Induction of the Stress Response In Vivo Decreases Nuclear Factor–Kappa B Activity in Jejunal Mucosa of Endotoxemic Mice

Timothy A. Pritts, MD; Quan Wang, MD, PhD; Xiaoyan Sun, MD, PhD; M. Ryan Moon, MD; David R. Fischer, MD; Josef E. Fischer, MD; Hector R. Wong, MD; Per-Olof Hasselgren, MD

**Background:** Results of previous studies suggest that the stress response protects cells and tissues by regulating pro-inflammatory mediators. The transcription factor nuclear factor-kappa B (NF-κB), normally sequestered in the cytoplasm by its inhibitory protein, IκB, regulates many genes involved in inflammatory responses to critical illness. Endotoxemia is associated with increased NF-κB activity in intestinal mucosa, but the effect of the stress response on endotoxin-induced NF-κB activation in intestinal mucosa is not known.

**Hypothesis:** Induction of the stress response inhibits NF-κB DNA binding activity in jejunal mucosa during endotoxemia.

**Methods:** The stress response was induced in mice by hyperthermia (42°C) or injection with sodium arsenite (10 mg/kg). After 2 to 5 hours, mice were injected with endotoxin (lipopolysaccharide, 12.5 mg/kg) or a corresponding volume of sterile saline. One hour later, jejunal mucosa was harvested for preparation of nuclear and cytoplasmic extracts.

**Results:** Mucosal levels of heat shock protein–72 increased after hyperthermia or treatment with sodium arsenite, consistent with induction of the stress response. The increase in NF-κB DNA binding activity and decrease in IκB-α levels seen after endotoxin injection were inhibited by previous induction of the stress response.

**Conclusion:** The protective effects of the stress response in vivo might, at least in part, be due to inhibited NF-κB activation.


---

The intestinal mucosa is now recognized as an active participant in the inflammatory response to sepsis and endotoxia. In recent studies, mucosal production of the proinflammatory cytokines interleukin (IL)-1β and IL-6, and the acute phase proteins complement component C3 and serum amyloid A3 was increased during acute inflammation. Results of other studies suggest that the intestine might be a significant source of serum tumor necrosis factor–α during shock.

Nuclear factor–kappa B (NF-κB) is an important transcription factor that regulates many genes involved in the inflammatory response, including the genes for IL-1, IL-6, tumor necrosis factor, and several acute phase proteins (for review, see Ghosh et al6). Nuclear factor–kappa B is normally sequestered in the cytoplasm by the inhibitory protein IκB. In response to a stimulus, IκB is phosphorylated, ubiquitinated, and degraded by the 26S proteasome. This allows NF–κB to translocate to the nucleus, where it binds to its target sequences and induces gene transcription.

Recently, we found that IκB-α, the major form of IκB, was degraded, and that NF-κB DNA binding activity was increased in intestinal mucosa during endotoxia. In other studies, NF-κB binding activity was decreased in vitro in different cell types by induction of the stress, or heat shock, response. The stress response is found in virtually all cells and tissues as a response to diverse cytotoxic stimuli (for review, see Wong and Wispe10). Central to the stress response is the induction of heat shock proteins (HSPs). Although these proteins were originally identified in cells exposed to elevated temperatures, expression of HSPs has subsequently been demonstrated in cells and tissues in response to nonthermal stress, including sodium arsenite treatment.

The effect of the stress response on NF-κB activation in intestinal mucosa in vivo has not been reported, to our knowledge. We hypothesized that induction of the stress response would decrease NF-κB activation...
**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

Male A/J mice weighing 18 to 23 g (Jackson Laboratory, Bar Harbor, Me) were housed for 1 week before experiments in a room at 25°C and with a 12-hour light-dark cycle. 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. All experiments were performed at least 3 times to ensure reproducibility.

