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The role of Nitric Oxide in Intestinal Epithelial Injury and Restitution in Neonatal NEC

Nikunj K. Chokshi, MD [Research Fellow],

Department of Pediatric Surgery, Childrens Hospital Los Angeles, Los Angeles, CA 90027

Catherine J. Hunter, MD [Research Fellow],

Department of Pediatric Surgery, Childrens Hospital Los Angeles, Los Angeles, CA 90027

Yigit S. Guner, MD [Research Fellow],

Department of Pediatric Surgery, Childrens Hospital Los Angeles, Los Angeles, CA 90027

Anatoly Grishin, PhD [Research Associate Professor], and

Department of Pediatric Surgery, Childrens Hospital Los Angeles, Los Angeles, CA 90027

Henri R. Ford, MD [Vice-President and Surgeon-in-Chief]

Childrens Hospital Los Angeles, Professor and Vice-Chairman, Department of Surgery, Keck School of Medicine, University of Southern California

Abstract

Necrotizing Enterocolitis (NEC) is the most common life-threatening gastrointestinal disease encountered in the premature infant. Although the inciting events leading to NEC remain elusive, various risk factors including prematurity, hypoxemia, formula feeding and intestinal ischemia have been implicated in the pathogenesis of NEC. Data from our lab and others suggest that NEC evolves from disruption of the intestinal epithelial barrier, as a result of a combination of local and systemic insults. We postulate that nitric oxide (NO), an important second messenger and inflammatory mediator, plays a key role in intestinal barrier failure seen in NEC. Nitric oxide and its reactive nitrogen derivative, peroxynitrite, may affect gut barrier permeability by inducing enterocyte apoptosis (programmed cell death) and necrosis, or by disrupting tight junctions or gap junctions that normally play a key role in maintaining epithelial monolayer integrity. Intrinsic mechanisms that serve to restore monolayer integrity following epithelial injury include enterocyte proliferation, epithelial restitution via enterocyte migration, and re-establishment of cell contacts. This review focuses on the biology of NO and the mechanisms by which it promotes epithelial injury while concurrently disrupting the intrinsic repair mechanisms.

INDEX WORDS

Necrotizing Enterocolitis; Nitric Oxide; Intestinal inflammation; Intestinal restitution

Necrotizing Enterocolitis (NEC) is the most common life-threatening gastrointestinal disease encountered in the premature infant. The incidence of NEC approaches 1 per 1000 live-births, with approximately 1 out of 7 affected neonates succumbing to the disease¹. The mortality rate

*Correspondence should be addressed to: Henri R. Ford, MD, Department of Pediatric Surgery, 4650 Sunset Blvd, Mailstop #72, Los Angeles, CA 90027, Tel: 323-361-2104, Fax: 323-361-8678.

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for the most severe form of the disease, which is characterized by involvement of the entire intestine, approaches 100%². Although the inciting events leading to NEC remain elusive, various risk factors including prematurity, hypoxemia, formula feeding and intestinal ischemia have been implicated in the pathogenesis of NEC. Advances in neonatology over the last few decades have increased survival of very low birth weight infants. As a result, the population of patients at risk for developing NEC is increasing. Given the decreased mortality from respiratory distress syndrome³, current trends predict that NEC may soon become the leading cause of death in premature infants⁴. Because of the increasing incidence of NEC and the associated mortality and long-term complications seen in surviving patients, the primary focus of research in NEC has shifted to the prevention and treatment of the disease.

Our group and others have shown that the pathogenesis of NEC involves disruption of the intestinal epithelial barrier, due to a combination of local and systemic insults⁵. The intact intestinal barrier comprises several layers of defense against bacterial invasion such as the mucus layer, the epithelium, and the underlying mucosal immune system. The intestinal epithelial barrier promotes immune surveillance against intraluminal microorganisms and macromolecules, by facilitating the continuous sampling of luminal antigens. However, pathologic or dysregulated transfer of antigens across the intestinal barrier can lead to various diseases, including inflammatory bowel disease in adults, and NEC in premature infants^{6, 7}. Intestinal epithelial cells function as both antigen processing and immune effector cells, producing pro-inflammatory cytokines and other mediators when stimulated⁸. Clinical studies have shown that inflammatory mediators, such as TNF α , IL-1, platelet activating factor, and nitric oxide (NO), produced by enterocytes and macrophages, contribute to the pathogenesis of NEC^{9–11}.

