Stress Response Decreases NF-κB Nuclear Translocation and Increases I-κBα Expression in A549 Cells

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Abstract

The stress response and stress proteins confer protection against diverse forms of cellular and tissue injury, including acute lung injury. The stress response can inhibit nonstress protein gene expression, therefore transcriptional inhibition of proinflammatory responses could be a mechanism of protection against acute lung injury. To explore this possibility, we determined the effects of the stress response on nuclear translocation of the transcription factor NF-κB, an important regulator of proinflammatory gene expression. In A549 cells induction of the stress response decreased tumor necrosis factor-α (TNF-α)–mediated NF-κB nuclear translocation. TNF-α initiates NF-κB nuclear translocation by causing dissociation of the inhibitory protein I-κBα from NF-κB and rapid degradation of I-κBα. Prior induction of the stress response inhibited TNF-α-mediated dissociation of I-κBα from NF-κB and subsequent degradation of I-κBα. Induction of the stress response also increased expression of I-κBα. We conclude that the stress response affects NF-κB-mediated gene regulation by two independent mechanisms. The stress response stabilizes I-κBα and induces expression of I-κBα. The composite result of these two effects is to decrease NF-κB nuclear translocation. We speculate that the protective effect of the stress response against acute lung injury involves a similar effect on the I-κB/NF-κB pathway. (*J. Clin. Invest.* 1997. 99:2423–2428.) Key words: heat shock response • inflammation • transcription factors • stress proteins • lung

Introduction

Virtually all cells respond to severe environmental stress by synthesizing a specific group of proteins called heat shock proteins (1, 2). This highly conserved response, the heat shock response, protects cells against a variety of potentially lethal factors, including hyperthermia, oxidants, and inflammation (3–5). The terms "heat shock proteins" and "heat shock response" are used because hyperthermia was the first described and is still the most consistent inducer of this response. Alternative terms for this response and the associated proteins are "stress response" and "stress proteins," respectively (2). These terms are considered to be more appropriate because (*a*) a variety of nonthermal stressors such as sodium arsenite, heavy metals, and oxidants can induce the same response, and (*b*) this response protects against both thermal and nonthermal forms of cellular and tissue injury.

Previous studies demonstrated that the stress response protected against acute lung injury in vivo (6, 7) and in vitro (8). Induction of the stress response protected rats against acute lung injury caused by either intratracheal administration of phospholipase-A_1 (6) or systemic injection of endotoxin (7). The stress response protected cultured pulmonary artery endothelial cells against endotoxin-mediated apoptosis (8). Increased expression of a specific stress protein [heat shock protein-70 (HSP-70)][1] by plasmid-directed gene transfer also protected cultured pulmonary artery endothelial cells against endotoxin-mediated apoptosis (8).

Acute lung injury is a complex process involving several injury mechanisms and multiple cellular targets (9). Inflammation plays a central role in lung injury, either as a primary injury process or acting to exacerbate an acute injury. Proinflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukins are increased during lung injury, and are thought to mediate humoral and cellular inflammatory responses of the lung. Inflammatory damage to the respiratory epithelium is another important component of acute lung injury. Therefore, modulation of proinflammatory responses in respiratory epithelium is likely to affect the course of acute lung injury.

An important feature of the stress response is its ability to transiently inhibit nonstress protein gene expression. This inhibitory effect seems to be particularly true with respect to proinflammatory gene expression. For example, the stress response inhibited cytokine gene expression in mononuclear cells (10, 11) and fibroblasts (4), and cytokine-mediated inducible nitric oxide synthase gene expression in cultured hepatic (12, 13), and neuronal cells (14).

The mechanisms by which the stress response protects against acute lung injury are not well understood. We postulated that stress response-mediated modulation of proinflammatory responses could be a mechanism of protection. Support for this hypothesis is derived from studies in which the stress response: (*a*) inhibited proinflammatory responses of cultured lung cells (15–17), (*b*) inhibited proinflammatory responses of whole rat lungs during endotoxemia (18), and (*c*) decreased in vitro NF-κB nuclear translocation (13, 14, 17). To begin testing this hypothesis, we sought to identify pathways by which the stress response modulates proinflammatory responses in cultured A549 cells, a lung adenocarcinoma epithelial cell line.

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1. *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; EMSA, electromobility shift assay; HSP-70, heat shock protein-70; TTBS, Tween tris-buffered saline.

Recent data (13, 14), including our own (17), demonstrated that the stress response inhibited NF-κB nuclear translocation. NF-κB is a pluripotent transcription factor involved in the regulation of many proinflammatory responses (19). NF-κB nuclear translocation was recently demonstrated to occur during acute lung injury in humans (20). NF-κB is retained in the cytosol by a family of inhibitory proteins called I-κB that mask NF-κB nuclear translocation sequences (19). Agents such as TNF-α, endotoxin, and interleukin-1β initiate a phosphorylation-dependent dissociation of I-κB from NF-κB, causing a rapid degradation of I-κB and subsequent nuclear translocation of NF-κB. The I-κBα gene contains several NF-κB binding sites in its promoter that are involved in the regulation of I-κBα expression (21, 22). Thus, an autoregulatory feedback loop exists whereby nuclear translocation of NF-κB increases expression of I-κBα, increasing cytosolic I-κBα, which is thought to prevent ongoing NF-κB nuclear translocation.