The stress response was induced by hyperthermia or injection of sodium arsenite. Mice subjected to hyperthermia were anesthetized with xylazine hydrochloride (2.5 mg/kg) and ketamine hydrochloride (60 mg/kg) and placed in a heated (45°C), humidified chamber. Rectal temperature was monitored with a thermal probe (model YSI-423; Yellow Springs Instrument Co, Yellow Springs, Ohio) and a 3-channel thermometer (model 8802-16; Cole Parmer Instrument Co, Chicago, Ill) at 1-minute intervals. Animals were heated to a rectal temperature of 42°C for 3 minutes, then removed from the heating chamber, resuscitated with 50 mL/kg body weight of sterile saline, and placed on a heating blanket until their rectal temperature had returned to 40.5°C. The mice were then returned to their cages and kept at room temperature. Rectal temperature was above 40.5°C for approximately 15 minutes. Control animals were anesthetized and resuscitated with 50 mL/kg body weight of sterile saline, but remained at room temperature. Heated and control animals were allowed to recover for 2 hours before induction of endotoxemia.

Chemical induction of the stress response was achieved by intraperitoneal injection of sodium arsenite, 10 mg/kg (Sigma-Aldrich Corp, St Louis, Mo), dissolved in 0.5 mL of sterile water. Control animals were injected with a corresponding volume of vehicle. The animals were allowed to recover for 5 hours before induction of endotoxemia.

Recovery periods after induction of the stress response by hyperthermia (2 hours) or sodium arsenite injection (5 hours) were based on previous experiments in our laboratory showing high levels of heat shock protein-72 (HSP-72) in intestinal mucosa at these times (Q.W. and T.A.P., unpublished observations, 1998).

Endotoxemia was induced as described previously. Briefly, mice were injected subcutaneously with lipopolysaccharide (LPS), 12.5 mg/kg (Escherichia coli endotoxin 0111:B4; Calbiochem Co, La Jolla, Calif), dissolved in 0.5 mL of sterile water. Control animals were injected with the same volume of saline. Control mice were injected with the same volume of 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.

One hour after injection of saline solution or LPS, animals were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and the abdomen was opened through a midline incision. The jejunum was excised, placed on an ice-cold board, flushed with ice-cold saline solution, and opened along the antimesenteric border. 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 previously, we found that the response to endotoxemia, with regard to IL-6 production and NF-κB activation and IkB-α degradation (T.A.P. and P.-O.H., unpublished data, 1998) was more pronounced in the jejunum than in other parts of the gastrointestinal tract. Mice were studied 1 hour after induction of endotoxemia because previously, we found that NF-κB binding activity and IkB-α degradation in the jejunal mucosa were maximal at this time.

**PREPARATION OF NUCLEAR AND CYTOPLASMIC FRACTIONS**

Nuclear and cytoplasmic fractions were prepared as described previously, with minor modifications. All steps were carried out on ice. Tissue samples were homogenized 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 phenylmethylsulfonyl fluoride), incubated on ice for 10 minutes, and then centrifuged at 850 g for 10 minutes at 4°C. The supernatants were discarded and the pellets were resuspended in 1 mL of buffer B with 0.1% Triton X-100 per milligram of tissue, incubated for 10 minutes on ice, and centrifuged as described above. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 750 µL of buffer A, centrifuged as described above, and resuspended in 1-mL/mg of tissue of a buffer consisting of 20-mmol/L HEPES (pH 7.9), 25% glycerol (volume per volume), 420-mmol/L sodium chloride, 1.5-mmol/L magnesium chloride, and 0.2-mmol/L EDTA. After incubation for 30 minutes on ice, the nuclear fraction was recovered by centrifugation at 20 000g for 15 minutes.

Continued on next page

in intestinal mucosa during endotoxemia. Our results suggest that induction of the stress response by hyperthermia or treatment with sodium arsenite decreases NF-κB DNA binding activity in the jejunal mucosa during endotoxemia, possibly by preserving cytoplasmic IkB-α levels.