We postulate that NO, an important second messenger and inflammatory mediator, plays a key role in intestinal barrier failure seen in NEC. This review focuses on the biology of NO and the mechanisms by which it mediates intestinal epithelial injury.

Biology of NO

NO is a short-lived reactive molecule that plays an important role in many physiological and pathophysiological processes. It was first described as endothelial derived relaxing factor (EDRF); a potent, short-lived vasodilator. Subsequent studies revealed that in addition to its role as a regulator of blood vessel tone, NO also modulates a variety of physiological processes including tissue homeostasis, neurotransmission, and inflammation^{12, 13}. Nitric oxide is the product of NO synthase (NOS), which catalyzes the conversion of arginine and oxygen (O₂) into NO and citrulline (see Figure 1). There are three isoforms of NOS, each encoded by a different gene. Two of these isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), are expressed constitutively at low levels; these enzymes produce picomolar concentrations of NO. Endothelial NOS and nNOS contain a calmodulin-binding domain and are activated by calmodulin in a calcium-dependent manner. The third isoform, inducible NOS (iNOS), is calcium-independent, as it binds calmodulin with high affinity under calcium replete physiological conditions¹⁴. In contrast, iNOS is not expressed under normal conditions, but is induced in high levels during inflammation. Once iNOS is expressed, it produces NO in nanomolar to micromolar concentrations¹⁵.

A thorough knowledge of the chemical properties of NO is important for understanding its biologic effects. Nitric oxide is an intermediate oxidation product of nitrogen; it has an unpaired electron and therefore can be considered a free radical. In the presence of other free radicals, NO propagates free radical induced damage. It reacts readily with oxidants to form more stable, higher nitrogen oxides such as nitrite and nitrate, which accounts for the short life span of NO under physiologic conditions. Furthermore, NO diffuses rapidly away from production sites.

Because it is soluble in both water and lipids, NO can efficiently diffuse across biologic membranes and through cells without requiring cell receptors or channels¹⁶.

Nitric oxide signaling is complex, and much of its functionality is based on the rapid movement of the molecule. Because it is freely permeable, NO can rapidly move in and out of neighboring cells over the span of a second. It has a limited life span under physiologic conditions (estimated half-life of 1 sec), and acts locally at a distance equivalent to 4–5 cells. Thus, the paracrine effects of NO decrease with distance as a result of this diffusion capacity rather than actual consumption of the molecule. The combination of rapid diffusion and short life span allows NO to synchronously stimulate and activate groups of cells¹⁷.

In vivo, NO is scavenged mainly by reacting with hemoglobin in red blood cells. Because of scavenging by hemoglobin, endothelial cells need to produce 100nM NO to achieve 5–10nM concentrations in adjacent targets, such as smooth muscle cells¹⁸. The best understood physiological effects of NO are mediated by cyclic GMP (cGMP). Soluble guanylyl cyclase, the enzyme that catalyzes production of cGMP from GTP contains protoporphyrin IX, with iron in the ferrous state, which binds NO with 10,000 fold greater affinity than molecular oxygen¹⁹. Binding of NO activates soluble guanylyl cyclase. Production of cGMP leads to activation of cGMP-dependent protein kinases and cytosolic calcium depletion in target cells. It is believed that cGMP mediates most physiologic effects of NO in many cell types.

The relative specificity and safety of NO are evidenced by the widespread presence of this molecule throughout the vasculature and nervous system. Lack of NO toxicity under normal conditions is due to the fact that eNOS and nNOS do not produce high concentrations of NO. Moreover, NO produced by eNOS rapidly diffuses into red blood cells, where it is converted into nitrate upon reaction with oxyhemoglobin. However, NO produced under inflammatory conditions may be toxic for several reasons. First, induction of iNOS during inflammation leads to local concentrations of NO in the micromolar range, as opposed to nanomolar range under normal conditions¹⁵. Second, unlike eNOS-derived NO, iNOS-derived NO cannot be rapidly scavenged by red blood cells. Third, in the absence of the reaction with hemoglobin that produces relatively non-toxic nitrate species, NO may react with superoxide anion, which produces the highly toxic oxidation intermediate peroxynitrite²⁰. Like NO, superoxide is produced at inflammatory sites as a consequence of the induction of oxidative enzyme complexes, such as NADPH oxidases and xanthine oxidase.

