Nuclear Factor–κB Is Activated in Intestinal Mucosa During Endotoxemia

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**Background:** The transcription factor nuclear factor–κB (NF-κB) regulates a large number of genes involved in the inflammatory response to critical illness. The intestinal mucosa plays an active role in the inflammatory and metabolic response to sepsis and endotoxemia, but it is not known if NF-κB is activated in the mucosa during these conditions.

**Objective:** To test the hypothesis that endotoxemia in mice activates NF-κB in intestinal mucosa.

**Methods:** Mice were injected subcutaneously with lipopolysaccharide, 12.5 mg/kg, or a corresponding volume of saline. At various intervals following injection, jejunal mucosa was harvested and nuclear and cytoplasmic fractions were prepared. The nuclear fractions were analyzed by electrophoretic mobility shift assay for NF-κB and by Western blot analysis for the NF-κB subunits p50 and p65. Cytoplasmic fractions were analyzed by Western blotting for the NF-κB inhibitory proteins IκB-α and IκB-β.

**Results:** Electrophoretic mobility shift assay showed that NF-κB was activated in jejunal mucosa 1 hour after injection of lipopolysaccharide and persisted for at least 4 hours. The NF-κB subunits p50 and p65 were present in nuclear fractions of mucosa from endotoxemic mice at the corresponding time points. Cytoplasmic levels of the inhibitory proteins IκB-α and IκB-β decreased during endotoxemia, and the proteins were nearly absent 60 minutes after injection of lipopolysaccharide.

**Conclusions:** The results suggest that IκB is degraded and NF-κB is activated in intestinal mucosa during endotoxemia. The findings support the concept that the intestinal mucosa is an important component of the inflammatory response to sepsis and endotoxemia.

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There is increasing evidence that the intestinal mucosa plays an important role in the inflammatory and metabolic response to sepsis, endotoxemia, and other critical illness. In recent studies from our and other laboratories, the local mucosal production of the proinflammatory cytokines tumor necrosis factor, interleukin (IL)-1, IL-6, and IL-10 was increased in endotoxemic and septic mice. In other studies, we found that sepsis and endotoxemia were associated with mucosal synthesis of the acute-phase proteins complement component C3 and serum amyloid A. The genes for several proinflammatory cytokines and acute-phase proteins are regulated by the transcription factor nuclear factor–κB (NF-κB), but it is not known whether NF-κB is activated in intestinal mucosa during sepsis and endotoxemia.

Nuclear factor–κB is a dimer consisting of various subunits, most commonly p50 and p65, and is normally sequestered in an inactive form in the cytoplasm by the inhibitory factor IκB (for review, see Baldwin). After appropriate stimuli, IκB is phosphorylated, ubiquitinated, degraded by the 26S proteasome, and dissociated from NF-κB. Several species of IκB have been described, including IκB-α and IκB-β. After dissociation of IκB from NF-κB, the activated transcription factor translocates to the nucleus, where it binds to DNA and up-regulates gene transcription.

Although there is evidence that NF-κB may be activated by different stimuli in cultured enterocytes, the participation of NF-κB in the inflammatory response in intestinal mucosa in vivo is not known. The present experiments were designed to test the hypothesis that endotoxemia results in activation of NF-κB in intestinal mucosa measuring mucosal levels of IκB and NF-κB activation in the jejunum of endotoxemic mice. We found that endotoxemia was associated with degradation of mucosal IκB-α and IκB-β and increased DNA binding activity of NF-κB.
MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male A/J mice weighing 18 to 23 g (Jackson Laboratory, Bar Harbor, Maine) were housed in a room with a temperature of 25°C and with a 12-hour light-dark cycle for 1 week before experiments. Endotoxemia was induced by the subcutaneous injection of lipopolysaccharide (LPS), 12.5 mg/kg (Escherichia coli endotoxin 0111:B4, Calbiochem Co, La Jolla, Calif) dissolved in 0.5 mL of sterile saline. Control mice were injected with the same volume of sterile saline. The mice had free access to drinking water throughout the experiments, but food was withheld after LPS or saline injection to avoid the influence of any difference in food intake between the groups of mice on mucosal responses. The dose and route of LPS administration used were based on a recent study in which mucosal IL-6 production was increased in endotoxemic mice. The animals were cared for in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati, Cincinnati, Ohio.

