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Stress-Induced Inhibition of the NF-κB Signaling Pathway Results From Its Insolubilization of the IκB Kinase Complex following Its Dissociation from Heat Shock Protein 90

Jean-Francois Pittet, Hyon Lee, Melissa Pespeni, Allison O’Mahony, Jeremie Roux, and William J. Welch

Activation of the stress response attenuates proinflammatory responses by suppressing cytokine-stimulated activation of the NF-κB signaling pathway. In this study, we show that the activation of the cellular stress response, either by heat shock treatment or after exposure to sodium arsenite, leads to a transient inhibition of IκBα phosphorylation. Inhibition of IκBα phosphorylation after stress was associated with the detergent insolubilization of the upstream kinases, IκB kinase α (IKKα) and IκB kinase β, components involved in IκBα phosphorylation. Pretreatment of cells with glycerol, a chemical chaperone that reduces the extent of stress-induced protein denaturation, reduced the stress-dependent detergent insolubility of the IKK complex and restored the cytokine-stimulated phosphorylation of IκB. The stress-dependent insolubility of the IKK complex appeared reversible; as the cells recovered from the heat shock treatment, the IKK complex reappeared within the soluble fraction of cells and was again capable of mediating the phosphorylation of IκBα in response to added cytokines. Treatment of cells with geldanamycin, an inhibitor of heat shock protein 90 (Hsp90) function, also resulted in IKK detergent insolubility and proteasome-mediated degradation of the IKK complex. Furthermore, while IKKα coprecipitated with Hsp90 in control cells, coprecipitation of the two proteins was greatly reduced in those cells early after stress or following exposure to geldanamycin. Stress-induced transient insolubilization of the IκB kinase complex following its dissociation from Hsp90 represents a novel mechanism by which the activation of the stress response inhibits the NF-κB signaling pathway in response to proinflammatory stimuli. The Journal of Immunology, 2005, 174: 384–394.
cells leads to transient (but reversible) change in the solubility of cytosolic and nuclear proteins (16–18). We show in this study that activation of the cellular stress response does indeed lead to changes in the distribution of IκB protein kinase complex. Specifically, either sublethal heat shock treatment or exposure of cells to sodium arsenite leads to the transient detergent insolubilization of both IκKα and IκKB and the subsequent inhibition of cytokine-mediated phosphorylation of IκBα. Pretreatment of cells with glicerol, a small molecule known to reduce the extent of stress-induced protein denaturation, or rendering the cells thermotolerant attenuated this stress-dependent detergent insolubility of the IκK complex. After heat shock treatment, both the glicerol-treated cells and the thermotolerant cells exhibited rapid phosphorylation of IκBα and nuclear translocation of the p50/p65 Rel proteins in response to added cytokines. Similar to heat shock treatment, exposure of the cells to geldanamycin, an inhibitor of Hsp90 function, also resulted in the rapid movement of the IκK complex into the detergent-insoluble fraction and its degradation by the proteasome. Furthermore, while IκKα coprecipitated with Hsp90 in control cells, coprecipitation of the two proteins was greatly reduced in those cells following exposure to geldanamycin. These observations implicate Hsp90 as a critical component that regulates the stability, solubility, and functionality of the IκK complex.

Materials and Methods

Cell culture

A549 cells, an alveolar epithelial cell line that shares many characteristics with primary alveolar epithelial cells (19), were plated in F-12K medium containing 10% FCS and penicillin/streptomycin/amphotericin (Invitrogen Life Technologies). Rat alveolar epithelial type II (ATII) cells were isolated by elastase digestion and selective adhesion plating by rat IgG, as described previously (19). The cells were plated on tissue-culture-treated wells in DMEM-H21 medium with 10% FCS and penicillin/streptomycin/amphotericin (Invitrogen Life Technologies).

Reagents

Recombinant TNF-α, IL-1β, and IFN-γ; protein G-Sepharose beads; and cationic liposomes (FuGENE 6) were purchased from Roche Biochemicals, and the chemiluminescence kit was obtained from Amersham. Rabbit polyclonal anti-IκBα, anti-IκKα, anti-IκKB, anti-IκKγ (NF-κB essential modulator), and anti-JNK Abs were purchased from Upstate Biotechnology and Santa Cruz Biotechnology. GST-Jun and anti-IκBα/anti-phosphorylated IκBα (Ser27) were obtained from Cell Signaling Technology. Goat anti-rabbit, anti-rat, and anti-mouse secondary Abs conjugated with HRP and Cy3 were purchased from Valeant Pharmaceuticals. γ-[32P]ATP for kinase assays was purchased from PerkinElmer Life Sciences. NF-κB consensus oligonucleotide probes and luciferase activity kit were purchased from Boehringer. Oligonucleotides were purchased from Integrated DNA Technologies. UniGene probes and nuclear translocation of the p50/p65 Rel proteins in 10 ng/ml each of TNF-α, IL-1β, IFN-γ. For nuclear protein isolation, cells were washed 20 times with lysis buffer, the beads were washed with 1 ml of kinase reaction buffer containing 20 mM HEPES, 2 mM MnCl2, 10 mM MgCl2, 25 mM glicerol-2-phosphate, 0.1 mM Na2VO4, 4 mM NaF, and 1 mM EDTA. Following centrifugation and removal of the supernatant, the beads were