In the current study we determined the proximal events involved in stress response-mediated inhibition of NF-κB nuclear translocation. We demonstrate that the stress response decreases NF-κB nuclear translocation by inhibiting dissociation of I-κBα from NF-κB and degradation of I-κBα. We also demonstrate for the first time that the stress response increases I-κBα expression, thus providing another potential mechanism to decrease NF-κB nuclear translocation.

Methods

Cell culture. All experiments involved A549 cells (American Type Culture Collection, Bethesda, MD) which are a human lung adenocarcinoma cell line representative of distal respiratory epithelium. Cells were maintained in a room air/5% CO_2 incubator at 37°C using DME (Gibco, BRL, Gaithersburg, MD) containing 8% fetal bovine serum and penicillin/streptomycin (Gibco, BRL).

Stress response models. Two different stimuli were used to induce the stress response. One group of cells were incubated at 43°C for 1 h (heat shock) in a room air/5% CO_2 incubator, then returned to 37°C. Another group of cells were treated with 1 mM sodium arsenite at 37°C for 2 h, then returned to basal growth media. Induction of the stress response was confirmed by Western blot analysis for HSP-70.

NF-κB nuclear translocation model. NF-κB nuclear translocation was induced by treating cells with human TNF-α (Boehringer Mannheim, Indianapolis, IN) at a concentration of 100 U/ml. NF-κB nuclear translocation was detected by electromobility shift assay (EMSA) 30 min after treatment with TNF-α. To determine the effect of the stress response on TNF-α-mediated NF-κB nuclear translocation, the stress response was induced 1 to 24 h before treatment with TNF-α.

Nuclear protein extraction. Nuclear protein extracts were prepared from treated cells grown to 80% confluence in 100 mm^2 dishes. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with PBS and harvested by scraping into 1 ml of PBS and pelleted at 6,000 rpm for 5 min. The pellet was washed twice with PBS, resuspended in one packed cell volume of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% vol/vol Nonidet P-40, 1 mM DTT, and 0.1 mM PMSF), and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 rpm, one cell pellet volume of extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 0.1 M EDTA, 1.5 mM $MgCl_2$, 25% vol/vol glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. The nuclear proteins were isolated by centrifugation at 14,000 rpm for 15 min. Protein concentrations were determined by Bradford assay (BioRad, Hercules, CA) and stored at −70° C until used for EMSA.

EMSA. The NF-κB oligonucleotide probe used for EMSA (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The probe was labeled with γ-[^{32}P]ATP using T4 polynucleotide kinase (Gibco, BRL) and purified in Bio-Spin chromatography columns (BioRad).

For EMSA 10 μg of nuclear proteins were preincubated with EMSA buffer (12 mM Hepes, pH 7.9, 4 mM Tris-HCl, pH 7.9, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly [d(I-C)], 12% glycerol vol/vol, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide probe for an additional 10 min. The specificity of the binding reaction was determined by coincubating duplicate samples with either 100-fold molar excess of unlabeled oligonucleotide probe or an anti-NF-κB antibody (anti-p65; Santa Cruz Biotechnology). Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3M paper (Whatman, Inc., Clifton, NJ), dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −70°C with an intensifying screen.

Western blot analysis. Treated cells were washed once in PBS and lysed in ice-cold buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, and PMSF (100 μg/ml). Protein concentrations were determined using the Bradford assay. Whole cell lysates were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and 50 μg of protein loaded per lane on an 8–16% Tris-glycine gradient gel (Novex, San Diego, CA). Proteins were separated electrophoretically and transferred to nitrocellulose membranes (Novex) using the Novex Xcell Mini-Gel system. For immunoblotting, membranes were blocked with 10% non-fat dried milk in Tris-buffered saline (TBS) for 1 h. Primary antibodies against either the inducible isoform of HSP-70 (monoclonal, SPA-810; Stressgen, Victoria, British Columbia, Canada), Iκ-Bα (polyclonal; Santa Cruz Biotechnology), or NF-κB-p65 (polyclonal, Santa Cruz Biotechnology) were applied at appropriate dilutions for 2 h. After washing two times in TBS containing 0.05% Tween 20 (TTBS), secondary antibodies (peroxidase-conjugated goat anti-mouse or anti-rabbit IgG; Sigma Chemical Co., St. Louis, MO) were applied at appropriate dilutions for 1 h. Blots were washed in TTBS two times over 30 min, incubated in commercial enhanced chemiluminescence reagents (ECL; Amersham, Buckinghamshire, England), and exposed to photographic film.

