Journal of Pediatric Surgery 53 (2018) 1208–1214

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Loss of endothelial nitric oxide synthase exacerbates intestinal and lung injury in experimental necrotizing enterocolitis☆

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ARTICLE INFO

Article history:
Received 16 February 2018
Accepted 27 February 2018

Key words:
Necrotizing enterocolitis
Animal model
Nitric oxide
Neonatal
Intestine
Ischemia

ABSTRACT

Background: Necrotizing enterocolitis (NEC) continues to be a devastating condition among preterm infants. Nitric oxide, which is synthesized in the intestine by endothelial nitric oxide synthase (eNOS), acts as a potent vasodilator and antioxidant within the mesentery and may play a role in prevention of NEC. We hypothesized that loss of endothelial nitric oxide would worsen both intestinal and associated lung injury and increase local and systemic inflammation during experimental NEC.

Methods: NEC was induced in five-day-old wild type (WT) and eNOS-knockout (eNOSKO) mouse pups. Experimental groups (n = 10) were formula fed and subjected to intermittent hypoxic and hypothermic stress, while control groups (n = 10) remained with their mother to breastfeed. Pups were monitored by daily clinical assessment. After sacrifice on day nine, intestine and lung were assessed for injury, and cytokines were measured in tissue homogenates by ELISA. Data were compared with Mann–Whitney, and $p<0.05$ was significant.

Results: Each NEC group was compared to its respective strain's breastfed control to facilitate comparisons between the groups. Both NEC groups were significantly sicker than their breastfed controls. eNOSKO NEC animals had a median clinical assessment score of 3 (IQR = 1–5), and the WT NEC animal's median score was 3 (IQR = 2–5). Despite similar clinical scores, intestinal injury was significantly worse in the eNOSKO NEC groups compared to WT NEC groups (median injury scores of 3.25 (IQR = 2.25–3.625) and 2 (IQR = 1–3), respectively ($p = 0.0474$). Associated lung injury was significantly worse in the eNOSKO NEC group as compared to the WT NEC group (median scores of 8.5 (IQR = 6.75–11.25) and 6.5 (IQR = 5–7.5), respectively, $p = 0.0391$). Interestingly, cytokines in both tissues were very different between the two groups, with varying effects noted for each cytokine (IL-6, IL-1β, VEGF, and IL-12) in both tissues.

Conclusion: Nitric oxide from eNOS plays a key role in preventing the development of NEC. Without eNOS function, both intestinal and lung injuries are more severe, and the inflammatory cascade is significantly altered. Further studies are needed to determine how eNOS-derived nitric oxide facilitates these beneficial effects.

Necrotizing enterocolitis (NEC) is one of the most common and morbid conditions in the premature neonatal population [1]. It affects about 7% of preterm infants, and unfortunately has proven difficult to eradicate over the years. Mortality continues to range from 20% to 30%, and is highest in patients with surgical disease [2]. Extensive surgical resection remains the mainstay of therapy for advanced disease, and often leads to long term morbidity secondary to short bowel syndrome [3]. New therapies for this disease are needed, and further understanding of the mechanism of disease is integral for further research in this direction.

Unfortunately, the sequelae of NEC are not limited to the intestine. Associated lung injury is a well-described phenomenon in NEC patients and animal models [4]. One of the highest risk factors for development of chronic lung disease in premature infants is the presence of necrotizing enterocolitis [5]. Additionally, lung disease in patients with NEC is commonly more severe than in matched patients without NEC [6].

The development of NEC is multifactorial, but seems to be directly related to intermittent insults secondary to hypoxia, hypothermia and enteral feeding in an already susceptible intestine [7]. The pathogenesis is complex and not completely understood. While intestinal mucosal

☆ This work was made possible with support from:1) KL2TR001106 (A. Shekhar, PI) from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award2) Indiana University Health, Indianapolis, IN.

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https://doi.org/10.1016/j.jpedsurg.2018.02.087
0022-3468/

epithelial injury is a defining characteristic of NEC, microvascular injury, and specifically, endothelial alterations, are thought to play a role as well [8].

Endothelial nitric oxide synthase (eNOS) is a constitutively expressed enzyme that produces nitric oxide [9]. NO is a gaseous signaling molecule endogenously produced in the endothelium of all tissues by eNOS and has been identified as the most important vasodilator in the perinatal period in animal models [10]. Mice with homozygous knockout of eNOS (eNOSKO) were found to have worse experimental NEC pathology in the intestine [9,11]. Another study demonstrated that L-arginine, an eNOS substrate, reduced injury, while nitric oxide inhibitors such as N-nitroarginine methyl ester (L-NAME) worsened injury in a neonatal piglet model of NEC [12]. The role of eNOS has not yet been thoroughly evaluated in the lungs of animals with experimental NEC.

In addition to direct impact on the affected tissues, as a free radical, NO is intimately involved in the inflammatory signaling seen in necrotizing enterocolitis [13]. Specifically, certain cytokines are known to have a close relationship with endothelial NO as a mediator in the inflammatory cascade. For example, interleukin 6 (IL-6) has been shown to be modulated by NO in some tissues [14]. Vascular endothelial growth factor (VEGF) is an endothelial specific marker that is typically elevated during endothelial cell injury or hypoxia, but is dependent on nitric oxide for release [15]. Interleukin 1β (IL-1β) is also typically released by the endothelium in response to stress [16]. Finally interleukin 12 (IL-12) has been shown in vitro to be upregulated in the endothelium by inhibition of eNOS [17]. These are just a few of the cytokines involved in the complex systemic response to inflammation, of which endothelial NO is an integral mediator.

