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Inhibition of Nuclear Factor-κB Ameliorates Bowel Injury and Prolongs Survival in a Neonatal Rat Model of Necrotizing Enterocolitis

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ABSTRACT: Necrotizing enterocolitis (NEC) is a major cause of morbidity and death in premature infants. NEC is associated with increased levels of pro-inflammatory cytokines in plasma and tissues that are regulated by the transcription factor nuclear factor-κB (NF-κB). It remains unknown, however, whether NF-κB mediates injury in neonatal NEC. We therefore examined the activation status of NF-κB perinatally in the small intestine and in a neonatal rat model of NEC. We found that intestinal NF-κB is strongly activated at birth and, in dam-fed newborn rats, is down-regulated within a day. In contrast, NF-κB remains strongly activated at both d 1 and d 2 in stressed animals, and this is accompanied by a significant decrease in the levels of the endogenous NF-κB inhibitor protein IκBα and IκBβ at d 2. To determine the importance of elevated NF-κB activity in intestinal injury in NEC, we administered the NEMO-binding domain (NBD) peptide that selectively inhibits the critical upstream IκB kinase (IKK). NBD but not a control peptide decreased mortality and bowel injury in this model, supporting the hypothesis that bowel injury in NEC results from elevated NF-κB activity. Our findings therefore lead us to conclude that selective NF-κB inhibition represents a promising therapeutic strategy for NEC. (***Pediatr Res* 61: 716–721, 2007**)

NEC remains a major cause of morbidity and mortality in neonates (1). A neonatal rat model of NEC, reproducing typical risk factors of clinical NEC including formula feeding, hypoxia, and cold stress, has been used by recent investigators to gain insight into the pathogenesis of NEC (2,3).

In animal models (4), as well as in clinical NEC (5), cytokine gene expression is up-regulated. This may result from the translocation of commensal bacteria and their products through an immature or compromised mucosal barrier, triggering an inflammatory cascade that leads to the release of cytokines and chemokines (6,7). Alternatively, bacteria can initiate the pro-inflammatory response by activating TLR4 on the surface of stressed epithelium, leading to intestinal inflammation and necrosis (8). It is possible that, in premature neonates, the inflammatory response is not properly down-regulated, resulting in excessive cytokine production. This leads to neutrophil recruitment, activation, reactive oxygen species release, and intestinal damage (7). However, the mechanisms regulating the inflammatory response in the neonatal intestine remain poorly understood.

NF-κB, a transcription factor that regulates the expression of many inflammatory genes including pro-inflammatory cytokines, chemokines, and leukocyte adhesion molecules (9,10), consists of five subunits (p50, p65, p52, cRel, and RelB) (9,10) that homo- or heterodimerize to form active NF-κB. The dimers of NF-κB mostly found in intestinal tissues are p50-p50 and p50-p65 (11,12). NF-κB is constitutively present in the cytoplasm of most cells, binding to inhibitory proteins named IκB. Following stimulation, IκB proteins are phosphorylated by an upstream IKK complex (9). This complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory component, NEMO (13). Phosphorylation of IκB by the IKK complex leads to its ubiquitination and subsequent degradation by the 26S proteasome, leaving NF-κB free to translocate to the nucleus and to regulate gene expression (9,13).

NF-κB is aberrantly up-regulated in many chronic inflammatory diseases, including inflammatory bowel disease, and inhibition of NF-κB activation has been shown to decrease bowel injury (14). We previously demonstrated that NF-κB is constitutively present at low levels in adult rat intestine, and is activated in acute bowel injury induced by PAF, a potent endogenous phospholipid mediator thought to play a role in NEC pathogenesis (1). Nonetheless, the regulation of NF-κB in the neonatal intestine and its role in NEC remain unknown. We hypothesize that a persistent and exaggerated NF-κB activation in neonatal animals plays an important role in the development of NEC.