**RESULTS**

In initial experiments, we examined cytoplasmic extracts for evidence of induction of the stress response by performing Western blotting for HSP-72. This protein is highly inducible and often used to document induction of the stress response. Little or no HSP-72 was present in mucosa from control mice or mice injected with endotoxin (Figure 1). In contrast, strong signals for HSP-72 were seen on Western blots from mice subjected to hyperthermia or treated with sodium arsenite, documenting induction of the stress response in the intestinal mucosa of these animals.

We recently reported that endotoxemia in mice resulted in increased NF-κB DNA binding activity in the jejunal mucosa. To confirm these results, and to determine which NF-κB subunits are involved in this response, we next performed electrophoretic mobility shift assay, competition reactions, and supershift analysis on nuclear fractions from the jejunal mucosa of endotox-
at 4°C. The nuclear and cytoplasmic fractions were assayed for protein concentration (Bio-Rad Laboratories, Hercules, Calif) and stored at -80°C until further analysis.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Nuclear factor–kappa B gel shift oligonucleotide 5’ AGT TGA GGG GAC TTT CCC AGG C 3’ (Santa Cruz Biotechnology, Santa Cruz, Calif) was end labeled with (32P) γ adenosine triphosphate (γATP) using polynucleotide kinase T4 (Gibco BRL, Grand Island, NY). End-labeled probe was purified from unincorporated (32P) γATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer (pH 7.4). Equivalent amounts of nuclear proteins (7.5 µg of protein) were incubated in a buffer containing 12% (vol/vol) glycerol, 12-mmol/L HEPES (pH 7.9), 4-mmol/L Tris-HCl (pH 7.9), 1-mmol/L EDTA, 1-mmol/L dithiothreitol, 25-mmol/L potassium chloride, 5-mmol/L magnesium chloride, 0.04-µg/µL poly[d(1-C)] (Boehringer Mannheim, Indianapolis, Ind), and Tris-EDTA buffer (pH 7.4). Labeled probe was added, and the samples were incubated for 20 minutes on ice. Where indicated in the “Results” section, an excess (20 ng) of unlabeled NF-κB consensus oligonucleotide or unlabeled mutant NF-κB oligonucleotide (5’-AGT TGA GGG GAC TTT CCC AGG C 3’; 1 base pair substitution underlined) (Santa Cruz Biotechnology) was added for competition reactions. For supershift reactions, 2 µL of antibody to the NF-κB subunit p50 or p65 (Santa Cruz Laboratories) were added 2 hours before addition of the radiolabeled probe. Samples were then subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 200 V using Tris-borate EDTA buffer (Tris-borate, 0.45-mmol/L; sodium phosphate EDTA [1 sodium chloride–sodium citrate], 15-mmol/L) and 0.1% sodium dodecyl sulfate (SDS). The samples were incubated for 2 hours on ice. Where indicated in the “Results” section, an excess (20 ng) of unlabeled NF-κB consensus oligonucleotide or unlabeled mutant NF-κB oligonucleotide (5’-AGT TGA GGG GAC TTT CCC AGG C 3’; 1 base pair substitution underlined) (Santa Cruz Biotechnology) was added for competition reactions. For supershift reactions, 2 µL of antibody to the NF-κB subunit p50 or p65 (Santa Cruz Laboratories) were added 2 hours before addition of the radiolabeled probe. Samples were then subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 200 V using Tris-borate EDTA buffer (Tris-borate, 0.45-mmol/L; sodium chloride, 25-mmol/L; sodium phosphate, 0.01-mmol/L; and EDTA, 0.001-mol/L). Blots were dried overnight and analyzed by exposure to x-ray film (X-Omat AR; Eastman-Kodak, Rochester, NY).

