Intestinal and hepatic expression of BNIP3 in necrotizing enterocolitis: regulation by nitric oxide and peroxynitrite

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Necrotizing enterocolitis (NEC) is the most frequent and lethal disease affecting the gastrointestinal system of premature infants (16). The overall mortality rate for NEC ranges between 10 and 70% (25) and approaches 100% for patients with the most severe form of the disease, which is characterized by involvement of the entire bowel (pan-necrosis; see Ref. 42). Although the exact etiology of NEC remains undefined, numerous risk factors, including prematurity (53), hypoxia (29), formula feeding (18), bacterial infection (43), and intestinal ischemia (1), have been implicated in its pathogenesis. These factors, together with the increasing number of associated inflammatory mediators (7, 21, 38), make NEC one of the most complex diseases in the neonatal population.

Among the proinflammatory mediators associated with the development of NEC is nitric oxide (NO), produced by the inducible NO synthase (iNOS). The end products of NO metabolism have been measured in newborn infants and in adult patients with clinical sepsis (40, 45). In an animal model of NEC induced by formula feeding and a brief period of hypoxia (3), we have recently found increased formation of endogenous nitroso species (mostly S-nitrosothiols, nitrosamines, and nitrosothiols), indicative of nitrosative stress (59). We have previously shown that NO modulates intestinal changes in septic shock (17, 46). Furthermore, iNOS is upregulated in the intestine of infants with acute NEC, and this expression is downregulated by the time of stoma closure when the infant has recovered from the acute inflammatory insult (17).

Hypoxia leads to the production of oxygen radicals (5) and elevated iNOS expression (58). The reaction product of NO with superoxide (54), or through the action of neutrophil myeloperoxidase (23, 44) is peroxynitrite (ONOO−; see Refs. 4 and 37). NEC is preceded by increased epithelial apoptosis, which results in loss of gut barrier function and thus predisposes the host to bacterial invasion and subsequent bowel necrosis (27). ONOO− produced from iNOS-derived NO has been implicated in this process because it causes apoptotic cell death of enterocytes subjected to the inflammatory stimuli that characterize NEC (17).

An elevated level of NO has been shown to induce apoptosis in some cell types (e.g., macrophages) but to suppress apoptosis in others (hepatocytes; see Refs. 50 and 51). The upregulation of iNOS in the liver serves an anti-apoptotic function during experimental endotoxemia, a phenomenon that is mediated by S-nitrosation of caspases, production of cGMP, and modulation of apoptosis-related genes in endotoxemia. In states of redox stress such as hypoxia/ischemia reperfusion injury (50, 51), however, iNOS is hepatotoxic. We have shown that NO suppresses expression of the Bcl-2 binding protein BNIP3, a pro-apoptotic member of the Bcl-2 family of apoptotic factors, in hepatocytes (57). Both apoptotic and necrotic cell death mechanisms have been attributed to BNIP3 (9, 49), but the exact pathway(s) by which BNIP3 expression induces cell damage remains unknown.

The foregoing studies suggest a link between hypoxia and the intestinal production of nitroso species with the histopathological changes seen in NEC. Furthermore, these events may be modulated, in part, by BNIP3. In the present study, we pursued the hypothesis that BNIP3 is elevated in the intestine and liver of neonatal rats subjected to hypoxia and formula feeding. We show that both BNIP3 mRNA and protein are
upregulated in the ileum and liver of formula-fed newborn rats exposed to hypoxia but not in breast-fed controls. In rats, this upregulation is reversed by an iNOS-specific inhibitor. We also show that BNIP3 is expressed in the human intestine and upregulated in intestinal tissue from human infants with acute NEC. Finally, ONOO− causes a time- and dose-dependent increase in BNIP3 protein in human enterocytes. We propose that hypoxia and subsequent formation of reactive oxygen and nitrogen species promote gut barrier failure in NEC by inducing enterocyte apoptosis, in part caused by elevated expression of BNIP3.