Observations in animal studies suggest that eNOS has a critical role in the development of NEC intestinal injury. We therefore hypothesized that eNOS would not only be a key component in limiting intestinal injury, but would also play a key role in limiting lung injury and altering the systemic inflammatory response during experimental NEC.

1. Materials and methods

1.1. Experimental NEC model

Indiana University Institutional Animal Care and Use Committee approved the experimental protocol and animal use. Wild type (WT) mouse pups (C57BL/6J, bred in house from adult mice, Stock No: 00664, Jackson Laboratory, Bar Harbor, ME) and eNOSKO mice (B6.129P2-Nos3tm1Unc/J, bred in house from adult mice, Stock No: 002684, Jackson Laboratory, Bar Harbor, ME) were permanently separated from their mother on postnatal day (P) five. Pups were housed in a neonatal incubator with humidity 40% and temperature 32 °C from P5 to P9. All groups had 10 pups per group. The control groups of both strains remained with their mother and breastfed ad libitum. Experimental groups were gavage fed hyperosmolar formula three times daily with a 2-French silicone catheter. Formula was prepared using 4 g of Esbilac canine supplement and 6 g Similac in 20 mL of nanopure filtered water (Barnstead Nanopure, APS Water Services Inc., Van Nuys, CA). Formula was newly prepared every 48 h. Animals were fed 300 kcal/kg/day and all feeds were supplemented with 8 mg/kg/day lipopolysaccharide (LPS, lipopolysaccharides from *Escherichia coli* O111: B4, Sigma-Aldrich Company LLC, Dorset, UK).

Before each feed, pups were stressed in a chamber with 5% O_2 and 95% N_2 for 10 min. Twice daily, after the morning and evening feeds, pups were placed in the 4 °C refrigerator for 10 min. Because of the high rate of very low birth weight in the knock out strain specifically, litters with pups less than 1.5 g in weight were excluded owing to the technical difficulties of gavage feeding animals this small. Mice who died less than 24 h into the protocol were excluded, as their death was more likely because of other causes than NEC. Any animal whose tissue was liquefied or unusable for histologic evaluation (i.e. died overnight and discovered hours later) was excluded completely from evaluation. Other animals that died during the study but were identified immediately were still included in analysis for all data points. This included 1 in the eNOSKO NEC group and 2 in the WT NEC group.

1.2. Clinical assessment

While control groups were weighed and assessed daily to minimize maternal separation, experimental groups were weighed daily but reassessed with each feed. Assessment proceeded in a systematic fashion to ensure consistency. The reported score is the pup's last score prior to death or euthanasia. Clinical sickness score was slightly modified from Zani et al. [18] to include specific criteria for scoring as previously reported.

1.3. Intestinal histologic evaluation

On P9, pups were sacrificed by decapitation and the terminal ileum and distal jejunum of each animal were then formalin fixed, paraffin embedded, and stained with hematoxylin and eosin. Two blinded authors evaluated degree of injury, and scores were averaged for each specimen. The scoring system used was published by Zani et al. [18] with scores ranging from 0 to 4: 0 = normal intestine; 1 = disarrangement of villus enterocytes, villus-core separation; 2 = significant disarrangement of villus enterocytes, villus-core separation down sides of villi, blunting of villi; 3 = epithelial sloughing of villi, loss of villi; 4 = intestinal necrosis or perforation. A score of 2 or higher was considered consistent with development of experimental NEC. Severe NEC was defined as grade 3 or 4 intestinal injury.

1.4. Lung histologic evaluation

At the time of euthanasia, the right lower lobe of the lung was formalin fixed, paraffin embedded, and stained with hematoxylin and eosin. Two blinded authors evaluated degree of injury using a modified version of the scale developed by the American Thoracic Society (ATS). The scale was modified to be more appropriate for immature lungs (Table 1). Scores from 0 (normal) to 2 (acute lung injury) are given in six different parameters and added together to yield a score between 0 and 12, with 0 representing normal lung and 12 indicating severe acute injury with hemorrhage [19].

1.5. Cytokine analysis

Following euthanasia, sections of small intestine and lung were snap frozen in liquid nitrogen and stored at −80 °C. Tissue was thawed and homogenized with the Bullet Blender (Next Advance, Averill Park, NY) in RIPA buffer (Sigma, St. Louis, MO) with 1:100 dilutions of both phosphatase and protease inhibitors (Sigma, St. Louis, MO). After homogenization, samples were centrifuged at 12,000 rpm and supernatants were collected for further analysis. Total protein was quantified with the Bradford Assay using a spectrophotometer (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA).

Table 1
Lung injury scoring system

Parameter	Score per field (400× magnification)		
	0	1	2
Neutrophils in the alveolar space	None	1-5	>5
Neutrophils in the interstitial space	None	1-5	>5
Hyaline membranes	None	1	>1
Proteinaceous debris filling the airspaces	None	1	>1
Alveolar septal thickening	<2×	2×–4×	>4×
Red blood cells in alveolar space	Few	Half filled	Filled

Lung injury score = sum of all scores, range from 0 to 12.