Immunoprecipitation. Treated cells were washed twice with PBS and lysed in 1 ml of RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing PMSF (100 μg/ml). Lysates were collected in microcentrifuge tubes, centrifuged at 5,000 rpm for 5 min to remove cellular debris, and pre-cleared overnight at 4°C with 30 μl of Protein G sepharose (BioRad) and 5 μl of normal rabbit serum. Samples were subsequently centrifuged at 3,000 rpm for 5 min to remove the Protein G sepharose. 30 μl of Protein G sepharose and 1 μg of anti-NF-κB antibody were then added to the supernatants and incubated overnight at 4°C with continuous rocking. The samples were subsequently centrifuged and the resulting pellets were washed four times with PBS containing 1.0 M NaCl. 30 μl of electrophoresis buffer were added to each pellet and the samples were boiled for 2 min. After centrifugation, 5 μl of the supernatant from each sample were analyzed by Western blot as described above.

Northern blot analysis. Total cellular RNA was recovered using the Trizol reagent (Gibco, BRL). RNA was quantified by spectrophotometry (260 nM) and 15 μg of total RNA per condition underwent electrophoresis on a 1% agarose gel containing 3% formaldehyde. The integrity of the RNA after electrophoresis was confirmed by ethidium bromide staining and brief UV illumination. RNAs were transferred to nylon membranes (Micron Separations Inc., Westboro, MA) and UV auto-crosslinked (UV Stratalinker 1800; Stratagene, La Jolla, CA). After a 4-h prehybridization at 42°C, membranes were hy-

bridized overnight at 42°C with a human I-κBα radiolabeled cDNA probe (a kind gift of Dr. Albert S. Baldwin, Jr., University of North Carolina). The cDNA was labeled with α-[^{32}P]dCTP (sp act 3,000 Ci/mM; New England Nuclear Research Products, Boston, MA) by random priming (Pharmacia/LKB Instruments, Piscataway, NJ). The hybridized filters were serially washed at 53°C using 2× sodium citrate/sodium chloride/0.1% SDS and 25 mM $NaHPO_4$/1 mM EDTA/0.1% SDS solutions. After washing, exposure was carried out overnight and analyzed using a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). To normalize results for loading differences, membranes were stripped with boiling 5 mM EDTA and rehybridized with an endlabeled [γ-^{32}P]dATP oligonucleotide probe for 18s rRNA.

Metabolic labeling and immunoprecipitation of I-κBα. Immediately after heat shock, cells were treated with methionine- and cysteine-deficient MEM for 45 min. Cells were subsequently labeled for 4 h with 0.5 mCi/ml [^{35}S]methionine/cysteine (NEN). After 4 h, the cells were lysed with solubilization buffer (60 mM Tris, pH 7.4, 190 mM NaCl, 6 mM EDTA, and 4% SDS), followed by the addition of 1 vol of water and 8 vol of dilution buffer (50 mM Tris, pH 7.4, 190 mM NaCl, 6 mM EDTA, and 2.5% Triton X-100). Cell lysates were cleared overnight at 4°C with 5 μl of normal rabbit serum, and 30 μl of a 1:1 suspension of protein G sepharose and immunoprecipitation buffer (1 vol of solubilization buffer, 1 vol of water, 8 vol of dilution buffer). Protein G was removed by centrifugation, then 10 μl of anti-I-κBα antibody (Santa Cruz) and 30 μl of protein G sepharose were added to the supernatants, and incubated overnight at 4°C. Protein G–antibody–protein complexes were collected by centrifugation, washed four times with wash buffer I (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.2% SDS), and twice with wash buffer II (50 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM EDTA). The pellet was resuspended in 15 μl of electrophoresis sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue), heated to 100°C for 3 min, centrifuged for 1 min, and separated electrophoretically on an 8–16% polyacrylamide gradient gel (Novex). Gels were fixed in 40% methanol, 10% acetic acid, and 3% glycerol for 60 min, soaked in Enlightening (DuPont NEN, Boston, MA) containing 3% glycerol for 30 min, and dried. After drying gels were exposed and analyzed using a Phosphorimager screen.

Results

Heat shock and sodium arsenite induce the stress response. A549 cells were subjected to heat shock or treated with sodium arsenite to induce the stress response. Heat shock and sodium arsenite are well known inducers of the stress response. To document induction of the stress response we determined intracellular expression of HSP-70, a major protein expressed during the stress response. Incubation of A549 cells at 43°C, for 1 h, increased expression of HSP-70 peptide compared to control cells incubated at 37°C (Fig. 1, lane *2* vs lane *1*). Treatment of A549 cells with 1 mM sodium arsenite, for 2 h, also increased expression of HSP-70 compared to untreated cells (Fig. 1, lane *3* vs lane *1*). These data demonstrate that heat shock and sodium arsenite induce the stress response in cultured A549 cells.