MATERIALS AND METHODS

Animal model of NEC. All experiments were carried out following an animal protocol approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh. Pregnant timed-dated Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were induced at term using a subcutaneous injection (1–2 U/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), gavaged with a special rodent formula (0.2 ml, see below) two times per day, and subjected to 10 min of hypoxia (5% O2-95% N2; Prax Air, Pittsburgh, PA) three times daily in a modular chamber (Billups-Rothenberg, 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. In some cases, the rats were treated daily after the first feeding with the iNOS inhibitor L-NAME-(1-iminoethyl)lysine (L-NIL, 1 mg/kg ip; Alexis Biochemicals, San Diego, CA). The formula composition consisted of 15 g Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml Esbilac canine milk replacer (Pet-Ag, Hampshire, IL) as described by Barlow et al. (3) and was designed to approximate the protein and caloric content of rat breast milk. The rats were killed as indicated, and segments of the terminal ileum were isolated. The mucosa was scraped gently from each segment and immediately placed in cold lysis buffer containing 62.5 mM Tris (pH 6.6), 10% glycerol, 1% SDS, and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin, 2 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride, all from Sigma, St. Louis, MO). The samples were then homogenized and boiled for 1 min followed by centrifugation at 10,000 g for 30 min to remove cellular debris. Protein concentration in the supernatant was determined using the bicinchoninic acid Protein Assay kit from Sigma, with BSA as standard. Protein samples (equivalent to 50 μg) were resolved on 12% SDS-polyacrylamide gels using a Bio-Rad mini-gel system (Hercules, CA) and then electroblotted on polyvinylidene difluoride membranes (Millipore, Bedford, MA). After being blocked for 1 h with milk (5% in PBS with 0.1% Tween 20) at room temperature, the membranes were probed for 1 h at room temperature with the primary antibody (polyclonal rabbit anti-human BNIP3 from BD PharMingen, San Diego, CA) dissolved in 1% milk PBS-Tween at 1:2,000 dilution. The membranes were then thoroughly washed and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG; Pierce, Rockford, IL) at 1:15,000 dilution (in PBS-Tween with 1% milk) for 1 h before detection. Protein bands were visualized using a Supersignal chemiluminescence substrate (Pierce) according to the manufacturer’s instructions. For normalization, the membranes were stripped and reprobed with an anti-β-actin antibody from Sigma.

RNA isolation and Northern blot analysis. Total RNA was isolated from the ileum or ileal mucosal scrapings, colon, and liver samples using the RNAlater reagent (Ambion, Austin, TX) as per the manufacturer’s protocol. Northern blotting and analysis of BNIP3 mRNA levels in rat samples was carried out using a probe obtained as described previously (57). The relative amount of mRNA is presented as the ratio of BNIP3 mRNA to 18S RNA.

Cell culture. The human fetal nonmalignant primary intestinal cell line (H4 cells) was used to represent immature fetal enterocytes and was generously provided by Dr. W. Allan Walker (Massachusetts General Hospital-East, Charlestown, MA). The H4 cells (from passages <30) were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, and 0.2 U/ml insulin in a humid atmosphere of 95% O2-5% CO2 at 37°C. ONOO− (Alexis Biochemicals) was added to the culture plates at different concentrations, and, for experiments under hypoxic conditions, culture plates were placed in a hypoxic chamber (1% O2) for the times indicated.

Viability assay. Cell viability was determined by the crystal violet method, as described previously (34). Briefly, cells were stained with 0.5% crystal violet in 30% ethanol/3% formaldehyde for 10 min at room temperature. Plates were washed six times with tap water. After being dried, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well microplate reader. Cell viability was calculated from relative dye intensity and presented as percentages relative to control samples.

Statistical analysis. Results are expressed as means ± SE or SD as indicated. Differences among groups were analyzed by the Student’s t-test or one-way ANOVA followed by Tukey’s Test or Fisher’s Least Significance Difference Test where appropriate (SigmaStat 2.03; SPSS, Chicago, IL). Statistical significance was determined at the 95% confidence level (P < 0.05) in all cases.
RESULTS

Formula feeding plus hypoxia upregulate BNIP3 mRNA expression in an animal model of NEC. Based on our observation that moderate to severe epithelial damage occurs in the intestine of formula-fed rats exposed to hypoxia (FFH) by day 4 after birth, we examined the expression of BNIP3 in the intestine of FFH and BF newborn rats (control). Mucosal scrapings of the terminal ileum and segments of the colon and liver from each rat were harvested on day 4 and processed for RNA isolation followed by Northern blotting analysis. BNIP3 mRNA was detected as a 1.7-kb band and was significantly upregulated in the ileum (Fig. 1A), colon (Fig. 1B), and liver (Fig. 1C) of FFH animals compared with BF controls (P < 0.001 by Student’s t-test). Similar upregulation of BNIP3 mRNA was found in the whole ileum of FFH compared with BF neonatal rats as determined by real-time PCR (data not shown).