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Abbreviations: EMSA, electrophoretic mobility shift assay; **IκB,** inhibitor protein κB; **IKK,** IκB kinase; **iNOS,** inducible nitric oxide synthase; **LPS,** lipopolysaccharide; **NBD,** NEMO-binding domain; **NEC,** necrotizing enterocolitis; **NEMO,** NF-κB essential modulator; **NF-κB,** nuclear factor-κB; **PAF,** platelet-activating factor

The goal of this study was therefore to investigate 1) the developmental regulation of NF-κB activation and inhibitory proteins IκB in the neonatal rat intestine; 2) the change of NF-κB activity in experimental neonatal NEC, and 3) the effects of selective NF-κB inhibition on NEC incidence in this model.

METHODS

Animal experiments. The study was approved by the Children's Memorial Research Center institutional animal care and use committee.

To study the perinatal activation of NF-κB and iNOS gene expression, rat fetuses were delivered *via* abdominal incision of time-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) at E18, E19, E20 or allowed to deliver naturally and left with the dams for different time periods. Their small intestines were removed, snap frozen in liquid nitrogen, and kept at −80°C until nuclear protein or RNA extraction.

To study the regulation of NF-κB in the NEC model, NEC was induced using the following protocol as previously described (3):

Time-pregnant Sprague-Dawley rats were delivered by an abdominal incision on d 21 of gestation following anesthesia-euthanasia in a CO_2 chamber for 60 s followed immediately by cardiotomy. Neonatal pups were dried and placed in a humidified (37°C) neonatal incubator (Air-Shield Vickers Medical, Hatboro, PA). Once recovered (about 1 h), the pups were gavaged with 7×10^7 colony forming unit (CFU) of a standardized bacterial mixture diluted in 50 μL of NS. Neonatal rats were fed Esbilac formula (33 g of powder diluted in 100 mL of water) by orogastric gavage using a 1.9 F silastic catheter. Gavage feedings were initiated with a volume of 0.1 mL every 3 h. The volume was then increased by 0.05 mL every 12 h. Pups were exposed to a brief episode of asphyxia (60 s in 100% N_2) followed by cold stress (4°C for 10 min), once on the day of birth and then twice daily. The standardized commensal bacterial mixture used in the NEC model was prepared as follows: the cecum content of three healthy adult rats was mixed 1:1 with regular growth media, vortexed, and cultured overnight at 37°C. The bacterial suspension was mixed 1:1 with 50% glycerol-bacterial culture medium and multiple aliquots frozen at −80°C. The day before each experiment, a bacterial aliquot was thawed, cultured for 16 h at 37°C in culture medium, then diluted to the appropriate concentration. In a set of preliminary experiments, we have found that adding commensal bacteria to the protocol significantly increased the frequency and severity of NEC. The number of rats with histologic NEC score grade 0, 1, 2, and 3 was 12, 2, 1, and 3, respectively, in the group without bacteria ($n = 18$), and 4, 3, 5, and 3, respectively, in the group with bacterial inoculation ($n = 15$).

To evaluate the effect of NF-κB inhibition on NEC, three litters were randomized into two groups around 3 h of life. Seven pups were excluded because of cyanosis (4) or very low birth weight (<4 g) (3). Healthy appearing pups in each litter were weighed and grouped so each group would be constituted of pups of similar weight and genetic background. Group 1 ($n = 14$) was treated with NBD (20 mg/kg, i.p.) and group 2 ($n = 15$) with control peptide (20 mg/kg, i.p.) 1 h before the asphyxia/cold stress on d 0 and d 1. Animals were observed closely and euthanized by decapitation when showing signs of distress (severe abdominal distension, respiratory distress, lethargy) or at 72 h. Their small intestines were collected and fixed in buffered formalin for histologic examination. While the daytime technician could not be blinded for practical reasons, the night technician was kept unaware of the group identity.