WESTERN BLOT ANALYSIS

Aliquots of the cytoplasmic fractions containing 25 µg of protein were boiled in equal volumes of loading buffer (125-mmol/L Tris-hydrochloride [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, and 1% 2-mercaptoethanol) for 3 minutes, then separated by electrophoresis on an 8% to 16% Tris-glycine gradient gel (Novex, San Diego, Calif). A protein ladder (See-Blue Standard; Novex) was included as a molecular weight marker. The proteins were transferred to nitrocellulose membranes, which were blocked with 10% nonfat dried milk in Tris-buffered saline solution (pH 7.6), containing 0.05% Tween-20 for 1 hour, then incubated with a polyclonal rabbit antimouse antibody to IkB-α (Santa Cruz Biotechnology) or HSP-72, the inducible form of HSP-70 (StressGen Biotechnologies Corp, Victoria, British Columbia) for 45 minutes. After washing twice in Tris-buffered saline solution containing Tween-20, the blots were incubated with a peroxidase-conjugated goat antirabbit IgG secondary antibody for 15 minutes. Blots were washed in Tris-buffered saline solution containing Tween-20 for 5 minutes 3 times then in Tris-buffered saline solution for 5 minutes, incubated in enhanced chemiluminescence reagents (Amer sham Life Sciences, Buckingham, England), exposed on radiographic film (Eastman-Kodak), and quantitated by densitometry.

NORTHERN BLOT ANALYSIS

For determination of IkB-α messenger RNA (mRNA) levels, total cellular RNA was isolated and extracted by a modification of the acid guanidinium thiocyanate-phenol-chloroform method using a commercially available reagent (Trizol; Gibco BRL). Aliquots containing 20 µg of total RNA were fractionated by electrophoresis on a 1% agarose gel containing 16% formaldehyde, then transferred to a nylon membrane (Nytran; Schleicher and Schuell Inc, Keene, NH). RNA was immobilized by baking the membranes at 80°C for 2 hours in a vacuum oven. Blots were prehybridized for 4 hours at 42°C in a mixture containing 50% formamide, 5 × Denhardt’s solution, 5 × sodium chloride sodium phosphate EDTA (1 × sodium chloride sodium phosphate EDTA = sodium chloride, 0.15-mmol/L; sodium phosphate, 0.01-mmol/L; and EDTA, 0.001-mmol/L), 0.3% sodium dodecyl sulfate, and herring sperm DNA (0.25-mg/mL). Complementary DNA to IkB-α was labeled with cytosine triphosphate (γ-32P)dCTP) by random labeling (Pharmacia Biotech Inc, Piscataway, NJ). Blots were hybridized with the 32P-labeled IkB-α probe in the same buffer as described above at 42°C overnight. Blots were then serially washed with 2 × sodium chloride-sodium citrate (1 × sodium chloride–sodium citrate = sodium chloride, 0.15-mmol/L; sodium citrate, 15-mmol/L) and 0.1% sodium dodecyl sulfate at room temperature for 15 minutes twice, then 0.2 × sodium chloride–sodium citrate and 0.1% sodium dodecyl sulfate at 50°C. After washing, exposure was carried out for 4 hours using a PhosphorImager screen (Molecular Dynamics, Sunnyvale, Calif). Blots were stripped and rehybridized with a complementary DNA probe to glyceraldehyde-3-phosphate dehydrogenase to control for equal loading of RNA.

emic mice. One hour after endotoxin injection, a high level of NF-κB DNA binding activity was noted (Figure 2), confirming previously published results. The NF-κB band disappeared with the addition of excess unlabeled cold competitor to binding reactions but not with mutant competitor, confirming its specificity. Addition of antibody to the NF-κB subunit p50, but not p65, resulted in a supershift, suggesting that the NF-κB binding complex consisted of p50 homodimers or p50 heterodimers with other subunits. Although no supershift was seen with the addition of antibody to the p65 subunit, previous studies from our laboratory demonstrated that p65 is present in nuclear fractions of the jejunal mucosa from endotoxin-treated animals, suggesting that the activated NF-κB complex may contain p50 and/or p65 subunits.