Heat shock treatment and exposure to cytokinin

A549 and ATII cell monolayers were subjected to heat shock treatment (43°C for 60 min for A549 cells, and 43°C for 15 or 30 min for ATII cells) or exposed to sodium arsenite (0.25–4 mM for 2 h) or exposed to geldanamycin (ATII cells, 0.5 μM for 1–8 h, as indicated in the figure legends). After heat shock treatment, the cells were plated back at 37°C and allowed to recover for 1–12 h. Following exposure to sodium arsenite, the medium was removed and the cells were washed with and further incubated in fresh culture medium for the times indicated. Cells were exposed to a mixture of proinflammatory cytokines (cytomix) (IL-1β, TNF-α, IFN-γ) at a concentration of 10 ng/ml each for the times indicated in the figure legends. These concentrations of cytomix have been shown to stimulate a significant increase in the production of nitrite by ATII cell monolayers without eliciting cell death, as measured by the MTT assay (20). In some experiments, cycloheximide (20 μg/ml) was added to the cell medium 1 h before exposure of the cells to heat shock treatment or geldanamycin. In some experiments, the proteasome inhibitor MG132 (4 μM) was added to the cell medium 1 h before cell harvest. A549 cell monolayers were treated with glicerol (1 M) 1 h before and during the heat shock treatment.

EMSA

A549 cells were plated in 35-mm culture dishes at a density of 1 × 106 per well and were cultured for 24 h. Cells were subjected to heat shock treatment (as stated above) before their exposure to proinflammatory cytokines (TNF-α, IL-1β, IFN-γ). For nuclear protein isolation, cells were washed three times with cold PBS and then harvested by scraping. Cells were collected by centrifugation, and the cell pellets were resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.4% Nonidet P-40, and 0.5 mM PMSF). After incubation on ice for 10 min, crude nuclei were isolated by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was removed, three cell pellet volumes of a high salt extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 0.5 mM PMSF) were added, and the suspension was incubated on a rotary shaker for 30 min at 4°C. The sample was centrifuged at 16,000 × g for 30 min at 4°C. The supernatant (containing extracted nuclear proteins) was collected, protein concentration was determined, and the lysate was stored at −70°C until further use.

EMSA was performed using an NF-κB consensus oligonucleotide probe (5′-ATG TGA GGG GAC TTT CCC AGG C-3′) that was end labeled with γ-[32P]ATP. Nuclear protein (5 μg) was incubated with 100,000 cpm of [γ-32P]ATP-labeled NF-κB consensus nucleotide for 20 min in a binding buffer consisting of 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glicerol, and 1 μg of poly(dI-dC). The specificity of the DNA/protein binding was determined by competition reactions in which a 100-fold molar excess of unlabeled NF-κB oligonucleotide was added. Supershifts of the NF-κB dimers were performed by the addition of Abs for the p50 and p65 subunits to the extracted nuclear protein overnight at 4°C. After incubation, the samples were analyzed by nondenaturing electrophoresis, and the bands were visualized by autoradiography.

Detergent fractionation

Differential detergent solubility of IκKα and IκKB after heat shock was analyzed by washing the cell monolayers with cold PBS and then lysing the cells with PBS containing 5 mM MgCl2, 0.1% Triton X-100, and protease and phosphatase inhibitors. After a gentle vortex and incubation on ice for 10 min, the cell lysate was centrifuged at 16,000 × g at 4°C for 10 min. The supernatant was removed and adjusted to 1× Laemmli sample buffer. The pellet was resuspended in 1× Laemmli sample buffer. An equal amount of protein from the supernatant and the Triton-insoluble pellet were then analyzed for their relative content of IκKα, IκKB, and JNK-1 via Western blotting.

Western blot analysis

Western blot analysis was performed, as described previously (19). Primary Abs were detected via incubation with HRP-conjugated secondary Abs, and the protein bands were visualized using chemiluminescence. Quantification was done using a digital image analysis system (NIH Image).