Preparation of nuclear extracts. Frozen small intestines were ground into powder with a mortar in liquid nitrogen and nuclear extracts obtained following a standard protocol (11). For fetal tissues, two (E20) to three (E18-19) specimens were processed together to obtain sufficient amounts of nuclear extracts. Samples were stored in liquid nitrogen after determination of their protein concentration by the Bradford's method (15).

Determination of NF-κB-DNA binding activity by EMSA. Equal amounts of nuclear extract (5 μg/10 μL) were used for EMSA and supershift experiment as previously published (11). The gel was dried and analyzed with a Storm 860 phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). The intensity of the NF-κB complex was quantified by densitometry, using ImageQuant software.

Preparation of intestinal whole cell extracts and Western blot analysis. Small intestinal tissue lysates were prepared by homogenization on ice for 1 min in ice-cold lysis buffer (2.0 mM Tris-Cl, pH 7.6, 30 mM NaCl, 1 mM EDTA, 0.2 mM benzamidine, 1 mM DTT, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 μg/mL chymotrypsin and 1% Nonidet P-40). Samples were centrifuged at 3,000 for 10 min at 4°C to remove tissue debris. The protein concentration of the tissue lysates was determined by the Bradford's method (15), and samples frozen at −80°C. Western blot for IκBα, IκBβ, and β-Actin was carried out as previously published and densitometry analysis was performed using the Openlab software (16).

iNOS mRNA analysis. mRNA was analyzed by RT-PCR as previously described (17). The primers used were as follows: rat iNOS primer 1: 5′ ATG GCT TGC CCT TGG AAG TTT CTC 3′, primer 2: 5′ CTC CAG GCC ATC TTG GTG GCA AAG-3′, rat GAPDH primer 1: 5′-ATT CTA CCC ACG GCA AGT TCA ATG G-3′, primer 2: 5′-AGG GGC GGA GAT GAT GAC CC-3′.

Histologic analysis. Intestinal specimens were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Microscopic injury was graded by a blinded pathology study as follows: 0: intact villi; 1: superficial epithelial cell sloughing; 2: mid-villous necrosis; 3: complete villous necrosis; 4: transmural necrosis.

Statistical analysis. Two-sided *t* test was used for the comparison of two single groups (densitometric analysis). To evaluate the differences in the incidence and severity of NEC, and in 72 h-survival between the two groups, results were compared using χ^2 analysis.

RESULTS

NF-κB is activated in the intestine after birth but its activation declines within 24 h in dam-fed pups. We previously found that NF-κB is constitutively active at low level in the intestine of adult rats (11). In this study, we show that a very low level of constitutive activation of NF-κB becomes detectable in intestinal tissues at 20 d of gestation (normal gestation 21.5 d) (Fig. 1*A*). In comparison, we did not find any constitutive activation of NF-κB in the lung before or after birth (results not shown). Immediately after birth, NF-κB is significantly activated in intestinal tissues (Fig. 1*A*). The complex contains mostly p50 subunits and a small amount of p65 (Fig. 1*A*). In control, dam-fed pups, NF-κB activation is down-regulated within a day after birth in the small intestine (Fig. 1*A* and Fig. 3, *A* and *B*). Prenatally, the level of intestinal inhibitory protein IκBα and β did not change significantly