At different time points up to 4 hours after injection of saline or LPS, animals were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally), and the abdomen was opened through a midline incision. The jejunum was excised, flushed with ice-cold saline, and opened along the antimesenteric border. This procedure was done on an ice-cold board. The intestinal mucosa was harvested by scraping with a microscope slide, and the samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. The jejunum was studied because in previous reports we found that the response to sepsis and endotoxemia with regard to mucosal protein synthesis, IL-6 production, and acute-phase protein synthesis was more pronounced in the jejunum than in other parts of the gastrointestinal tract. Because in a recent study NF-kB was activated in liver of endotoxemic mice, in an initial experiment, the left lobe of the liver was excised in addition to the jejunum to compare the response to endotoxemia in the 2 tissues.

PREPARATION OF NUCLEAR AND CYTOPLASMIC FRACTIONS

Nuclear and cytoplasmic fractions were prepared as described elsewhere with minor modifications. All steps were carried out on ice. Tissue samples were homogenized (Power Gen 700, Fischer Scientific, Indianapolis, Ind) in 1 mL of ice-cold buffer A (10-mmol/L HEPES, pH 7.9, 1.5-mmol/L magnesium chloride; 10-mmol/L potassium chloride; 1-mmol/L dithiothreitol; and 1-mmol/L phenylmethylsulfonylfluoride) and allowed to incubate on ice for 10 minutes. The supernatants were then centrifuged at 850 g for 10 minutes at 4°C. The supernatants were discarded, and the pellets were resuspended in 400 µL of buffer A with 0.1% Triton X-100 (Sigma, St Louis, Mo), incubated for 10 minutes on ice, and centrifuged as above. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 750 µL of buffer A, centrifuged as above, and resuspended in 200 µL of a buffer consisting of 20-mmol/L HEPES, pH 7.9, 25% glycerol (vol/vol), 420-mmol/L sodium chloride, 1.5-mmol/L magnesium chloride (buffer B).

RESULTS

In an initial experiment, electrophoretic mobility shift assay (EMSA) was performed on liver tissue and jejunal mucosa 1 hour after injection of saline or LPS. Similar to a recent report, endotoxemia resulted in NF-kB activation in the liver (Figure 1). Nuclear factor-kB DNA binding activity was seen 1 hour after LPS injection in jejunal mucosa as well. Whereas no NF-kB activation was seen in the liver of saline-injected control mice, there was evidence of NF-kB activation in jejunal mucosa from both control and endotoxemic mice. In multiple experiments (n = 10), there was a clear increase in NF-kB activation in jejunal mucosa 1 hour after injection of LPS as compared with saline-injected animals. When excess unlabeled oligonucleotide was added, the NF-kB band disappeared (last lane in Figure 1), indicating that the complex seen reflected NF-kB binding activity.

We next examined NF-kB activation in jejunal mucosa at different time points after injection of saline or LPS. The results from that experiment showed evidence of maximal NF-kB activation 1 hour after injection of LPS, with a decline in binding activity during the next 3 hours (Figure 2). No increase in NF-kB binding activity was seen earlier than 1 hour after induction of endotoxemia.

To investigate the composition of the DNA-binding complex, supershift analysis was performed. A supershift was seen after incubation with antibody to the NF-kB subunit p50 (Figure 3). Little or no supershift was noted when antibodies to p52 and p65 were used. The greatest supershift was seen when antibodies to p50 and p65 were used together. These results suggest that the activated NF-kB complex was composed primarily of p50 subunits.

When Western blot analysis of the nuclear fraction of jejunal mucosa was performed, results showed that p50 and p65 appeared at high concentrations in this fraction 1 hour after injection of LPS and thereafter declined during the next 3 hours (Figure 4). This result is consistent with translocation of both p50 and p65 to the nucleus in endotoxemic mice.