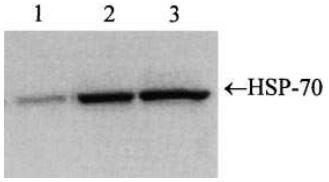

Figure 1. Representative Western blot analysis demonstrating that heat shock and treatment with sodium arsenite induce HSP-70 in cultured A549 cells. (Lane *1*) control cells; (lane *2*) 4 h after heat shock; and (lane *3*) 4 h after sodium arsenite. Similar results were obtained at 8 and 24 h after treatment (data not shown).

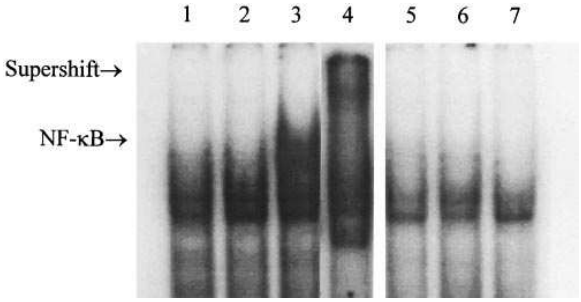

Figure 2. Representative EMSA demonstrating that the stress response decreases TNF-α-mediated nuclear translocation of NF-κB. (Lane *1*) control cells; (lane *2*) cells undergoing the stress response alone (heat shock); (lane *3*) cells treated with TNF-α alone for 30 min; lane *4*: nuclear extracts from cells treated with TNF-α alone were co-incubated with an anti-NF-κB antibody (p65); (lane *5*) nuclear extracts from cells treated with TNF-α alone were co-incubated 100-fold molar excess of cold oligonucleotide; (lane *6*) cells were heat shocked 1 h before treatment with TNF-α; and (lane *7*) cells were treated with sodium arsenite 1 h before treatment with TNF-α.

Induction of the stress response decreases TNF-α-mediated NF-κB nuclear translocation. It is known that induction of the stress response inhibits proinflammatory gene expression. Recent data from our laboratory, and other investigators, suggested that one mechanism of inhibition involves inhibition of NF-κB nuclear translocation. NF-κB is an important transcription factor involved in the regulation of many proinflammatory responses. We determined the effects of the stress response on TNF-α-mediated NF-κB nuclear translocation in cultured A549 cells. Treatment with the proinflammatory cy-

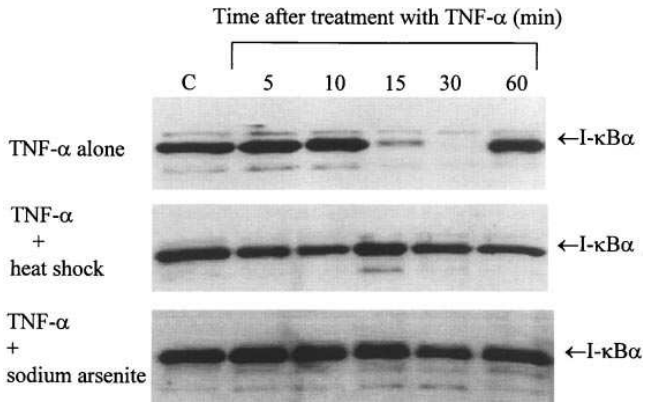

Figure 3. Representative Western blot demonstrating that the stress response inhibits the disappearance of immunoreactive I-κBα after treatment with TNF-α. "C" represents control cells treated with media only at 37°C. Other cells were treated with TNF-α and harvested for Western blot analysis at the indicated timepoints. The top panel represents cells treated with TNF-α alone. The middle panel represents cells that were heat shocked 1 h before treatment with TNF-α. The bottom panel represents cells treated with sodium arsenite 1 h before treatment with TNF-α.

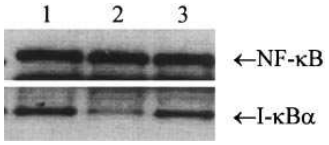

Figure 4. Representative immunoprecipitation and Western blot analysis (see text for details) demonstrating that the stress response inhibits dissociation of I-κBα from NF-κB. (Lane *1*) control cells treated with media only at 37°C; (lane *2*) cells treated with TNF-α alone for 10 min; (lane *3*) cells that were heat shocked 1 h before treatment with TNF-α.

tokine TNF-α (100 U/ml) increased nuclear translocation of NF-κB as demonstrated by formation of an NF-κB/DNA complex within 30 min of treatment (Fig. 2, lane *3*). Co-incubation of nuclear extracts with an anti-NF-κB (p65) antibody caused a supershift of the NF-κB/DNA complex, confirming the specificity of binding (Fig. 2, lane *4*). Co-incubation of nuclear extracts with 100-fold molar excess of cold oligonucleotide inhibited formation of the NF-κB/DNA complex after TNF-α treatment, further confirming the specificity of binding (Fig. 2, lane *5*). Prior induction of the stress response, by either heat shock or sodium arsenite, decreased formation of the NF-κB/DNA complex after TNF-α treatment (Fig. 2, lanes *6* and *7*, respectively). Control cells and cells undergoing the stress response alone did not have detectable NF-κB/DNA complexes (Fig. 2, lanes *1* and *2*, respectively). These data demonstrate that induction of the stress response by different stimuli (heat shock or sodium arsenite) decreases TNF-α-mediated NF-κB nuclear translocation in cultured A549 cells.