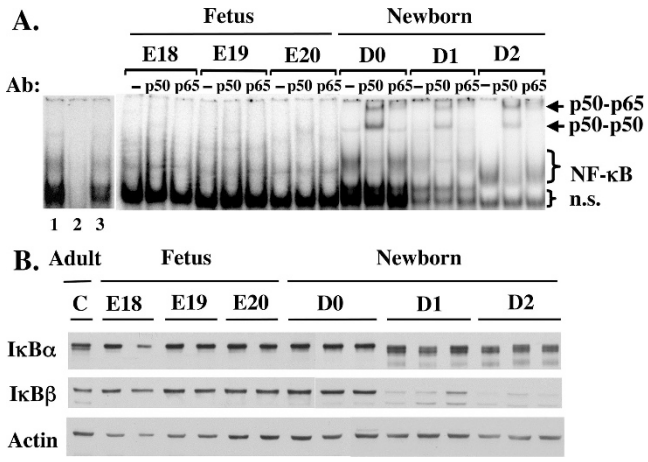

Figure 1. Intestinal NF-κB is activated at birth then down-regulated 24 h after birth. Small intestines of fetal rats delivered at different gestational ages (E18 to E20) and of neonatal pups (d 0, 1, and 2) were obtained. Nuclear extracts were prepared and NF-κB activity was assessed by EMSA of equal protein amounts of nuclear extracts (*A*). Supershift experiments with antibodies (*Ab*) against p50 and p65, the two subunits found in the intestine, were performed (similar results were obtained in three independent experiments). *n.s.,* Nonspecific complex with the NF-κB probe that is not supershifted by anti-NF-κB subunit antibodies. On the left (*lanes 1–3*), a competitive experiment is shown where the D0 sample (*lane 1*) is incubated with an excess (100×-fold) of unlabeled NF-κB probe (*lane 2*) or an excess of an unrelated (AP-1) probe (*lane 3*). In *B*, the levels of IκBα and β were assessed by Western blot.

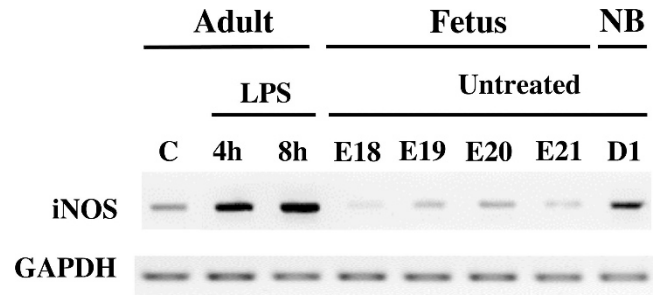

Figure 2. iNOS gene expression is up-regulated after birth and correlates temporally with the activation of NF-κB activation in small intestinal tissues. The small intestine of fetal and neonatal rats of different ages was removed, total RNA (mRNA) was purified, and semi-quantitative RT-PCR for iNOS and GAPDH was performed. Samples from adult rat treated or not with LPS 7 mg/kg for 4 and 8 h is shown in comparison. *NB,* newborn. A typical PCR gel is shown.

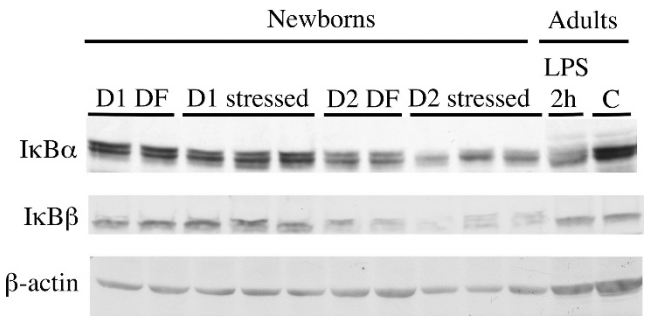

Figure 4. Western blot analysis of IκBα and IκBβ proteins in the rat ileum. Small intestinal tissue lysates from stressed pups or dam-fed controls (*DF*) were obtained at d 1 and 2, and (for comparison) from adult rats untreated (*C*) or treated with LPS 7 mg/kg intraperitoneally for 2 h. Equal amounts of protein from each sample were subjected to SDS-PAGE and the gels to immunoblot analysis with anti-IκBα, IκBβ, and β-actin antibodies.

with maturation (Fig. 1*B*). Just after birth (d 0), IκBα and β remained unchanged. In 1-d and 2-d dam-fed neonatal rats, the IκBα complex intensity decreased to 93% and 87% of d 0 value, respectively, and included a second band of faster migration. At d 1 and d 2, the level of IκBβ was significantly decreased (60% and 46% of d 0 value, respectively) (Fig. 1*B*).