Superoxide is normally detoxified by superoxide dismutase (SOD); however, NO reacts with superoxide faster than SOD, thus countering the protective effect of SOD and leading to the production of peroxynitrite instead. Because of the diffusion abilities of NO, superoxide and NO do not need to be produced in the same cell in order to form significant amounts of peroxynitrite. With the close proximity of NO and superoxide at inflammation sites, the formation of peroxynitrite is consistent, and is considered a hallmark of the inflammatory process²¹.

Although peroxynitrite is not a free radical, it is much more reactive than its parent molecules NO and O₂²⁰. Its half-life under physiologic conditions is only 10–20 ms, but this time is sufficient to allow diffusion across membranes, crossing one to two cell spans²¹. Peroxynitrite exerts its effects on proteins through two mechanisms. It can directly oxidize target protein moieties, including thiols, iron/sulfur centers, and zinc fingers²². These reactions are among the fastest known for peroxynitrite²³. In addition to its direct effects, peroxynitrite indirectly mediates oxidation of biomolecules by decomposing into highly reactive radicals, which include the hydroxyl radical and nitrogen dioxide (NO₂[•]). Peroxynitrite can nitrate tyrosine residues in proteins, resulting in the formation of nitrotyrosine. Because nitration of tyrosine

is an alternative to phosphorylation at key residues, it can affect a protein's enzymatic activity and interfere with intracellular signaling processes²⁴.

NO and NEC

Current theory regarding the pathogenesis of NEC suggests that the disease is initiated by perinatal insults to the immature intestine, including formula feeding, bacterial colonization of the gut, hypoxia, and hypoperfusion. These insults result in an initial epithelial injury that may cause intestinal inflammation with release of inflammatory mediators, leading to impaired gut barrier function. Luminal bacteria then translocate across the compromised barrier, further propagating the inflammatory cascade and exacerbating epithelial injury. Extensive barrier failure and ensuing intestinal tissue necrosis are the ultimate manifestations of NEC^{5, 25}. We have shown that NO plays an important role in intestinal epithelial injury in NEC. The role of iNOS-derived NO was strongly implicated first by the work of Ford and colleagues, who found an upregulation of iNOS mRNA and protein in infants undergoing laparotomy for NEC, as compared to those undergoing intestinal resection for other reasons¹¹.

Both constitutive and inducible isoforms of NOS are expressed in the gastrointestinal tract. The constitutive calcium-dependent eNOS isoform is expressed in the myenteric plexus, endothelial cells, gastric epithelial cells, and enterocytes^{26, 27}. Within the intestinal epithelium, NO is believed to modulate water and electrolyte transport, as well as mucosal permeability^{28, 29}. Inhibition of NOS leads to an increase in transepithelial passage of ⁵¹Cr-EDTA, suggesting a role for NO in maintaining mucosal integrity²⁹. NO also acts to enhance mucosal blood flow and maintain microvascular tone. NO inhibits platelet aggregation and adhesion of leukocytes³⁰. Guinea pigs treated with NOS inhibitors develop ileitis²⁵. These data suggest that low levels of NO produced by eNOS may be required for the maintenance of intestinal homeostasis¹⁵.

In contrast, sustained overproduction of NO as a result of iNOS upregulation may have cytopathic effects on the epithelium, leading to intestinal barrier failure. As discussed earlier, peroxynitrite, a product of the reaction of NO with superoxide, is largely responsible for the detrimental effects of NO during inflammation. Whereas iNOS is expressed in the ileum of mice colonized with bacteria³¹, endotoxin (LPS) stimulation significantly increases its expression^{27, 32}. iNOS mRNA and protein are also upregulated in the rat intestine after LPS administration, leading to derangement in intestinal barrier function. However, treatment with the iNOS inhibitor aminoguanidine ameliorated the epithelial damage and decreased bacterial translocation across the epithelium. Aminoguanidine also decreased bacterial translocation and prevented epithelial sloughing in mice challenged with high doses of LPS³³. The iNOS scavenger, NOX, has similar effects: it abrogates LPS-induced bacterial translocation, decreases the levels of enterocyte apoptosis, and inhibits the formation of 3-nitrotyrosine, the molecular footprint of peroxynitrite³⁴. Moreover, iNOS knockout mice exhibit lower levels of LPS-dependent bacterial translocation than their wild type littermates³⁵. Taken together, these data indicate a key role of iNOS-derived NO in the gut barrier failure during experimental inflammation.