Induction of the stress response inhibits TNF-α-mediated disappearance of immunoreactive I-κBα. I-κBα retains NF-κB in the cytosol by masking its nuclear translocation sequences. In response to proinflammatory signals, I-κBα dissociates from NF-κB and is rapidly degraded. We therefore determined the effects of the stress response on TNF-α-mediated I-κBα degradation. In cells treated with TNF-α alone, immunoreactive I-κBα was substantially decreased at 15 to 30 min after TNF-α treatment, and returned to basal levels by 60 min (Fig. 3, top). Prior induction of the stress response, with either heat shock (Fig. 3, middle) or sodium arsenite (Fig. 3, bottom), inhibited the disappearance of immunoreactive I-κBα after treatment with TNF-α. These data suggest that the stress response inhibits TNF-α-mediated degradation of I-κBα in A549 cells.

Induction of the stress response inhibits dissociation of I-κBα from NF-κB. Having demonstrated that induction of the stress response inhibits disappearance of immunoreactive I-κBα, we next determined if the stress response inhibits dissociation of I-κBα from NF-κB. To detect I-κBα/NF-κB complexes, cytosolic extracts were immunoprecipitated with an anti-NF-κB-antibody, and then the immunoprecipitates were immunoblotted with anti-I-κBα and anti-NF-κB antibodies. In untreated cells

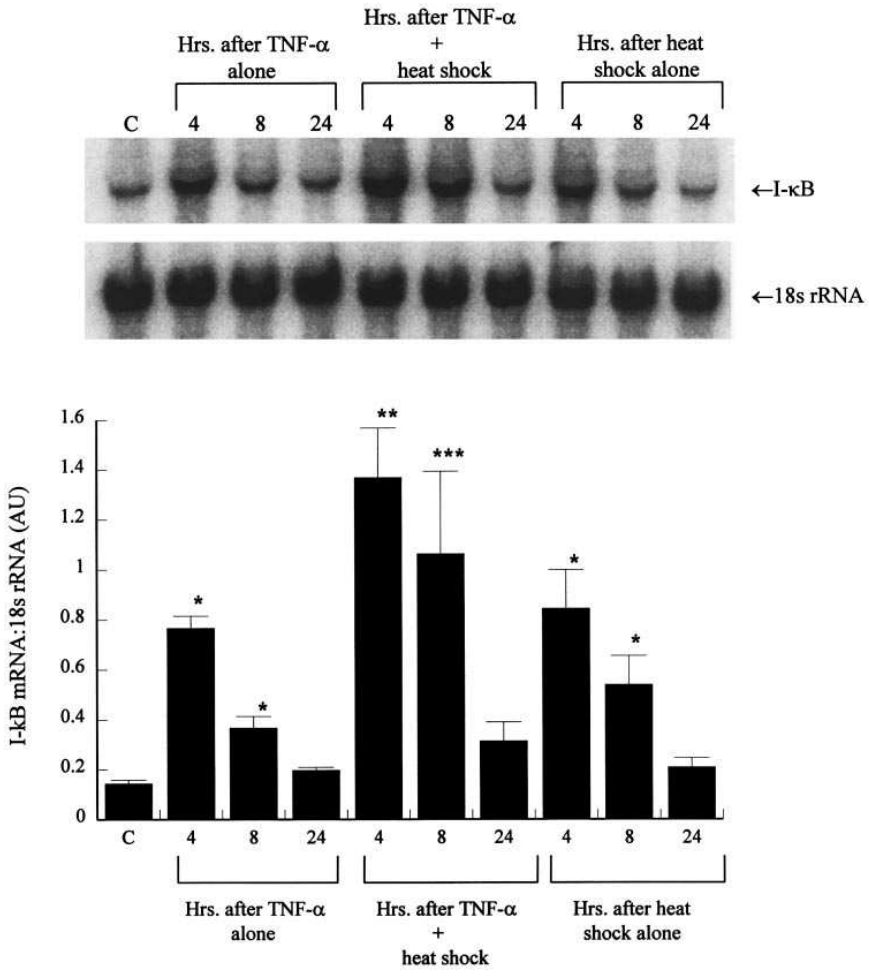

Figure 5. Representative Northern blot analysis demonstrating that the stress response increases I-κBα mRNA expression. Blots were probed with a radiolabeled oligonucleotide for 18s rRNA to control for loading differences. Relative mRNA expression, representing three separate experiments, is plotted as the ratio of I-κBα mRNA to respective 18s rRNA. $*P < 0.05$ vs control cells, $**P < 0.05$ vs 4 h after TNF-α alone, $***P < 0.05$ vs 8 h after TNF-α alone (ANOVA). "C" represents control cells treated with media only at 37°C. Band densities were determined by Phosphorimager scanning.