Expression of iNOS, a downstream target of NF-κB correlates temporally after birth with elevated NF-κB activity. In the fetal rat ileum, iNOS was minimally expressed (Fig. 2). However, following birth, its expression was strongly up-regulated (Fig. 2), similar to that in adult rats challenged with 7 mg/kg LPS intraperitoneally, a well-characterized activator of iNOS gene expression (16).

NF-κB is persistently activated in a neonatal rat model of NEC. In neonatal rats subjected to the stress of formula feedings, hypoxia and cold exposure (NEC model), NF-κB activation remains high at d 1 and 2 (Fig. 3). The levels reached 3.6-fold (±0.2) (mean ± SEM) of dam-fed control values on d 1 and 2.7-fold (±0.1) on d 2 (Fig. 3*C*) ($p < 0.001$). Although the NF-κB complexes observed on d 1 and 2 were mostly supershifted with anti-p50 antibodies, they also contained p65.

IκBα and IκBβ levels are decreased in the neonatal intestine in NEC at D2. While we did not find any differences in the level of IκBα and IκBβ at D1, their levels were decreased in the ileum of stressed animals at d 2 compared with those of controlled dam-fed newborns (93% of DF value for IκBα and 83% for IκBβ) (Fig. 4). Although equivalent amounts of protein were loaded, β-actin protein levels were consistently higher in adult tissues compared with those of newborns, suggesting that β-actin is developmentally regulated in the intestine. These differences were accounted for in the interpretation of IκB protein levels.

NBD peptide protects against bowel injury and decreases mortality in a neonatal rat model of NEC. Treatment with the NBD, but not a mutated control peptide, significantly decreased intestinal NF-κB activation in stressed neonatal animals (Fig. 5). Furthermore, animals that received the NBD peptide exhibited improved survival compared with controls (10/14 *versus* 5/15) ($\chi^2 = 4.2$; $p < 0.05$) (Fig. 6*A*).

Animals pretreated with the NBD peptide had a decreased incidence of NEC (any grade) compared with animals treated with control peptide (2/14 *versus* 9/15) ($\chi^2 = 6.42$; $p < 0.025$) (Figs. 6*B* and 7). This corresponds to a mean (±SEM) histologic score of 1.3 (±0.34) [95% confidence interval (CI), 0.61–1.99] for the control group and a mean (±SEM) histologic score of 0.14 (±0.1) (95% CI, 0.07–0.35) in the NBD group ($p < 0.01$). Also, none of the animals in the NBD group had severe NEC (defined as having a score of 2 or higher) compared with 5 severe NEC in the control peptide group (5/15, *versus* 0/14) ($\chi^2 = 5.64$; $p < 0.025$) (Figs. 6*B* and 7).

DISCUSSION

The pathogenic mechanism leading to NEC remains poorly understood. We hypothesized that, in the premature infant, a