Sustained NO production has also been found in human patients suffering from various intestinal inflammatory disorders. Patients with active ulcerative colitis have high levels of iNOS and increased 3-nitrotyrosine immunoreactivity in the colonic epithelium³⁶. *Helicobacter pylori*-induced gastritis is also associated with increased iNOS expression in antral biopsies performed during active disease, as evidenced by 3-nitrotyrosine immunostaining and enterocyte apoptosis³⁷. High levels of iNOS upregulation have been found in resected intestinal tissue from patients with acute NEC¹¹. Interestingly, the expression of iNOS mRNA and protein co-localized with areas of epithelial injury (enterocyte apoptosis

and necrosis) along the crypt-villus axis. Furthermore, immunoreactivity to 3-nitrotyrosine was noted in the epithelium and lamina propria at the sites of enterocyte apoptosis. At the time of stoma closure, when the infants had recovered from acute NEC and the inflammation had subsided, the expression of iNOS mRNA had returned to basal levels¹¹. Collectively, these data suggest a strong correlation between iNOS expression, NO and peroxynitrite production on the one hand, and mucosal injury on the other hand. The induction of intestinal barrier failure through enterocyte apoptosis and failure of appropriate restitution of the epithelium are believed to be key.

To better understand the role of NO in the pathogenesis of NEC, we employed an animal model. Many experimental models of NEC use invasive surgical procedures to induce ischemia, or intravenous infusion of pro-inflammatory mediators to induce inflammation. While these models may reproduce the morphological characteristics of human NEC, they do not parallel the pathophysiology of the disease. Our lab has developed a rat model of NEC whereby newborn rat pups are subjected to formula feeding and hypoxia thrice daily; control animals are breast-fed by their mothers. A high percentage of animals from the formula/hypoxia group develop varying degrees of intestinal inflammation, characterized by submucosal edema, neutrophil infiltration, and epithelial sloughing³⁸. This model relies on perinatal insults similar to those associated with, and reproduces pathology similar to, human NEC. It has also been a valuable tool to study the expression profile of inflammatory mediators and iNOS in experimental NEC. Importantly, the pattern of cytokine expression and iNOS upregulation in this model parallels that seen in human NEC. In the rat model of NEC, there is increased iNOS and IFN γ , with decreased IL-12 production³⁸. This model has recently been adapted to mice^{39–41}, allowing the use of genetically modified animals to address the roles of specific genes in the pathogenesis of NEC. We are currently using iNOS knockout mice to further define the role of iNOS-derived NO in the pathogenesis of NEC.

Effects of NO and peroxynitrite on the intestinal epithelium: Apoptosis, Proliferation and Migration

NO and Apoptosis pathways

The mucosal surface of the gastrointestinal tract consists of a vast epithelial monolayer that serves as a physical barrier to the entry of intraluminal antigens and microorganisms. Maintenance of intestinal barrier integrity is the result of a dynamic equilibrium between tissue injury and tissue repair mechanisms such as epithelial restitution and proliferation. Factors that may affect gut barrier integrity include enterocyte apoptosis (programmed cell death) and necrosis, as well as disruption of tight junctions or gap junctions that normally play a key role in maintaining epithelial monolayer integrity. Intrinsic mechanisms that serve to restore monolayer integrity following epithelial injury include enterocyte proliferation, epithelial restitution via enterocyte migration, and re-establishment of cell contacts. The mechanisms by which NO and its toxic nitrogen derivative adduct, peroxynitrite, promote their cytopathic effects while concurrently disrupting the repair mechanisms will be discussed.

Enterocyte apoptosis at the villus tip is a normal physiologic process that plays a key role in the continuous renewal of the intestinal epithelium. However, we and others have reported increased enterocyte apoptosis in both human and experimental NEC^{11, 38}. The increased enterocyte apoptosis co-localizes with upregulation of iNOS activity as well as 3-nitrotyrosine immunostaining, suggesting that peroxynitrite plays a role in this process. Peroxynitrite has been shown to induce apoptosis in a variety of cell types, including undifferentiated neural cells (PC12), intestinal epithelial cells (T84), HL-60 myeloid cells, macrophages, and neurons^{42–44}. To further delineate the mechanisms by which peroxynitrite exerts its cytopathic effects on the intestinal epithelium, we exposed IEC-6 cells, a non-transformed rat

epithelial cell line of crypt origin, to peroxynitrite, and examined the effects on apoptosis, proliferation and migration. Our data show that peroxynitrite induces apoptosis in the IEC-6 cells in a time- and dose-dependent manner, at concentrations that are readily attainable in vivo. Such enterocyte apoptosis involves activation of caspase-3⁴⁵.