Formula feeding plus hypoxia upregulate ileal BNIP3 protein expression in experimental NEC in an NO-dependent manner. Because one of the hallmarks of NEC is extensive epithelial damage involving the distal small bowel, we examined the expression of BNIP3 protein in the ileal mucosal scrapings of BF and FFH animals. As shown in Fig. 2A, BNIP3 protein is constitutively expressed at very low levels in the ileum of BF animals by the first day of life and remains essentially the same until death. In contrast, the levels of BNIP3 protein are slightly increased in the FFH group and significantly different from BF at day 4 (P < 0.05). Consistent with the upregulation of BNIP3 mRNA (Fig. 1A), formula feeding and hypoxia also led to upregulation of BNIP3 protein in the ileal mucosal scrapings of newborn rats (Fig. 2B). The increased expression of BNIP3 protein correlated with upregulation of other pro-inflammatory mediators in the mucosal scrapings of the ileal samples and with the characteristic pathological changes of experimental NEC by day 4 (59, 61). Interestingly, treatment with L-NIL (Fig. 2B) significantly suppressed the upregulation of BNIP3 protein in the FFH animals (P < 0.05) and the incidence of intestinal inflammation [28.6 ± 11.3 vs. 61.3 ± 8% in FFH without L-NIL, n = 3 independent experiments, total no. of animals/group (FFH + L-NIL/FFH): 28/31]. This observation, which stands in stark contrast to the downregulatory effect shown in hepatocytes (57), suggests that iNOS-derived reactive nitrogen intermediates participate in the upregulation of the ileal BNIP3 protein expression observed in experimental NEC.

Hypoxia and ONOO\(^-\) induce cell death and increase BNIP3 protein expression in human intestinal epithelial cells. Intestinal hypoxia is a major factor in the pathogenesis of NEC. Upregulation of iNOS and excess production of reactive nitrogen species have been associated with the development of this disease (17). To investigate the effects of hypoxia and reactive nitrogen species on the expression of BNIP3 in human intestinal epithelial cells, H4 cells were incubated in a hypoxic atmosphere.
BNIP3 EXPRESSION IN NEC

A

![Image](https://via.placeholder.com/150)

**Fig. 2.** Formula feeding plus hypoxia upregulate ileal BNIP3 protein expression in an animal model of NEC. Effect of nitric oxide (NO). Newborn rats were randomized into BF and FFH groups. The terminal ileum of each rat was harvested on the day indicated, and the mucosal scrapings were processed for protein isolation followed by Western blotting. Although short exposure to hypoxia was not cytotoxic to the cells, cell viability decreased to 40% after 24 h (Fig. 3A). Interestingly, the loss of viability observed after 24 h of exposure was associated with the highest expression of a protein recognized as a 60-kDa band by antibodies raised against human BNIP3 (Fig. 3B). Similarly, treatment with ONOO− led to a concentration-dependent increase in expression of the same protein (Fig. 4A). Furthermore, treatment with ONOO− induced cell death in a concentration-dependent manner after 24 h (Fig. 4B). At concentrations \( \geq 100 \mu M \), ONOO−-induced cell death was 100%, and no BNIP3 protein could be detected (likely because of protein degradation). Western blot analysis of cultured H4 cells treated with 0–100 \( \mu M \) ONOO− and exposed to hypoxia for several hours showed an upregulation of BNIP3 protein by 4 h of exposure and maximal expression at 24 h (Fig. 4C). Interestingly, the ONOO− concentration dependence seemed to be lost in hypoxic conditions, where even at supraphysiological ONOO− concentrations (50 \( \mu M \)), the levels of BNIP3 are much lower (Fig. 4A) than the levels induced by hypoxia alone (Fig. 4C). The combined treatment was more cytotoxic to the cells (data not shown) and resulted in the lack of detection of BNIP3, most likely because of specific degradation of BNIP3 protein and not of total protein, as shown by the normal β-actin levels. This effect was confirmed in rat intestinal epithelial cells (IEC-6 cells) using a different anti-BNIP3 antibody previously described (30). In IEC-6 cells, BNIP3 expression was increased at 12 h, was maximally increased after 24 h of exposure to hypoxia (1% \( O_2 \)), and then gradually decreased and was not detected after 60 h (data not shown). No change was detected in β-actin levels over the time course, again suggesting that specific degradation of BNIP3 protein and not of total protein accounted for the lack of detection at later time points.

