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Original Contribution

Nitrosative stress in an animal model of necrotizing enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is a disease of newborns characterized by gut barrier failure. We reasoned that upregulation of inducible nitric oxide synthase (iNOS) may result in nitrosative stress and accumulation of nitroso species in the intestine. Newborn rats were either breast-fed (BF), or formula-fed and additionally subjected to hypoxia (FFH). At Day 4 after birth, the distal ilea were harvested and processed for Western blot analysis and measurement of NO-related metabolites. While BF neonates showed normal morphology, FFH neonates developed signs of NEC by Day 4. These pathological changes correlated with upregulation of iNOS and increases in tissue nitrite, nitrosothiol, and nitrosamine concentrations. Enhanced nitroso levels were most prominent in the mucosal layers of the ileum and iNOS inhibition resulted in a significant decrease in both nitroso species and incidence of NEC. In contrast, increased nitrite levels were distributed evenly throughout the ileum and remained unchanged following iNOS inhibition. Similarly, specimens from NEC patients had higher intestinal levels of NO-related metabolites compared to non-NEC controls. This is the first report of tissue levels of nitroso species in the gut of an animal model of NEC and of human specimens. The results suggest that local nitrosative stress contributes to the pathology associated with NEC. Unexpectedly, the NO breakdown product nitrite, previously considered biologically inert, was found to be present throughout the ileal wall, suggesting that cellular NO metabolism is altered significantly in NEC. Whether nitrite plays a protective or deleterious role remains to be investigated.

Keywords: Nitric oxide; Nitrite; Nitrosation; Hypoxia; Inflammation; Necrotizing enterocolitis; Free radical

Introduction

Necrotizing enterocolitis (NEC) is the most frequent and the most lethal disease that affects the gastrointestinal system of the premature infant [1]. The overall mortality rate for patients with NEC ranges from 10 to 70% [2] and approaches 100% for patients with the most severe form of the disease, which is characterized by involvement of the entire bowel (pan-necrosis) [3]. In addition to the poor prognosis, NEC represents one of the most complex diseases seen in the neonatal population. Although numerous risk factors including prematurity [4], hypoxia [5], formula feeding [6], bacterial infection [7], and intestinal ischemia [8] have been implicated in the pathogenesis of NEC, the exact etiology of the disease remains undefined.

Numerous inflammatory mediators have been implicated in the cascade of events leading to the hemodynamic instability associated with sepsis. Elevated plasma levels of IL-6 (interleukin-6) and TNF-α (tumor necrosis factor-alpha) have been measured in infants with NEC, bacterial

Abbreviations: NEC, necrotizing enterocolitis; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; LPS, lipopolysaccharide; BF, breast-fed; FFH, formula-fed and subjected to hypoxia; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PAF, platelet activating factor; IL-6, interleukin-6; TNFα, tumor necrosis factor-alpha; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NNA, N^{ω}-nitro-L-arginine; ROS, reactive oxygen species; RNOS, reactive nitrogen oxide species.

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sepsis, or meningitis [9]. More recently, nitric oxide (NO) has been shown by our group to modulate intestinal changes in septic shock [10,11]. Furthermore, the end products of NO metabolism have been shown to be elevated in newborn infants as well as adult patients with clinical sepsis, indicating increased NO production [12,13]. Nitric oxide synthase (NOS) is the principal enzyme responsible for the formation of NO in biological systems. The inducible isoform, also called iNOS or NOS-2, is induced by a variety of stimuli and cellular stresses including hypoxia, lipopolysaccharide (LPS), and proinflammatory cytokines, and its role has been widely studied in different experimental and clinical settings [14,15]. In this respect, we have previously reported that iNOS mRNA is upregulated in the intestine of infants with acute NEC, but returns to basal levels by the time of stoma closure, when the patients have recovered from the acute illness [10].

In order to understand the pathogenesis of NEC, we have revisited an animal model originally described by Barlow et al. that uses formula feeding after a brief period of hypoxia to induce experimental NEC [16]. We have been able to establish a similar model in our laboratory and have used it to study the expression of various cytokines in the intestine [17]. The objective of the present study was twofold: first, we wanted to determine the expression of iNOS protein in the gut of newborn rats subjected to hypoxia and formula feeding. Second, because we hypothesized that upregulation of iNOS in the gut leads to excessive production of NO and perhaps other redox-related forms, we sought to measure the levels of NO-derived metabolites in the intestinal segments as possible biomarkers of nitrosative stress. We here show that hypoxia and formula feeding cause pathological changes in a rat model of NEC that are accompanied by an upregulation of iNOS expression and a concomitant increase in NO production leading to nitrosative stress and accumulation of endogenous nitroso species.

Materials and methods

Animal model of NEC

All the experiments were carried out following an animal protocol approved by the Animal Research and Care Committee (ARCC) of the Children's Hospital of Pittsburgh (Protocol No. 31/93). Pregnant time-dated Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were induced at term using a subcutaneous injection (1 to 2 U per animal) of Pitocin (Monarch Pharmaceuticals, Bristol, TN). Immediately after birth, the neonates were weighed and randomized into one of the different treatment groups. Group 1 consisted of neonatal rats left with their mother, and thus they were breast-fed (BF). Group 2 consisted of neonates separated from their mothers, housed in a temperature- and humidity-controlled incubator (Ohio Medical Products, Madison, WI) and gavaged with a special rodent formula (0.2 ml, see below) two times per day and subjected to 10 min of hypoxia (5% O_2, 95% N_2) (Prax Air, Pittsburgh, PA) thrice daily in a modular Chamber (Billups-Rothenberg Inc., Del Mar, CA) as follows: pups were fed in the morning posthypoxia, exposed to a second hypoxic insult after 4 h, and then subjected to the final hypoxic insult followed by the final feed (FFH). The formula composition consists of 15 g Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac canine milk replacer (Pet-Ag Inc., Hampshire, IL) as described by Barlow et al. [16], and was designed to approximate the protein and caloric content of rat breast milk. The rats were sacrificed on different days as indicated and the intestinal samples (segments of terminal ileum) were harvested for morphological studies and Western blotting as described below. For the time course studies, the animals grouped into Day 0 were sacrificed within 3 h after birth, and were neither fed nor subjected to the hypoxic insult.

Morphological evaluation of intestinal samples

Rats were sacrificed on different days as indicated. The intestines were inspected for gross necrotic changes, pneumatosis intestinalis, and the last 2 cm of terminal ileum was harvested for morphological studies. Hematoxylin and eosin (H&E) slides were prepared as per standard protocol [18] and examined by light microscopy. The presence of morphological changes in the intestinal epithelium, including separation of the villous core, submucosal edema, and epithelial sloughing, were determined and graded by a pathologist from Children's Hospital of Pittsburgh blinded to the experimental groups as previously described [17,19].

Collection of human intestinal specimens

The Human Rights Committee of Children's Hospital of Pittsburgh approved collection of operative specimens for experimental purposes (Protocol No. 02-208). We compared two newborn patients undergoing bowel resection for NEC with two neonates undergoing intestinal resection for inflammatory conditions other than NEC. Diagnosis of NEC was confirmed histologically by the hospital pathologist. At laparatomy, representative intestinal segments of the surgical specimen (jejunum and ileum, each from a different patient) were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Quantification of nitrite, nitrate, and nitroso compounds was performed as described below.

Western blot analysis

Newborn rats were sacrificed as indicated and segments of the terminal ileum or grossly diseased intestine were resected. The mucosa was gently scraped from each