In the next series of experiments, we examined the effect of the stress response on NF-κB DNA binding activity. When electrophoretic mobility shift assay for NF-κB was performed on nuclear fractions from animals subjected to hyperthermia before endotoxin injection, the NF-κB band was abolished (Figure 3, left). Similarly, in animals treated with sodium arsenite before endotoxin injection, the intensity of the NF-κB band was decreased compared with that of animals injected with vehicle and endotoxin (Figure 3, right). These data suggest...
that induction of the stress response before endotoxin injection resulted in decreased NF-κB DNA binding activity. Minimal NF-κB DNA binding activity was seen in mucosa from animals subjected to hyperthermia or treated with sodium arsenite alone.

Nuclear factor–kappa B is usually sequestered in the cytoplasm by the inhibitory protein IκB. In response to a stimulus, IκB is degraded, freeing NF-κB to translocate to the nucleus and bind to its target gene sequences. Western blot analysis of cytoplasmic fractions for IκB-α revealed a decreased intensity of the band 1 hour after endotoxin injection, consistent with rapid degradation of IκB-α (Figure 4). Western blot analysis performed on samples from animals that had undergone induction of the stress response by either hyperthermia (Figure 4, left) or sodium arsenite (Figure 4, right) before endotoxin injection revealed that IκB-α levels were maintained in these animals.

Preserved IκB-α levels in intestinal mucosa of endotoxemic mice that had undergone induction of the stress response might reflect increased synthesis, reduced breakdown of the protein, or both. To test the potential contribution of increased IκB-α synthesis, we next examined the expression of IκB-α mRNA in the different groups of mice. Levels of IκB-α mRNA increased in jejunal mucosa from animals injected with endotoxin (Figure 5). In addition, treatment of mice with either hyperthermia or sodium arsenite resulted in increased IκB-α mRNA expression (Figure 5).

In the present study, induction of the stress response in mice by hyperthermia or sodium arsenite injection resulted in increased HSP-72 levels in jejunal mucosa and was associated with maintained cytoplasmic IκB-α levels and decreased endotoxin-induced NF-κB binding activity. This is the first report, to our knowledge, of down-regulation of NF-κB binding activity by the stress response in vivo. The results are consistent with those observed previously in vitro in cultured respiratory tract and intestinal epithelial cells. The finding that a down-
ileitis. The results of the present study are important. Although our data do not allow us to ascribe a protective effect specifically to HSP-72, other studies provide more direct evidence of a role for this protein in cytoprotection. For example, transfec-
tion of intestinal epithelial cells with HSP-72 protected cells from oxidant and thermal injury. In other experiments, antisense oligonucleotides directed against HSP-72 blunted heat shock inhibition of nitric oxide synthase-2 activity in astroglial cells.

Under basal conditions, NF-κB is sequestered in the cytoplasm by its inhibitory protein, IκB (for review, see Perkins27). The best-studied member of the IκB family is IκB-α. Although IκB-α is degraded in the cytoplasm in response to appropriate stimuli, IκB-α levels are rapidly restored, partly because NF-κB activation induces IκB-α gene transcription. Consistent with this model, we found that endotoxemia resulted in increased expression of IκB-α mRNA. Interestingly, both hyperthermia and sodium arsenite treatment also increased IκB-α mRNA expression, despite decreasing NF-κB DNA binding activity.

Stress-responsive genes, such as HSP-72, are transcriptionally regulated by the transcription factor heat shock factor, and possess heat shock regulatory elements in their promoter. The finding in the present study of increased IκB-α mRNA expression after induction of the stress response is similar to a previous experiment involving respiratory epithelial cells. In that study, a potential heat shock element was discovered in the human IκB-α promoter. This represents a possible mechanism for increased IκB-α mRNA expression after induction of the stress response, and may explain why IκB-α levels were maintained after induction of the stress response, despite reduced NF-κB activity.