**BNIP3 is expressed in human NEC.** To determine whether BNIP3 is expressed in the diseased intestine of human infants undergoing intestinal resection for acute NEC, we compared intestinal segments from six newborn patients undergoing intestinal resection for acute NEC with six neonates undergoing intestinal resection for inflammatory conditions other than NEC (control) in two independent experiments. Western blot analysis using a polyclonal rabbit anti-human BNIP3 antibody showed an increased expression of the 60-kDa band in the intestinal segments of five of the six NEC patients compared with non-NEC controls (Fig. 5).

**DISCUSSION**

NEC is a complex, multifactorial disease of newborns characterized by intestinal epithelial cell apoptosis and necrosis and impaired enterocyte migration and proliferation that ultimately result in sustained gut barrier failure. In this study, we investigated the expression and regulation of the cell death-related protein BNIP3 (19, 41, 49) in the intestine and liver of newborn rats in an experimental model of NEC, in human intestinal epithelial cells in vitro, and in the diseased intestine of human infants with acute NEC.
BNIP3 has been shown to be overexpressed in human tumors and human tumor cell lines (12, 30, 47, 48) and in primary neonatal rat cardiac myocytes exposed to hypoxia (19, 31). Furthermore, at normal oxygen concentrations, most tissues have undetectable levels of BNIP3 but activate transcription during hypoxia through a 5'-promoter of a hypoxia-inducible factor 1 (HIF-1) binding site (see Ref. 52 for review). However, this is the first report of BNIP3 expression in the human intestine and in nontumor tissue in an experimental animal model.

BNIP3 is a membrane-associated protein localized to mitochondria and other cytoplasmic membrane structures and is widely expressed in a large number of mouse and human tissues (9). BNIP3 belongs to the BH3-containing Bcl-2 family proteins in which the BH3 domain plays an important role in eliciting apoptosis (55). However, a recent study shows that BNIP3 heterodimerizes with Bcl-2/Bcl-xl and induces cell death independent of a BH3 domain at both mitochondrial and nonmitochondrial sites (41). In addition to apoptosis, BNIP3 may also mediate a form of necrotic cell death after protein integration in the mitochondrial outer membrane and rapid mitochondrial permeability transition pore opening (49). Because NEC is characterized by an extensive hemorrhagic inflammatory necrosis of the distal small bowel and proximal colon (39) and is associated with epithelial apoptotic cell death (10, 27), it is not surprising that cell death-related genes such as BNIP3 are upregulated in the intestine of NEC patients. Interestingly, the expression of BNIP3 (both mRNA and protein) is induced and related to hypoxia-induced apoptosis in a number of human and animal cell lines, including: CHO-K1 (Chinese hamster ovary), CV-1 (monkey kidney), Rat-1 (rat fibroblast), PAM212 (human epithelial), Hep G2 (human hepatocellular carcinoma), and ECV-304 (human bladder carcinoma) cell lines (6). Moreover, BNIP3 and Nix (a BNIP3 homolog sharing both structural and functional similarity; see Ref. 8), are the only members of the Bcl-2 family of apoptotic factors induced in response to hypoxia (6). Whether the expression of BNIP3 is a relevant process in human NEC still needs to be investigated. We attempted to suppress the expression of BNIP3 in IEC-6 rat intestinal epithelial cells using a commercial small-interfering RNA (Silencer; Ambion) to determine the role of BNIP3 in enterocyte apoptosis, but were unsuccessful (data not shown).

