epithelial barrier function in an immature rat pup model. *Pediatr Res*. 2011; 69:395. [PubMed: 21263375]
40. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29:e45. [PubMed: 11328886]
41. Cerqueira NF, Hussni CA, Yoshida WB. Pathophysiology of mesenteric ischemia/reperfusion: a review. *Acta Cir Bras*. 2005; 20:336. [PubMed: 16186955]
42. Gourlay DM. The good and the bad of the innate immune response in necrotizing enterocolitis. *J Surg Res*. 2012; 175:51. [PubMed: 21920553]
43. Goepfert AR, Andrews WW, Carlo W, et al. Umbilical cord plasma interleukin-6 concentrations in preterm infants and risk of neonatal morbidity. *Am J Obstet Gynecol*. 2004; 191:1375. [PubMed: 15507968]
44. Romagnoli C, Frezza S, Cingolani A, et al. Plasma levels of interleukin-6 and interleukin-10 in preterm neonates evaluated for sepsis. *Eur J Pediatr*. 2001; 160:345. [PubMed: 11421413]
45. Minekawa R, Takeda T, Sakata M, et al. Human breast milk suppresses the transcriptional regulation of IL-1 β -induced NF- κ B signaling in human intestinal cells. *Am J Physiol Cell Physiol*. 2004; 287:C1404. [PubMed: 15229109]
46. Wedel T, Krammer HJ, Kuhnel W, Sigge W. Alterations of the enteric nervous system in neonatal necrotizing enterocolitis revealed by whole-mount immunohistochemistry. *Pediatr Pathol Lab Med*. 1998; 18:57. [PubMed: 9566283]
47. Edelson MB, Bagwell CE, Rozycki HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics*. 1999; 103:766. [PubMed: 10103300]
48. Emami CN, Chokshi N, Wang J, et al. Role of interleukin-10 in the pathogenesis of necrotizing enterocolitis. *Am J Surg*. 2012; 203:428. [PubMed: 22450025]
49. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993; 75:263. [PubMed: 8402911]
50. Claud EC, Lu L, Anton PM, Savidge T, Walker WA, Cherayil BJ. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci U S A*. 2004; 101:7404. [PubMed: 15123821]
51. Nanthakumar N, Meng D, Goldstein AM, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS One*. 2011; 6:e17776. [PubMed: 21445298]
52. Claud EC, Zhang X, Petrof EO, Sun J. Developmentally regulated tumor necrosis factor- α induced nuclear factor- κ B activation in intestinal epithelium. *Am J Physiol Gastrointest Liver Physiol*. 2007; 292:G1411. [PubMed: 17307728]
53. Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe*. 2007; 2:371. [PubMed: 18078689]
54. Blom E, Ali MM, Mortensen B, Huseby NE. Elimination of alkaline phosphatases from circulation by the galactose receptor. Different isoforms are cleared at various rates. *Clin Chim Acta*. 1998; 270:125. [PubMed: 9544450]
55. Meijer DK, Scholtens HB, Hardonk MJ. The role of the liver in clearance of glycoproteins from the general circulation, with special reference to intestinal alkaline phosphatase. *Pharm Weekbl Sci*. 1982; 4:57. [PubMed: 6180375]

56. Li Q, Liu Y, Che Z, et al. Dietary L-arginine supplementation alleviates liver injury caused by *Escherichia coli* LPS in weaned pigs. *Innate Immun*. 2012 Epub March 22.

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Fig. 1.

Serum cytokine levels on day of life 3 are decreased by supplemental enteral IAP administration. Rat pups were exposed to LPS and hypoxic stress in addition to formula feeding. Blood was collected on day of life 3 and serum assayed for cytokine levels using a multiplex bead-based immunoassay kit. Serum cytokine levels for (A) TNF-α, (B) IL-1β, (C) IL-6, and (D) IL-10 were determined in control (dam fed), NEC, and NEC + IAP40, 4, or 0.4 U/kg groups. Data are expressed as mean ± standard error of the mean. ****P 0.0001, *P 0.01.**

Fig. 2.

Hepatic iNOS and TNF- α mRNA expressions are unaltered by enteral IAP administration. Compared with the control group, the hepatic tissue in the NEC group has increased relative mRNA expression of both iNOS and TNF- α . Although there appears to be a decrease in the levels of both iNOS and TNF- α , these differences are not significant. (A) Relative iNOS mRNA expression, * $P = 0.001$. (B) Relative TNF- α liver mRNA expression, * $P = 0.005$.

Fig. 3.

Pulmonary iNOS and TNF- α mRNA expressions are unchanged by enteral IAP administration. Compared with the control group, the pulmonary tissue in the NEC group has increased relative mRNA expression of both iNOS and TNF- α . Although there appears to be a decrease in the levels of both iNOS and TNF- α , these differences are not significant. (A) Relative iNOS mRNA expression, * $P = 0.05$. (B) Relative TNF- α liver mRNA expression, * $P = 0.001$.