Nuclear factor-kB is usually activated by the degradation and dissociation of IκB from NF-kB. Western blot analysis showed that IκB-α and IκB-β levels in the cytoplasmic fraction of jejunal mucosa decreased in endotoxemic mice; the inhibitory proteins were almost not detectable at 1 hour after injection of LPS but reappeared at 2 hours (Figure 5). Notably, the changes in concentrations over time were almost identical for IκB-α and IκB-β. These results are consistent with degradation of the inhibitory proteins in intestinal mucosa during endotoxemia followed by a relatively rapid resynthesis of the proteins, similar to changes in IκB levels in other tissues after stimulus.
In the present study, endotoxemia in mice resulted in disappearance of IkB and activation of NF-kB in jejunal mucosa. Results from the experiments in which supershift assay and Western blot analysis of the nuclear fraction were performed suggest that the activated NF-kB consisted primarily of p50 subunits, but that p65 subunits were present as well. This is the first report of NF-kB activation in intestinal mucosa in vivo during endotoxemia. The results lend further support to the concept that the intestinal mucosa participates in the inflammatory response to endotoxemia.

In recent studies from our and other laboratories, the local production of tumor necrosis factor, IL-1, IL-6, and the acute-phase proteins C3 and serum amyloid A was increased in intestinal mucosa during sepsis and endotoxemia. Nuclear factor-kB activates the genes for several proinflammatory cytokines and acute-phase proteins (for review, see Baldwin). It may therefore be speculated that the results observed in the present study reflect a role of NF-kB in the transcriptional regulation of mucosal cytokines and acute-phase protein synthesis. Further experiments will be needed, however, to clarify the relationship between NF-kB activation and the synthesis of proinflammatory cytokines and acute-phase proteins in the intestinal mucosa.

The result of up-regulated NF-kB activity in the liver 1 hour after injection of LPS was similar to a recent report and suggests that NF-kB is activated in multiple organs and tissues during endotoxemia. The liver is an important production site for proinflammatory cytokines and acute-phase proteins during sepsis and endotoxemia, and the genes for these inflammatory products may be regulated by NF-kB in the liver as well.

The classic form of NF-kB is a p50/p50 dimer. Besides p50/p50, NF-kB may consist of homodimers and heterodimers of these and other subunits. In the present study, supershift analysis suggested that the NF-kB binding complex consisted mainly of p50 subunits. However, Western blotting indicated that p65 translocated to the nuclear fraction as well. These apparently conflicting results may reflect a greater abundance of p50 than p65 in the nuclear fraction, or a greater sensitivity of Western blotting than of supershift analysis to detect proteins present in the nuclear fraction.

Several members of the IkB family have been described, including IkB-α, IkB-β, IkB-γ, IkB-δ, and IkB-ε. The best-known IkB molecules are IkB-α and IkB-β. IkB-α is usually degraded rapidly after stimulus, whereas IkB-β degradation is delayed and more protracted and has been suggested to account for a more prolonged inflammatory response. The finding in the present study that IkB-α and IkB-β degradation (ie, disappearance...
Sepsis continues to be a major cause of morbidity and mortality in surgical patients. Recent studies have shown that the intestinal mucosa plays an important role in the inflammatory response to sepsis and endotoxemia, producing both proinflammatory cytokines and certain acute-phase proteins. The regulatory mechanisms of this response are not well understood. The transcription factor NF-κB regulates the transcription of the genes for a large number of inflammatory proteins. Previous studies in our laboratory have demonstrated that NF-κB is activated by different inflammatory stimuli in cultured enterocytes in vitro, but the role of NF-κB in the intestinal mucosa in vivo is not known. The present study provides evidence that NF-κB is activated in the intestinal mucosa in vivo during endotoxemia. Understanding the role of NF-κB in the inflammatory response in the intestinal mucosa during endotoxemia may eventually allow therapeutic manipulation of this response in the intestine.

Figure 1. Electrophoretic mobility shift assay for nuclear factor–κB (NF-κB) activity in liver and jejunal mucosa 1 hour after injection of saline or lipopolysaccharide (12.5 mg/kg) in mice. Lanes 1, 2, 5, and 6 represent saline-injected control mice, and lanes 3, 4, 7, and 8, lipopolysaccharide-injected mice. Lane 9 shows the effect of addition of excess unlabeled oligonucleotide competitor to binding reactions.

Figure 2. Electrophoretic mobility shift assay for nuclear factor–κB (NF-κB) activity in jejunal mucosa at different time points after injection of saline or lipopolysaccharide (LPS) in mice.