Fig. 3. Hypoxia causes cell death and upregulates BNIP3 protein expression in human intestinal epithelial cells. Primary human fetal intestinal epithelial cells (H4 cell line) were incubated under hypoxic conditions by placing the culture plates in a hypoxic chamber (1% O2) for the times indicated. After 6 or 24 h, the cells were harvested, and Western blotting was performed. Viability experiments were performed in parallel using the crystal violet method. A: viability of H4 cells exposed to hypoxia for 6 and 24 h. Results in bars represent means ± SE of 3 independent experiments performed in triplicate. *P < 0.01 vs. control (analyzed by 1-way ANOVA followed by Fisher’s least-significant difference [LSD] test). B: representative Western blot showing the expression of the BNIP3 60-kDa band. MW, mol wt. Results in bars represent means ± SE of 3 independent experiments. *P < 0.05 vs. control and 6 h hypoxia (analyzed by 1-way ANOVA followed by Tukey’s test).
Based on the findings that mucosal damage associated with enteral feeding and perinatal hypoxia are two major factors in the development of NEC, we used a reproducible experimental model in newborn rats developed in our laboratory (38) to study the expression of BNIP3. It has been previously shown that, in the liver, BNIP3 mRNA is expressed as a major transcript of 2.5 kb and as a minor transcript of 1.7 kb (9). Our results show that BNIP3 mRNA is expressed as a 1.7-kb transcript in the intestine of newborn rats and is increased in the FFH group compared with control animals on day 4. This finding correlates with the morphological changes characteristic of intestinal inflammation seen in ileal segments from FFH rats compared with ileal segments from BF controls. Consistent with the finding that the major intestinal damage in NEC occurs in the terminal ileum, we found a fourfold increase in BNIP3 mRNA in the ileal mucosal scrapings from FFH animals compared with only a twofold increase in colon samples from the same animals. It should be noted that, during the isolation of total RNA from ileal mucosal scrapings, the detectable yield was too low to perform individual analysis for each animal; therefore, the samples from the same experimental group had to be pooled for Northern analysis. Consequently, it is possible that the actual relative levels of BNIP3 mRNA in FFH animals may be higher than the values reported here.

In addition to intestinal epithelial injury, liver failure or dysfunction is also seen in severe NEC. Indeed, a role for hepatic inflammatory mediators contributing to intestinal damage in NEC has recently been postulated (20). The exact role of the liver in the pathogenesis of NEC, however, remains to be elucidated. Based on our demonstration that BNIP3 is expressed in liver hepatocytes isolated from adult rats subjected to pro-apoptotic stimuli (57), we hypothesized that BNIP3 is also expressed in the liver of newborn rats and that this expression is differentially regulated in FFH vs. BF animals. Our data show that formula feeding plus hypoxia upregulate BNIP3 mRNA expression in the liver of FFH animals compared with BF controls. The degree of increase was similar to that seen in the ileum, suggesting that BNIP3 is involved in the liver damage associated with NEC. Interestingly, our previous studies indicated that NO or its reactive metabolites suppress the expression of BNIP3 in hepatocytes in vitro (57), in contrast to our results in vivo in the liver of FFH rats. We speculate that the differential effects of NO in the liver may be context-dependent. Paradoxically, NO is either pro- or anti-apoptotic, depending on cell type, dose, and milieu. For example, low levels of NO tend to protect various cell types from apoptotic death, whereas high levels are generally toxic (14). In vivo, NO can protect the liver in endotoxemia (22), yet exposure to NO leads to liver injury during ischemia/reperfusion (33). NO derived from iNOS is also hepatotoxic in whole animal models of ischemia/reperfusion such as hemorrhagic

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Fig. 4. Peroxynitrite (ONOO⁻) alone or in combination with hypoxia causes cell death and upregulates BNIP3 protein expression in human intestinal epithelial cells. Primary human fetal intestinal epithelial cells (H4 cell line) were treated with ONOO⁻ (1–50 µM) for 4 or 24 h, and the cells were harvested for protein isoaltion and Western blotting analysis. For hypoxia experiments, the culture plates were placed in a hypoxic chamber (1% O₂) for the times indicated. Viability experiments were performed in parallel using the crystal violet method. A: Western blot showing the expression of the BNIP3 60-kDa band (duplicate samples/group). Results in bars represent means ± SD. *P < 0.05 vs. control and 1–25 µM ONOO⁻ (analyzed by 1-way ANOVA followed by Tukey’s test) B: viability of H4 cells exposed to ONOO⁻ (1–50 µM) for 24 h. Results in bars represent means ± SE of 5 independent experiments. *P < 0.01 and **P < 0.001 vs. control (analyzed by 1-way ANOVA followed by Tukey’s test). C: Western blot showing the effect of the combined treatment on BNIP3 protein expression. Results represent the band densitometry analysis expressed as degree of increase vs. hypoxia alone at the respective time point from 2 independent experiments. *P < 0.01 vs. hypoxia without ONOO⁻. **P < 0.05 vs. 25 µM ONOO⁻. ***P < 0.05 vs. 5 µM ONOO⁻ (analyzed by 1-way ANOVA followed by Fisher’s LSD Test).
shock (24, 26, 35, 36, 60), where regions of the liver are exposed to hypoxic conditions (C. A. McCloskey, Y. Vodovotz, and T. R. Billiar, unpublished observations). Many of the disparate roles of NO have been ascribed to differential chemistry that this radical can undergo when in the presence of other reactants. For example, NO may react with superoxide anion to form ONOO⁻, or with oxygen to form N₂O₃ (54). The former has been suggested to be toxic in various inflammatory settings (4), whereas the latter mediates the S-nitrosation of numerous low-molecular-weight compounds and proteins (including caspases; see Ref. 28).