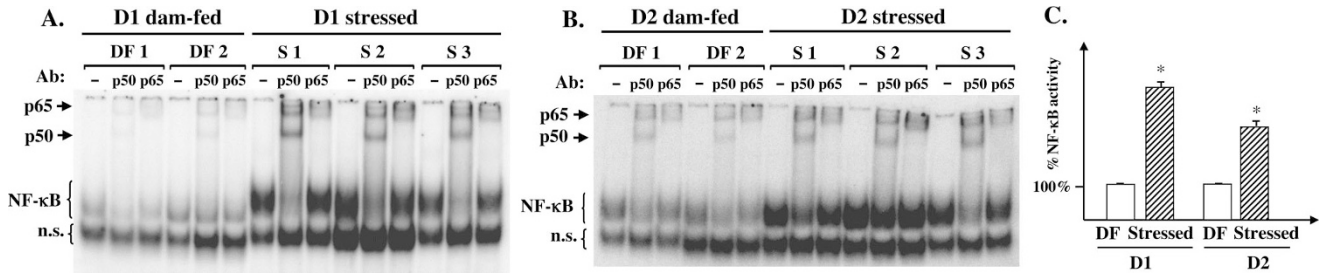

Figure 3. NF-κB is activated in a neonatal rat model of NEC. The small intestines of newborn rats submitted to formula feedings, hypoxia and cold stress (*S*) and of dam-fed control (*DF*) pups were obtained at d 1 (*A* and *C*) and at d 2 (*B* and *C*) and nuclear extracts prepared (six to eight samples per group). The NF-κB activity was assessed by EMSA and supershift experiments with antibodies (*Ab*) against p50 and p65 were performed. NF-κB–DNA binding activity was quantified by densitometry (*C*). A mean value was calculated for dam-fed control at d1 and results were expressed as a percentage of the dam-fed controls ± SEM (*$p < 0.001$).

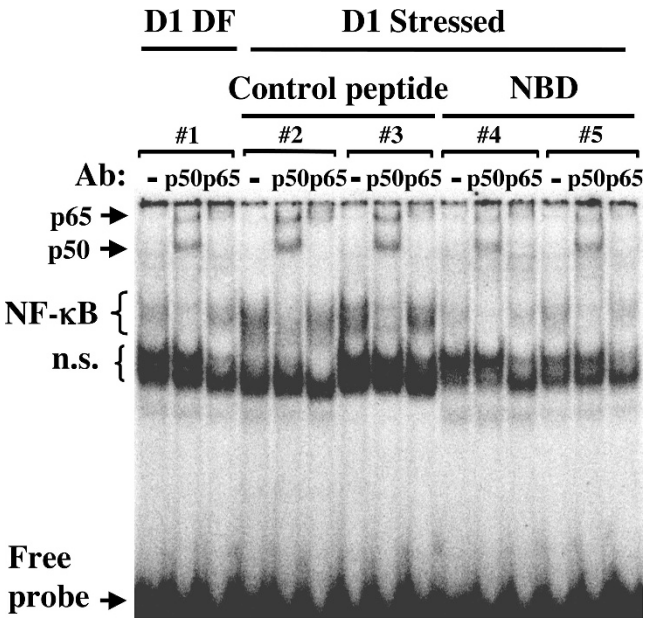

Figure 5. The NBD peptide inhibits NF-κB activation *in vivo* in the intestine. Small intestinal tissues of stressed pups (#1–5) treated with the NBD or control peptide were obtained at d 1 and were processed to obtain nuclear extracts. NF-κB activity was examined by EMSA and supershift experiments performed with anti-p50 and anti-p65 antibodies. A typical gel is shown here with the sample of a dam-fed control for comparison. Similar results were obtained in three independent experiments.

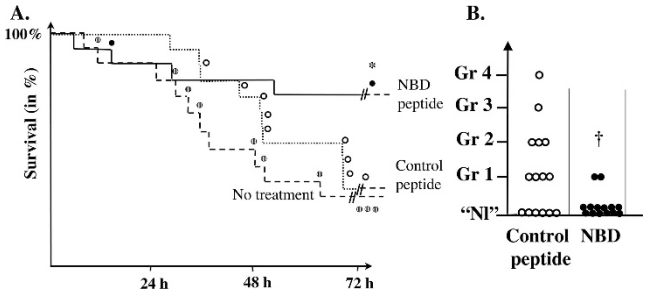

Figure 6. Effect of the NBD peptide on intestinal injury score and neonatal survival in a neonatal rat model of NEC. Neonatal rats were either untreated or treated with the NBD peptide (20 mg/kg, i.p.) ($n = 14$) or control peptide (20 mg/kg, i.p.) ($n = 15$) 1 h before the first stress at D0 and D1. Intestines were fixed in formalin immediately at time of death and sections were examined by a pathologist blinded to the groups. The survival curve (*A*) and the injury score (*B*) is presented here (*$p < 0.05$, †$p < 0.025$; grade 0: intact villi; 1: superficial epithelial cell sloughing; 2: mid-villous necrosis; 3: complete villous necrosis; 4: transmural necrosis). In *A*, ○: NEC and a survival curve of pups submitted to the NEC protocol without treatment is shown for comparison.