both NF-κB and I-κBα were detectable after immunoprecipitation, demonstrating that I-κBα was bound to NF-κB under basal conditions (Fig. 4, lane *1*). In cells treated with TNF-α for 10 min, NF-κB was detectable in the immunoprecipitates, but I-κBα was barely detectable, demonstrating that treatment with TNF-α caused dissociation of I-κBα from NF-κB (Fig. 4, lane *2*). In cells that were heat shocked prior to TNF-α treatment, both NF-κB and I-κBα were readily detectable in the immunoprecipitates (Fig. 4, lane *3*). These data demonstrate that the stress response inhibits TNF-α-mediated dissociation of I-κBα from NF-κB in cultured A549 cells.

Induction of the stress response increases I-κBα expression. Increased expression of I-κBα after NF-κB nuclear translocation is thought to be an important autoregulatory mechanism for limiting continued NF-κB nuclear translocation. We therefore determined the effects of the stress response (heat shock) on I-κBα expression. Induction of the stress response alone increased I-κBα mRNA expression in a time-dependent manner (Fig. 5, lanes *8–10*). Treatment with TNF-α alone also increased I-κBα mRNA expression in a time-dependent manner (Fig. 5, lanes *2–4*). Induction of the stress response, prior to TNF-α treatment, increased I-κBα mRNA expression above that of cells treated with TNF-α alone (Fig. 5, lanes *5–7*). To determine if increased I-κBα mRNA expression after heat shock results in increased I-κBα peptide expression, we metabolically labeled A549 cells with [^{35}S]methionine/cysteine after exposure to heat shock. Newly synthesized I-κBα peptide was detected by immunoprecipitation with an anti-I-κBα antibody. As shown in Fig. 6, heat shock increased I-κBα peptide expression above that of control cells. Collectively, these data demonstrate that (*a*) heat shock alone increases I-κBα expression, and (*b*) heat shock augments TNF-α-mediated I-κBα mRNA expression, despite decreasing TNF-α-mediated NF-κB nuclear translocation.

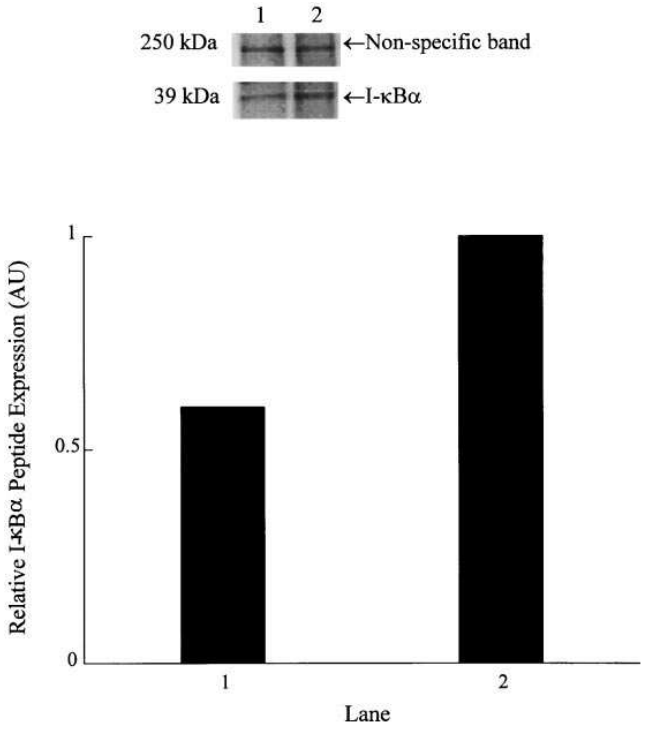

Figure 6. Cell lysates after metabolic labeling with [^{35}S]methionine/cysteine and immunoprecipitation with an anti-I-κBα antibody demonstrating that the stress response increases expression of I-κBα peptide. (Lane *1*) Control cells incubated at 37°C and labeled for 4 h; and (lane *2*) cells were heat shocked then labeled for 4 h immediately after heat shock. Graph represents relative band densities of lanes *1* and *2* normalized for the respective band densities of the nonspecific 250-kD bands that comigrated with I-κBα. Band densities were determined by Phosphorimager scanning.