The larger amount of BNIP3 protein detected in the ileal mucosa of FFH animals beginning at days 3–4 coincides with the maximal expression of iNOS protein. The development of NEC is associated with production of reactive nitrogen species derived from iNOS (17). We have previously shown that iNOS-derived NO suppresses BNIP3 expression in murine hepatocytes independent of any other inflammatory stimuli (57). More recently, NO was shown to induce BNIP3 expression, leading to cell death in murine macrophages (56). Our present results using the selective iNOS inhibitor l-NIL show that suppression of iNOS enzymatic activity attenuates BNIP3 protein expression in the ileum of FFH animals. This finding suggests that upregulation of BNIP3 contributes to the detrimental effect and intestinal injury caused by reactive nitrogen species after upregulation of iNOS in NEC.

The intestinal damage in NEC is more marked in the epithelial cell lining of the terminal ileum. Our experiments show that the increased expression of BNIP3 seen in the ileal mucosal scrapings can be reproduced in vitro in H4 human fetal enterocytic cells exposed to hypoxia, the reactive nitrogen product ONOO⁻, or a combination of both. Exposure of intestinal epithelial H4 cells to hypoxia for 24 h resulted in an increased expression of the 60-kDa band of BNIP3 protein, whereas shorter exposure (6 h) had no significant effect. The increased expression of BNIP3 was associated with loss of cellular viability, suggesting that BNIP3 upregulation contributes to the hypoxic damage of epithelial cells. Moreover, this time-dependent effect points to BNIP3 as an effector in the late stages of hypoxia rather than as an early mediator. Therefore, inhibition of BNIP3 may be a novel therapeutic target in NEC. Indeed, a recent study reported that accumulation of BNIP3 in prostate cancer cells is observed only after 4 days of hypoxic environment (32). Similarly, ONOO⁻, the reaction product of NO and superoxide believed to be responsible for the cytotoxic effects of NO in a number of cell types and experimental models (2, 11, 15), increased the expression of BNIP3 protein and induced cell death in a concentration-dependent manner in H4 cells. Interestingly, the ONOO⁻ concentration dependence seemed to be lost in hypoxic conditions, which can be explained by the fact that hypoxia is a much stronger inducer of BNIP3 than ONOO⁻. Therefore, in the context of hypoxia, the effect of ONOO⁻ on BNIP3 expression becomes less relevant. The combined treatment with ONOO⁻ and hypoxia resulted in upregulation of BNIP3 within 4 h of exposure and was more cytotoxic to the cells, resulting in the lack of detection of BNIP3 most likely because of increased protein degradation. It has already been proposed that the posttranslational control of BNIP3 through rapid protein degradation may constitute a mechanism for regulating the intracellular levels of a potentially lethal protein (9).

One reported anomaly regarding BNIP3 is that, despite its calculated molecular mass of 21.54 kDa, when transiently expressed it migrates on SDS-PAGE as a major band of 60 kDa and a minor band of 30 kDa (9). In our studies, BNIP3 also appeared at both molecular weights. However, we found that the two bands were not always expressed to the same extent. Similar observations have been reported in certain epithelial tumors, where the extent of upregulation of the 60-kDa band does not parallel that of the 30-kDa protein (48). At this time, we cannot fully explain this phenomenon, but differences in sample processing, time of sample collection, and lack of alternative commercially available antibodies may explain this issue.

Collectively, our studies add BNIP3 to the list of inflammatory mediators that may play a role in the pathogenesis of NEC.

Fig. 5. BNIP3 protein expression in human NEC. Representative ileal segments from 6 newborn patients undergoing bowel resection for NEC (4–6 and 10–12) and ileal specimens from 6 neonates undergoing intestinal resection for inflammatory conditions other than NEC (control, 1–3, and 7–9) were analyzed for the presence of BNIP3 in 2 independent experiments. The frozen intestinal samples were processed, and Western blotting and analysis using a rabbit polyclonal antibody against human BNIP3 was performed as described in MATERIALS AND METHODS with 50 μg protein/lane. Results in bars represent means ± SE (n = 6 patients/group). *P < 0.05 vs. control (analyzed by Student’s t-test).
and thereby may provide a new therapeutic target for this disease.

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