NO can induce apoptosis directly or indirectly. NO can reversibly inhibit enzymes that produce free radical intermediates, such as those involved in the mitochondrial electron transport chain (ETC). When NO inhibits cytochrome-c oxidase, superoxide may transiently leak from the ETC, leading to the formation of peroxynitrite⁴⁶. While the reactions of NO with enzymes are usually reversible, the reactions of peroxynitrite are not. Through oxidation of critical cysteine residues, peroxynitrite irreversibly inactivates many components of the ETC, including complex I, complex II, complex III and complex V⁴⁷, leading to inhibition of oxidative phosphorylation and ultimately, cell death. Experiments in our laboratory have shown that exposure of IEC-6 cells to peroxynitrite de-energizes the mitochondria as judged by 3,3'-dihexyloxacarbocyanine iodide (Mitosensor) staining (Ford HR, unpublished). Electron microscopy of peroxynitrite-treated cells reveals rounding of mitochondria and disruption of the internal mitochondrial cristae⁴⁸.

Exposure to peroxynitrite leads to cellular damage, ultimately leading to cell death via two possible mechanisms, necrosis or apoptosis. The classification of the type of cell death is based on morphological characteristics, enzymological criteria, functional aspects, or immunological characteristics; however, the distinction between necrosis and apoptosis is not always clear⁴⁹. Cell death primarily through inhibition of mitochondrial respiration and loss of ATP production is normally classified as necrotic. By contrast, apoptotic cell death is characterized by the ATP-dependent activation of cysteine proteases (caspases).⁵⁰ The caspases are a family of cysteine proteases, which may be triggered via the activation of death receptors (extrinsically) or by the permeabilization of the outer membrane of mitochondria (intrinsically).

Although the loss of mitochondrial function is a characteristic of necrosis, mitochondria are intricately involved in the execution of the apoptotic program. Mitochondrial outer membrane permeabilization (MOMP) leads to the release of cytochrome c (cyt c) into the cytosol. Cyt c is a key activator of caspases. Although peroxynitrite may cause necrosis by de-energizing the mitochondria, it can also lead to apoptotic cell death via activation of the caspase machinery. This has been shown in many cell types, including PC12⁵¹, fibroblasts⁵², neurons⁵³, neutrophils⁵⁴, and endothelial cells⁵⁵. While the induction of apoptosis in these varied cell types requires different experimental conditions, it appears that MOMP and the resulting efflux of proapoptotic signaling molecules occurs universally following peroxynitrite exposure. Cytosolic cyt c acts in concert with apoptosis activating factor-1 (Apaf-1) to activate pro-caspase 9. Active caspase 9 is an initiator caspase, which in turn activates effector caspases, such as caspases 3 and 7. Active effector caspases execute apoptosis by dismantling key proteins and cellular structures⁵⁶. Caspases execute apoptosis not only by activation of pro-apoptotic pathways, but also by mitigating survival signals, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI 3-K) pathways⁵⁷.

MAPK pathways are another class of targets in peroxynitrite-induced cell death. MAPKs are serine/threonine protein kinases that are activated by mitogens and stresses. MAPK signaling cascades regulate multiple cellular processes including proliferation, differentiation, survival, and apoptosis. There are three families of MAPK: extracellular response kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 stress response kinases.

The ERK pathway is activated by growth factors, as well as oxidants and free radicals⁵⁸. Peroxynitrite activates ERK in fibroblasts⁵⁹, neutrophils⁶⁰ and smooth muscle cells⁶¹. In

human neutrophils, the activation of ERK by peroxynitrite leads to upregulation and surface expression of β_2 -integrins, and increased neutrophil adhesion to endothelial cells. ERK activation in these cells also leads to enhanced oxidative burst upon stimulation⁶⁰. ERK-dependent activation of the oxidative burst may be relevant in NEC, where it may favor conversion of locally produced NO into peroxynitrite. ERK is also believed to play a role in apoptotic signaling, however the existing data are controversial. Depending on the cell type and nature of activating stimulus, ERK may either inhibit or promote apoptosis. Peroxynitrite-induced activation of ERK has been found to promote apoptosis via a p53-dependent mechanism^{62–64}.