There are several potential mechanisms by which the stress response might decrease NF-κB binding activity in the intestinal mucosa. In the present study, induction of the stress response resulted in increased IκB-α mRNA expression. Increased transcription and translation of IκB-α could augment cytoplasmic IκB-α pools, providing additional IκB-α protein to bind NF-κB, thus minimizing NF-κB dissociation and nuclear translocation. Heat shock proteins can also serve as molecular chap-
Acting in this fashion, HSPs could potentially stabilize IkB-α protein by inhibiting its phosphorylation or decreasing its degradation by the 26S proteasome. Further studies are needed to fully investigate potential mechanisms by which the stress response maintains cytoplasmic IkB-α levels and reduces NF-κB DNA binding activity during endotoxemia.

The present studies were performed on intestinal mucosa. This is a complex tissue, and it is not known which cell type(s) accounted for the increased HSP-72 levels and decreased NF-κB binding activity. In recent studies, IkB-α was degraded and NF-κB was activated in cultured enterocytes in response to IL-1β. In other studies, HSP-72 protein levels increased in intestinal epithelial cells after heat shock and sodium arsenite treatment. Thus, the increased HSP-72 levels and decreased NF-κB binding activity seen in the jejunal mucosa in the present study may reflect events in the enterocytes, although other cell types may have also been involved.

The present finding that NF-κB DNA binding activity in intestinal mucosa during endotoxemia was reduced by previous induction of the stress response may have important clinical and therapeutic implications. Because of the central role of NF-κB in the inflammatory response, much research is presently being devoted to different methods by which NF-κB activation can be reduced. For example, in recent studies, antioxidants reduced NF-κB activation and decreased endotoxin-induced liver injury, and inhibited NF-κB activation during septic shock in rats. Other studies found that somatic gene transfer with IkB-α decreased mortality from endotoxemia in mice. Recent evidence suggests that some anti-inflammatory compounds, including glucocorticoids and salicylate, may exert their effects, at least in part, by inhibiting NF-κB activation. In addition, local or systemic administration of antisense oligonucleotides to the NF-κB subunit p65 decreased intestinal inflammation in murine experimental colitis. Thus, there may be several potential avenues to modulate NF-κB activation during inflammation in addition to induction of the stress response.

Supported in part by a grant and a research fellowship from Shriners of North America, Tampa, Fla (Dr D. R. Fischer); Office of Research and Development, Medical Research Service, Department of Veterans Affairs, Washington, DC (Dr Hasselgren); training grant T32GM08478 from the National Institutes of Health, Bethesda, Md (Dr Pritts); and the Zeneca ICI Fellowship Award from the Surgical Infection Society (Dr Moon). IkB-α complementary DNA was the kind gift of Albert S. Baldwin Jr, PhD, University of North Carolina at Chapel Hill.

Presented as a poster at the 19th Annual Meeting of the Surgical Infection Society, Seattle, Wash, April 30, 1999.
Sepsis is a major cause of morbidity and mortality in surgical patients. Results of recent studies suggest that the intestinal mucosa plays an active role in the inflammatory response to sepsis and endotoxemia by producing pro-inflammatory cytokines and acute-phase proteins. Nuclear factor-kappa B is an important transcription factor that regulates many genes involved in the inflammatory response. Induction of the stress, or heat shock, response in vitro down-regulates NF-κB activation in several cell types, including enterocytes, but the effect of the stress response on NF-κB activation in vivo is not known. The current evidence suggests that induction of the stress response in vivo decreases NF-κB activation in intestinal mucosa during endotoxemia. The results are important clinically because they suggest that the protective effects of the stress response in vivo may, at least in part, be due to inhibited activation of NF-κB.

References

30. Parikh AA, Salzman AL, Kane CD, Fischer JE, Hasselgren PO. IL-6 production in human intestinal epithelial cells following stimulation with IL-1β is associated with activation of the transcription factor NF-κB. J Surg Res. 1997;69:139-144.