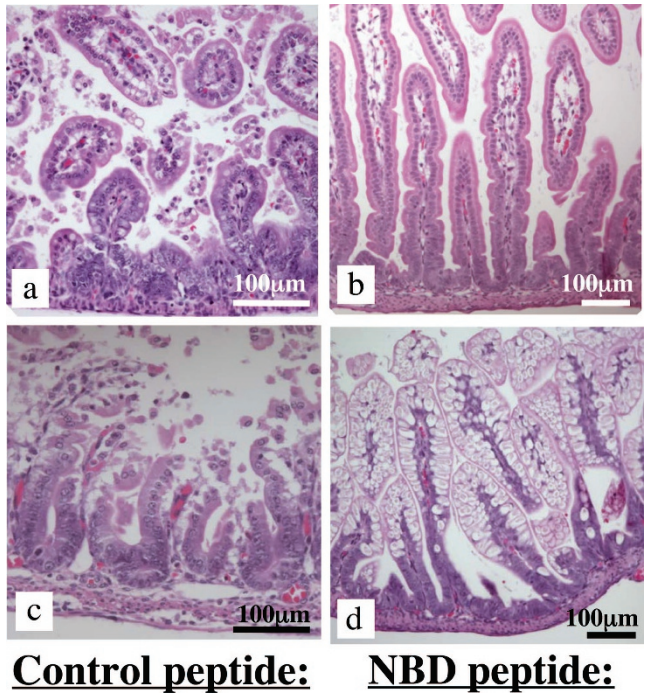

Figure 7. Small intestinal microscopic changes following the NEC protocol. Various degrees of experimental necrotizing enterocolitis were observed in the control animals (*a* and *c*), whereas minimal injury was found in the animals treated with NBD before the NEC protocol (*b* and *d*).

persistent and excessive activation of the transcription factor NF-κB could lead to persistent intestinal inflammation, injury, and NEC. In this study, we found that 1) NF-κB was persistently activated in stressed neonatal animals, 2) IκBα was decreased compared with dam-fed or adult animals at d2, and 3) the NBD peptide, a potent and selective IKK/NF-κB inhibitor, reduced intestinal NF-κB activation and the incidence of NEC and death in neonatal rats. These findings therefore support the important role of persistent NF-κB activation in the pathogenesis of NEC.

A common feature of NEC is the production and release of inflammatory mediators, including PAF (18) and NF-κB target gene products such as TNF (5,6) and the chemokine CXCL2, both of which are pivotal in mediating experimental bowel necrosis (17,19).

NF-κB is activated in many disease processes, including septic shock (20) and ulcerative colitis (21), and we previously demonstrated that it is activated in a model of acute bowel injury induced by PAF (11). However, very little is known about the activation of NF-κB during the perinatal/neonatal period and its role in the pathogenesis of NEC. In this study, we found a very low level of constitutive NF-κB activation in intestinal tissues during late gestation, but strong activation immediately after birth. This activation correlated temporally with the expression of iNOS, a well-documented NF-κB target gene (22). In normal, dam-fed animals, this activation is down-regulated within 24 h. In contrast however, we find that NF-κB remains persistently active in animals subjected to the induction of NEC. Transient activation of NF-κB at birth might be due to the exposure to bacterial products and to oxidative stress-inducing signals associated with delivery. Although transient activation of NF-κB at the time of birth might be protective and important for mounting a limited pro-inflammatory response that allows proper control of bacterial colonization, a persistent and exaggerated NF-κB activation might be detrimental, causing excessive cytokine release and intestinal injury.