The JNK pathways are activated by environmental stresses. When activated, JNK phosphorylates and thereby activates c-jun, which dimerizes with c-fos to form the transcription factor AP-1. The apoptotic effects of JNK depend on the cell type, inciting stimulus, and local milieu. However, most studies indicate that JNK is pro-apoptotic. Peroxynitrite has been shown to activate JNK in several cell types^{65–67}, and dominant negative JNK may protect cells from apoptosis⁶⁸. The contribution of JNK pathways into peroxynitrite-induced apoptosis in the intestinal epithelium remains unclear.

The activation of p38 MAPK by peroxynitrite has been linked to apoptosis in a variety of cell lines⁵⁸. Even at low concentrations, peroxynitrite increases the activating phosphorylation of p38 within minutes of exposure^{66, 69}. The activation of p38 causes upregulation of apoptotic effectors, such as growth arrest and DNA damage (GADD) genes⁷⁰. In many of the cell systems studied, pharmacologic inhibition of p38 leads to attenuation of the apoptotic response^{63, 70}. However, like ERK and JNK, p38 may affect apoptosis in opposite ways. We have shown that p38 upregulates cyclooxygenase-2, a rate-limiting enzyme in the biosynthesis of prostanoids⁷¹, leading to increased prostanoid production. Prostanoids play an important role in intestinal epithelial homeostasis and inflammation^{72, 73} and are known to protect intestinal cells from apoptosis^{74, 75}. Therefore, peroxynitrite-activated p38 may have complex effects on apoptosis, cell survival, and inflammation.

NO in Proliferation and Migration signaling pathways

Proliferation and migration of enterocytes are the two major repair processes in the gut epithelium. Although it is known that proliferation of enterocyte precursors in the crypts is dynamically regulated by the physiologic state of the epithelium, the underlying regulatory mechanisms are not well understood⁷⁶. The early observations that NO could modulate critical cell signaling processes through the S-nitrosylation of critical cysteine residues provided a clue to understanding the role of redox regulation in signal transduction⁷⁷. However, the subsequent discovery of peroxynitrite's ability to nitrate tyrosine residues suggested that NO might, via formation of peroxynitrite, alter proliferative signaling mediated by protein tyrosine kinases. This shifted attention to phosphorylation cascades, as nitrosylation of these residues leads to modulation of cell signaling processes which rely on tyrosine phosphorylation⁷⁸. One such proliferation-stimulating tyrosine kinase is epidermal growth factor receptor (EGFR), which is known to play an important role in epithelial restitution.

It has been shown, in a rat model of colitis, that EGF significantly reduces colonic ulceration and inflammation, and also promotes healing of peptic ulcers⁷⁹. The possibility that peroxynitrite can modulate EGF signaling is strong, as EGF acts through a receptor tyrosine kinase. Binding of EGF to the receptor (EGFR) initiates a tyrosine and serine/threonine phosphorylation cascade, leading to gene transcription, cellular proliferation and differentiation⁸⁰. In the intestinal cell line Caco-2, exposure to peroxynitrite results in nitration of EGFR, which decreases EGFR tyrosine phosphorylation and proliferation in response to EGF⁸¹. The inhibition of the EGFR signaling cascade in vivo would result in decreased enterocyte proliferation and differentiation, which, in combination with the apoptosis induced

by peroxynitrite, may contribute to the formation and persistence of bare areas at the villus tips.

Another group of proliferative protein tyrosine kinases is the Src kinase family. This non-receptor kinase family includes Src, Fyn, Yes, Lck, Hck, Fgr, Blk, and Lyn. Src kinases are regulated by phosphorylation of tyrosine residues at positive (Tyr416) and negative (Tyr527) regulatory sites. Src members contain a tyrosine kinase domain and two conserved protein-protein interaction domains, SH2 and SH3, which create docking sites for phosphotyrosine and proline-rich helices⁸². Activated Src family members participate in multiple signaling processes; they promote cell survival, stimulate proliferation, and inhibit apoptosis. Downstream effectors include Shc, PI3K, phospholipase C, and focal adhesion kinase (FAK)⁸². We have demonstrated that exposure of enterocytes to peroxynitrite inhibits serum- and EGF-induced proliferation, decreases Src activating phosphorylation, and increases Src nitration. Moreover, peroxynitrite blunts induction of proliferation by the constitutively active v-Src transgene⁸³.