Discussion

In the current study we demonstrate that the stress response decreases TNF-α-mediated NF-κB nuclear translocation in cultured A549 cells. This conclusion is purely qualitative since we can not directly measure the extent to which nuclear translocation is inhibited using EMSA. The stress response also inhibits TNF-α-mediated dissociation of I-κBα from NF-κB, and disappearance of immunoreactive I-κBα. I-κBα is the major protein which retains NF-κB in the cytosol. These data therefore indicate that the stress response decreases NF-κB nuclear translocation by stabilizing the association between I-κBα and NF-κB.

The mechanisms involved in the stabilization of I-κBα remain to be elucidated. Stress proteins are known to function as molecular chaperones. They can stabilize and refold damaged intracellular proteins during times of stress (1). Therefore, it is possible that stress proteins stabilize I-κBα by direct protein-protein interactions. A recent report by Feinstein et al. (14) suggests that a specific stress protein, HSP-70, may be involved in this process. These authors demonstrated that increased expression of HSP-70 by plasmid-directed gene transfer decreased NF-κB nuclear translocation. However, protein-protein interactions between HSP-70 and I-κBα were not addressed in that study. We have not been able to colocalize HSP-70 and I-κBα in cell extracts from A549 cells (Wong, H., unpublished observations). Therefore, the mechanisms involved in I-κBα stabilization by HSP-70 and other stress proteins remain unclear at this time.

I-κBα expression is regulated by NF-κB (21, 22). The human I-κBα promoter contains several NF-κB binding sites that are important in the regulation of I-κBα expression. NF-κB-mediated I-κBα expression is consequently able to limit continued NF-κB nuclear translocation. We were surprised to find that although the stress response decreased NF-κB nuclear translocation, it did not inhibit TNF-α-mediated induction of I-κBα mRNA expression. In fact, the stress response augmented TNF-α-mediated I-κBα mRNA expression above that seen with TNF-α alone. Taken together these data suggest that in A549 cells, TNF-α may increase I-κBα mRNA expression by mechanisms involving factors in addition to NF-κB. Furthermore, the activity of these undefined factors seems to be enhanced by the stress response.

Induction of the stress response alone increased I-κBα mRNA and peptide expression. These data demonstrate for the first time that I-κBα is a stress responsive gene, at least in A549 cells. Classic stress responsive genes, such as HSP-70, are transcriptionally regulated via heat shock regulatory elements in the promoter region of the gene (23). Heat shock regulatory elements are defined by a 5-nucleotide periodicity consisting of contiguous nGAAn blocks (n denoting less conserved nucleotides) arranged in alternating orientations (nGAAn or nTTCn). Functional heat shock regulatory elements include at

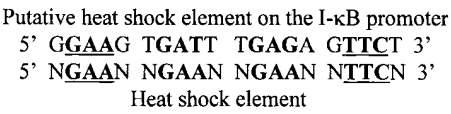

Figure 7. Putative heat shock responsive element located from 5' 606 to 625 3' bp of the human I-κBα promoter, compared to a representative heat shock element consisting of 4 tandem repeats of nGAAn and nTTCn units. Two imperfect units (bold) are flanked by two perfect units (bold and underlined).

least three such blocks and some of the blocks can have substitutions. For example, nGAGn and nCTCn are frequently found (23).

Having demonstrated that heat shock increases I-κBα expression, we examined the published human I-κBα promoter sequence for potential heat shock responsive elements (22). Beginning at position 606 bp, the human I-κBα promoter contains a contiguous 20-bp segment that matches well with naturally occurring heat shock responsive elements. This segment contains two imperfect nGAAn units flanked by a perfect nGAAn unit and a perfect nTTCn unit (Fig. 7). One of the imperfect nGAAn units is replaced with one of the known substitutions listed above (nGAGn). We are currently studying this 20-bp segment in the human I-κBα promoter to determine if it is a functional heat shock-responsive element.

NF-κB-mediated gene regulation is an important component of many lung proinflammatory responses. We now propose a new model for the gene regulatory effects of the stress response based on our results. This model, although incomplete, will serve to focus important areas for further investigation. Components of the stress response stabilize I-κBα, thereby inhibiting dissociation of I-κBα from NF-κB. Stabilization prevents I-κBα degradation and NF-κB nuclear translocation, thus potentially blocking the gene regulatory effects of NF-κB. The stress response also increases I-κBα expression and augments TNF-α-mediated I-κBα mRNA expression. These effects may provide an additional cytosolic pool of I-κBα to further attenuate NF-κB nuclear translocation. The key elements of this model are currently being investigated using the cell system described here.

In summary, we show that the stress response inhibits NF-κB nuclear translocation, possibly by effects on I-κBα. Given the importance of NF-κB as an effector of proinflammatory gene expression, we suggest that this effect may be a component of the protective effects of the stress response against acute lung injury.

Acknowledgments

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References

1. Welch, W.J. 1992. Mammalian stress response: cell physiology structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev.* 72:1063–1081.