Several clinical interventions have been shown to decrease the incidence of NEC including prenatal steroids (23), breast milk (24), and probiotics (25), all of which have been shown to down-regulate NF-κB. Dexamethasone suppresses NF-κB activation by inducing the NF-κB inhibitor IκB (26) and by direct protein-protein interactions that prevent NF-κB from

inducing transcription in a promoter site-specific fashion (27). Breast milk might contribute to decreased intestinal inflammation, as human breast milk has been shown to suppress the activation of the IL-8 gene promoter induced by IL-1β through NF-κB in intestinal epithelial cells (28). In these cells, human breast milk induces the production of IκBα (28). Probiotics have also shown promise as a prophylactic intervention to prevent NEC (25), and part of their effects may be NF-κB inhibition in colonic epithelial cells through proteasome inhibition (29).

Studies suggest that NF-κB might be developmentally regulated in various organs or cell-types. For example, IL-8 production and NF-κB activation in response to TNF is higher in neonatal polymorphonuclear leukocytes than adult cells (30). Of interest to neonatal intestinal disease, the expression of IκBα was found to be lower in immature *versus* mature enterocytes (31) with concomitant increased production of IL-8 in response to LPS and IL-1β in these fetal epithelial cell explants (32).

We did not find any significant prenatal changes in constitutive IκBα and IκBβ with maturation. Although NF-κB is strongly activated immediately after birth, the IκBα and IκBβ levels remain unchanged. This lack of correlation was also observed in IEC cells (16): A drop in the IκBα level could be detected only when cells were also co-incubated with cycloheximide, which inhibited IκBα resynthesis. This occurs because IκBα is a downstream gene target of NF-κB and is rapidly resynthesized upon NF-κB activation (33). We found that, in 1-d and 2-d dam-fed neonatal rats, whereas IκBα was only slightly decreased, the level of IκBβ dropped significantly. The level of IκBα and of IκBβ further decreased in stressed animals compared with dam-fed controls at d 2 of life.

Whereas NF-κB activation has been demonstrated in several experimental models of pro-inflammatory disease, its role in neonatal NEC has not been determined. We studied the importance of NF-κB activation in NEC using a specific NF-κB inhibitory peptide that contains the C-terminal NBD sequence from IKKα and IKKβ (a hexapeptide sequence required for interaction of the IKK with NEMO) coupled with the cell permeable antennapedia domain (34). By binding to NEMO, it blocks its association with the IKK complex and inhibits NF-κB activation without interrupting the constitutive activity of NF-κB (34). The NBD peptide has been shown to inhibit cytokine-induced NF-κB activation and NF-κB-dependent gene expression in Hela cells (34), and we previously demonstrated that it blocks LPS-induced MIP-2/CXCL2 gene expression in enterocytes (35). Importantly, the NBD peptide effectively ameliorates inflammation *in vivo* in models such as ear edema (34), acute peritonitis (induced by intraperitoneal injection of zymosan), and experimental acute pancreatitis (36).

In this study, we found that the NBD peptide inhibited intestinal NF-κB activation, improved neonatal survival, and decreased the incidence of histologically confirmed bowel injury in neonatal NEC. This observation therefore strongly suggests that bowel injury in NEC may result from a dysregulation of IKK activity. Furthermore, although our data are derived from a limited sample size and must be viewed as preliminary, they provide extremely compelling evidence supporting further in-depth *in vivo* investigations of the NBD peptide in animal models of NEC.

In summary, our study demonstrates that NF-κB is persistently activated in a neonatal model of NEC and that dysregulated IKK activity mediates these effects. Our data suggest that, although limited NF-κB activation in specific cells of the intestine is likely to be protective, a prolonged activation of NF-κB above a certain threshold might be detrimental. Further studies are required to better characterize the role of NF-κB activation in NEC before we consider NF-κB inhibition as a therapeutic approach to NEC. However, our *in vivo* findings provide a compelling argument for aggressively pursuing NF-κB as therapeutic target.

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