It should be noted, however, that peroxynitrite may activate rather than inhibit Src. This has been shown in pancreatic adenocarcinoma cells⁸⁴, endothelial cells⁸⁵, and astrocytes⁸⁶. Opposing effects of peroxynitrite may be due to different steric accessibility of target sites in Src kinase signaling complexes, or differences between Src family members. Two distinct mechanisms of src kinase activation by peroxynitrite have been identified. For example, Hck is activated by peroxynitrite-dependent oxidation of a specific cysteine residue, whereas Lyn can be activated via nitrotyrosine formation at the inhibitory site Tyr527^{87, 88}. In summary, it is possible that modulation of the tyrosine kinase signaling cascades by peroxynitrite decreases enterocyte proliferation, which, in combination with peroxynitrite-induced apoptosis, may contribute to gut barrier failure by the formation of denuded areas of the mucosa.

Enterocyte migration is another potential target of NO in NEC. We have demonstrated that enterocyte migration is inhibited in experimental NEC³⁹, and that enterocyte migration in vitro depends on signaling cascades of the small GTPases Rho, Rac, and Cdc42. Exposure to endotoxin activates RhoA, leading to phospho-FAK expression and the enhancement of focal adhesions. This entrenchment of the enterocyte would effectively prevent cell movement³⁹. Preliminary studies in our lab have shown that Cdc42 is activated during enterocyte migration, using an IEC-6 monolayer wound model. We've also found that in enterocytes, Cdc42 acts through PAK (p21 kinase) (Ford, unpublished data). NO affects migration in a Rho-, focal adhesion kinase (FAK)-, and N-terminal src homology (SH₂)-containing tyrosine phosphatase-2 (SHP-2)-dependent fashion. Furthermore, NO stimulates phosphorylation of FAK in vivo and in vitro, and inhibits formation of lamellipods by causing accumulation of stress fibers and focal adhesions in vitro³⁹. These findings provide an insight into the contribution of NO to the inhibition of enterocyte migration seen in NEC. Effects of NO/peroxynitrite on enterocyte migration machinery warrant further investigation.

Conclusion

Necrotizing enterocolitis is a complex disease characterized by intestinal barrier failure. Such derangement in intestinal barrier function plays a critical role in the progression and severity of the disease. Once barrier failure occurs, the lamina propria is exposed to increased levels of endotoxin and other bacterial products as a result of bacterial translocation. The net result is activation of the neonatal immune system, which triggers an inflammatory signaling cascade that leads to an exuberant pro-inflammatory response characterized by the release of cytokines, prostanoids, and NO. The reaction of NO with superoxide leads to the production of peroxynitrite, a potent oxidant. These two molecules exert their cytopathic effects through various molecular pathways outlined above, which lead to enterocyte apoptosis or necrosis and

impairment of both enterocyte proliferation and epithelial restitution through enterocyte migration. This imbalance between tissue injury and repair propagates the inflammatory cascade leading to full blown NEC. The ultimate consequence of these insults is further epithelial injury, intestinal perforation, and systemic sepsis, which can eventually lead to death in the most severe cases. Novel therapies that are designed to neutralize these pro-inflammatory processes are key areas of research in the continued effort to decrease the incidence and severity of NEC in neonates.

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Figure 1. NO Production

Nitric oxide synthase (NOS) catalyzes the reaction between arginine and oxygen, with the consumption of 1.5 moles of NADPH per mole of NO produced. Citrulline and water are also produced during the reaction.

Figure 2. Effects of NO on the intestinal barrier

Perinatal insults and hypoxia lead to mucosal damage. NO and its reactive intermediary, peroxynitrite (ONOO^-), are produced. This leads to a cascade of events, including apoptosis at the villus tip, bacterial translocation, and immunocyte activation. Activated immunocytes and inflamed enterocytes continue to produce cytokines and inflammatory mediators such as NO. Unchecked, this cycle will continue and lead to systemic sepsis, and can eventually lead to death in the most severe cases.