Figure 3. Electrophoretic mobility supershift assay of jejunal mucosa 1 hour after injection of lipopolysaccharide. Comp indicates addition of excess unlabeled competitor; p50, p52, and p65, the presence of antibody to that specific nuclear factor–κB (NF-κB) subunit.

Figure 4. Western blot analysis of the nuclear fraction of jejunal mucosa at different time points after injection of saline or lipopolysaccharide (LPS) in mice. Antibodies against p50 (top) and p65 (bottom) were used in these Western blots.

Figure 5. Western blot analysis of the cytoplasmic fraction of jejunal mucosa at different time points after injection of saline or lipopolysaccharide (LPS) in mice. Antibodies against IκB-α (top) and IκB-β (bottom) were used in the analysis.

ent results at least in part reflected NF-κB activation in enterocytes, although other mucosal cells may have participated in the response as well.

Jejunal mucosa was studied in the present report because we found previously that this segment of the gastrointestinal tract is particularly sensitive to the effects of sepsis and endotoxemia with respect to the production of IL-6, total proteins, and acute-phase proteins. It will be important in future experiments to test whether sepsis and endotoxemia result in NF-κB activation in other parts of the gastrointestinal tract as well.
The intracellular pathways involved in NF-κB activation in intestinal mucosa are not known from the present study. In a recent report we found that NF-κB activation in cultured human enterocytes required serine protease activity but not tyrosine kinase activity or oxygen radicals.20 This was different from several other cell types in which these mechanisms were involved in NF-κB activation.21,22 Thus, NF-κB activation may be regulated by different intracellular mechanisms in different cell types and tissues. The intracellular signaling pathways involved in the regulation of NF-κB activation in intestinal mucosa will be an important area for future research considering the potential consequences of mucosal NF-κB activation during sepsis, endotoxemia, and perhaps other conditions as well.

In recent in vitro experiments, we found that NF-κB was activated in cultured human intestinal epithelial cells by IL-1β.23 It remains to be determined whether IL-1β plays a similar role in intestinal mucosa in vivo.

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REFERENCES


DISCUSSION

Mitchell Fink, MD, Boston, Mass: The results of these carefully performed experiments speak for themselves: NF-κB is activated in jejunal mucosa following the injection of lipopolysaccharide in mice. The intestinal mucosa, however, is a complex tissue and consists of myriad cell types, including enterocytes, intraepithelial and subepithelial lymphocytes, intraepithelial and subepithelial lymphocytes of various subtypes, plasma cells, polymorphonuclear leukocytes, macrophages, mast cells, endothelial cells, and specialized antigen-processing, or M cells. Even among the enterocytes themselves are multiple subtypes, including primordial and pluripotent crypt cells, villus-absorptive enterocytes, goblet cells, Paneth cells, and enterochromaffin cells. Thus the obvious question to ask is, which mucosal cell populations is NF-κB being activated in? Have the authors done any studies using cluted epithelial cells in an effort to narrow the field at least partially?

Finally, let’s assume for the moment that endotoxin activates NF-κB in enterocytes in vivo. What do the authors think the mechanism might be, since in vitro cultured enterocytes do not seem to respond to LPS even in the presence of serum? Does LPS activate other cells, say macrophages in the lamina propria, triggering the production of interferon gamma and possibly other cytokines that in turn activate NF-κB in enterocytes? Or is it possible that, unlike cultured, immortalized enterocytic cell lines, native enterocytes are actually responsive to LPS?

Dr Pritts: The intestinal mucosa is indeed a very complex tissue composed of many cell types. We have not yet examined which specific cell type (or types) are activated during endotoxemia in vivo. As you also noted, we have previously shown that NF-κB can be activated in cultured enterocytes in vitro, and in other studies we have also used immunohistochemistry to show that the enterocytes produce IL-6 in vivo in the intestinal mucosa. We feel that this provides circumstantial evidence that NF-κB may be activated in enterocytes in vivo, but we will need to address this in future studies.

You also asked about potential mechanisms of NF-κB activation in the intestinal mucosa in vivo, pointing out that cultured enterocytes, under at least most conditions, do not appear to react to endotoxin directly in vitro. Further studies will be needed to address the mechanisms leading to in vivo NF-κB activation in detail. It is our suspicion that IL-1 and TNF-α may at least play a role.