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

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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.

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 5-channel thermometer (model 8502-16; 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 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 saline solution. 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 IκB-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 IκB-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 phenylmethylsulfonylfluoride), 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 A 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.

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 those 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-
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 (α-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[dI-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, and EDTA, 0.001-mol/L; [pH 8.3]). Blots were dried overnight and analyzed by exposure to x-ray film (X-Omat AR; Eastman-Kodak, Rochester, NY).

NORTHERN BLOT ANALYSIS

For determination of IκB-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 blocked with 10% nonfat dried milk in Tris-buffered saline solution containing Tween-20 and 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.

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 10% 2-mercaptoethanol) for 3 minutes, then separated by electrophoresis on a 10% bisacrylamide gel at 200 V. 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 antiserum antibody to IκB-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.

For determination of IκB-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 IκB-α was labeled with cytosine triphosphate (α-[32P]dCTP) by random labeling (Pharmacia Biotech Inc, Piscataway, NJ). Blots were hybridized with the [32P]-labeled IκB-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, and 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.

J. Immunol. 163:2227–2233, 1999. The NF-κB complex is activated by a variety of stimuli, including tumor necrosis factor, interleukin-1, and LPS. The NF-κB complex is formed by the association of the RelA subunit (p65) with the IkBα subunit (p105). The complex is localized in the cytoplasm of the cell, where it is sequestered by the IkBα subunit. Upon activation, the NF-κB complex is released from IkBα and translocates to the nucleus, where it binds to specific DNA sequences called NF-κB sites.

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).

**COMMENT**

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-

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**Figure 1.** Heat shock protein–72 (HSP-72) levels in jejunal mucosa of mice in which stress response was induced by hyperthermia (42°C for 3 minutes) (left) or injection of sodium arsenite (10 mg/kg) (right). Afterward, endotoxemia was induced by subcutaneous injection of endotoxin, 12.5 mg/kg. Nonendotoxemic mice were treated with a corresponding volume of normal saline. RT indicates room temperature; HT, hyperthermia; VEH, vehicle; SA, sodium arsenite; NS, normal saline; and LPS, lipopolysaccharide.

**Figure 2.** Nuclear factor–kappa B (NF-κB) DNA binding activity determined by electrophoretic mobility shift assay in jejunal mucosa. First lane shows electrophoretic mobility shift assay for NF-κB 1 hour after treatment with endotoxin, 12.5 mg/kg. LPS indicates lipopolysaccharide; C, competitor; M, mutant competitor; and p50 and p65, antibodies to those subunits.

**Figure 3.** Effect of stress response, as induced by hyperthermia (left) or injection of sodium arsenite (right), on lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) DNA binding activity in jejunal mucosa of mice. Experimental conditions and abbreviations are same as those listed in the Figure 1 legend.

**Figure 4.** Heat shock protein–72 (HSP-72) levels in jejunal mucosa of mice in which stress response was induced by hyperthermia (42°C for 3 minutes) (left) or injection of sodium arsenite (10 mg/kg) (right). Afterward, endotoxemia was induced by subcutaneous injection of endotoxin, 12.5 mg/kg. Nonendotoxemic mice were treated with a corresponding volume of normal saline. RT indicates room temperature; HT, hyperthermia; VEH, vehicle; SA, sodium arsenite; NS, normal saline; and LPS, lipopolysaccharide.

**Figure 5.** Nuclear factor–kappa B (NF-κB) DNA binding activity determined by electrophoretic mobility shift assay in jejunal mucosa. First lane shows electrophoretic mobility shift assay for NF-κB 1 hour after treatment with endotoxin, 12.5 mg/kg. LPS indicates lipopolysaccharide; C, competitor; M, mutant competitor; and p50 and p65, antibodies to those subunits.
regulation of NF-κB DNA binding activity can be induced by the stress response in vivo may have important clinical implications considering the role of NF-κB in the regulation of many inflammatory genes.

Although the exact mechanisms are not fully understood, there is substantial evidence that the stress response exerts a protective effect against various noxious influences at a cellular, tissue, and whole body level. For example, animals in which the stress response was induced before induction of sepsis or endotoxia exhibited decreased mortality. Although our data do not allow us to ascribe any effect of the stress response specifically to HSP-72, which is often used as a marker of the stress response, and may explain why HSP-72 blunted heat shock inhibition of nitric oxide synthase–2 activity in astroglial cells.

Figure 4. Effect of stress response, as induced by hyperthermia (left) or injection of sodium arsenite (right), on lipopolysaccharide (LPS)-induced reduction of IkB-α levels in jejunal mucosa of mice, determined by Western blotting. Experimental conditions and abbreviations are same as those listed in Figure 1 legend.
erones. 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.

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Figure 5. Effect of stress response, as induced by hyperthermia (left) or injection of sodium arsenite (right), and endotoxemia on IkB-α messenger RNA (mRNA) levels in jejunal mucosa of mice, determined by Northern blotting. Experimental conditions and abbreviations are same as those listed in Figure 1 legend. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase. Blots were stripped and reprobed for GAPDH.
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 provides strong indication 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


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