2. Minowada, G., and W.J. Welch. 1995. Clinical implications of the stress response. *J. Clin. Invest.* 95:3–12.

3. Li, G.C., and Z. Werb. 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc. Natl. Acad. Sci. USA.* 89:3218–3222.

4. Bellmann, K.A., A. Wenz, J. Radons, V. Burkart, R. Leemann, and H. Kolb. 1995. Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin. *J. Clin. Invest.* 95:2840–2845.

5. Jäätelä, M., D. Wissing, P.A. Bauer, and G.C. Li. 1992. Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* 11:3507–3512.

6. Villar, J., J.D. Edelson, M. Post, J.B. Mullen, and A.S. Slutsky. 1993. Induction of heat stress proteins is associated with decreased mortality in an animal model of acute lung injury. *Am. Rev. Respir. Dis.* 147:177–181.

7. Villar, J., S.P. Ribeiro, J.B.M. Mullen, M. Kuliszewski, M. Post, and A.S. Slutsky. 1994. Induction of the heat shock response reduces mortality rate and organ damage in a sepsis-induced acute lung injury model. *Crit. Care Med.* 22: 914–922.

8. Wong, H.R., R.J. Mannix, J.M. Rusnak, A. Boota, H. Zar, J.S. Lazo, and B.R. Pitt. 1996. The heat shock response attenuates lipopolysaccharide-mediated apoptosis in cultured sheep pulmonary artery smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 15:745–751.

9. Kollef, M.H., and D.P. Schuster. 1995. The acute respiratory distress syndrome. *N. Engl. J. Med.* 332:27–37.

10. Schmidt, J.A., and E. Abdulla. 1988. Down-regulation of IL-1β biosynthesis by inducers of the heat-shock response. *J. Immunol.* 141:2027–2034.

11. Snyder, Y.L., L. Guthrie, G.F. Evans, and S. Zuckerman. 1992. Transcriptional inhibition of endotoxin-induced monokine synthesis following heat shock in murine peritoneal macrophages. *J. Leukocyte Biol.* 51:181–187.

12. deVera, M.E., J.M. Wong, J.Y. Zhou, E. Tzeng, H.R. Wong, T.R. Billiar, and D.A. Geller. 1996. Cytokine-induced nitric oxide synthase gene transcription is blocked by the heat shock response in human liver cells. *Surgery (St. Louis).* 120:144–149.

13. deVera, M.E., Y.M. Kim, H.R. Wong, Q. Wang, T.R. Billiar, and D.A. Geller. 1996. Heat shock inhibits cytokine-inducible nitric oxide synthase expression in rat hepatocytes. *Hepatology.* 24:1238–1245.

14. Feinstein, D.L., E. Galea, D.A. Aquino, G.C. Li, H. Xu, and D.J. Reis. 1996. Heat shock protein 70 suppresses astroglial-inducible nitric oxide synthase expression by decreasing NF-κB activation. *J. Biol. Chem.* 271:17224–17232.

15. Wong, H.R., J.D. Finder, K. Wasserloos, and B.R. Pitt. 1995. Expression of inducible nitric oxide synthase in cultured rat pulmonary artery smooth muscle cells is inhibited by the heat shock response. *Am. J. Physiol.* 269:L843–L848.

16. Wong, H.R., M. Ryan, S. Gebb, and J.R. Wispé. 1997. Selective and transient in vitro effects of heat shock on alveolar type II cell gene expression. *Am. J. Physiol.* 272: L132–L138.

17. Wong, H.R., M. Ryan, and J.R. Wispé. 1997. The heat shock response inhibits inducible nitric oxide synthase gene expression by blocking Iκ-B degradation and NF-κB nuclear translocation. *Biochem. Biophys. Res. Commun.* 231: 257–263.

18. Hauser, G.J., E.K. Dayao, K. Wasserloos, B.R. Pitt, and H.R. Wong. 1996. Heat shock protein induction inhibits inducible nitric oxide synthase mRNA expression and attenuates hypotension in endotoxin-challenged rats. *Am. J. Physiol.* 271:H2529–H2535.

19. Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649–681.

20. Schwartz, M.D., E.E. Moore, F.A. Moore, R. Shenkar, P. Moine, J.B. Haenel, and E.A. Abraham. 1996. Nuclear factor-κB is activated in alveolar macrophages from patients with acute respiratory distress syndrome. *Crit. Care Med.* 24:1285–1292.

21. de Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F.H. Bach. 1993. Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NFκB. *EMBO J.* 12:2773–2779.

22. Ito, C.Y., A.G. Kazantsev, and A.S. Baldwin, Jr. 1994. Three NF-κB sites in the IκB-α promoter are required for induction of gene expression by TNFα. *Nucleic Acids Res.* 22:3787–3792.

23. Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of heat shock regulatory elements. *Mol. Cell Biol.* 8:3761–3769.