Immunoprecipitation and kinase activity assay

Cells were harvested in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glicerol, 1 mM DTT, phosphatase inhibitors (1 mM each of Na2VO4, β-glycerophosphate, sodium pyrophosphate, and NaF), and protease inhibitors. Following centrifugation at 16,000 × g for 10 min at 4°C, the supernatant was removed and used for the immunoprecipitation reactions. Abs specific for Hsp90, IκKγ, or IκKB-1 were added, and the samples were incubated on a rotary shaker overnight at 4°C. Ag-Ab complexes were captured via addition of protein G-Sepharose for 2 h and centrifugation at 16,000 × g for 1 min. Following three 1-ml washes with lysis buffer (see above), 20 μl of a 2× Laemmli sample buffer was added to the beads, which were then immediately heated at 95°C for 3 min. For the kinase activity assay, following the three 1-ml washes with lysis buffer, the beads were washed with 1 ml of kinase reaction buffer containing 20 mM HEPES, 2 mM MnCl2, 10 mM MgCl2, 25 mM glicerol-2-phosphate, 0.1 mM Na2VO4, 4 mM NaF, and 1 mM DTT. Following centrifugation and removal of the supernatant, the beads were

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resuspended in 20 µl of kinase buffer. A total of 0.5 µg of GST-IκBα or GST-cJun along with 10 µCi of [γ-32P]ATP was added to the beads, and the samples were incubated for 30 min at 30°C. The kinase reaction was stopped by the addition of Laemmli sample buffer and subsequent heating at 95°C for 3 min. Analysis of phosphoproteins was performed by SDS-PAGE and autoradiography.

**NF-κB reporter cells and luciferase assay**

A549 or ATII cells were transiently transfected with a plasmid containing the luciferase gene under the control of three tandem NF-κB-binding motifs, followed by a minimal IFN-γ promoter, which has previously been shown to demonstrate NF-κB activation (19). Cells were transfected in triplicate, in 12-well plates (cell density of 1 × 10⁵ cells/well), by incubation with cationic liposomes. Forty-eight hours later, the cells were exposed to a mixture of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, each at 10 ng/ml) or they were subjected to heat shock treatment and then allowed to recover at 37°C for either 1 or 12 h before exposure to the proinflammatory cytokines. Twenty-four-hour after cytokine exposure, cell extracts were prepared and analyzed for luciferase activity, according to the manufacturer's instructions (Promega), using a luminometer. Luciferase activity was corrected for total cellular protein and reported as fold induction over the control cells (cells that were transfected, but not treated with cytokine). Cells were cotransfected with a β-galactosidase plasmid, and β-galactosidase activity was determined to verify the efficiency of transfection between experiments.

**Immunofluorescence**

A549 cells were seeded on glass coverslips at a concentration of 5 × 10⁵ cells per 35-mm dish. Cells were treated with 1 µl glycercol for 1 h before and during the heat shock treatment, as described above, and then treated with cytoxim for 30 min. The coverslips were washed briefly in PBS and immediately fixed in 3.7% paraformaldehyde for 20 min at room temperature. Following fixation, the cells were permeabilized in 0.1% Triton X-100 for 3 min and blocked in 10% normal goat serum and 1% BSA for 30 min. The coverslips were incubated with p65 Ab overnight at 4°C. The coverslips were washed three times in PBS and then incubated for 1 h with a Cy3-conjugated secondary Ab and 4′,6′-diamidino-2-phenylindole (1:1000, 1 mg/ml stock). Coverslips were mounted on slides using 1 M n-propylgallate and visualized on a Nikon fluorescence microscope.

**Cell viability**

Cell viability after exposure to different experimental conditions was measured by Alamar blue assay. Rat ATII or A549 cells were left at 37°C or subjected to a 30- or 60 min 43°C heat shock treatment. The heated cells were returned to 37°C for 1, 10, or 22 h. Cell medium was then collected and read on a medium containing a 1:10 mixture of Alamar blue and placed at 37°C for 1 h later no longer resulted in the binding of the p50/p65 to the DNA probe (Fig. 1, A and C).

**Heat shock treatment renders the IKK complex detergent insoluble and prevents IκBα phosphorylation following cytokine stimulation**

Previously studies have shown that heat shock treatment increases the amount of proteins that partition into the detergent-insoluble fraction. Over time, as the cells recover from the stress event, many of the proteins rendered insoluble are observed to partition back into the detergent-soluble fraction (17, 18, 21, 22). Because the 43°C/60-min heat shock treatment blocked the activation of p50/p65 DNA binding (Fig. 1), we examined whether the solubility of upstream kinases involved in IκBα phosphorylation might be affected following the heat shock treatment. To corroborate previous findings regarding IκBα phosphorylation after heat shock, the status and kinetics of IκBα phosphorylation were first examined in the A549 cells before and after heat shock treatment. Cells were either maintained at 37°C, or first subjected to a 43°C/60-min heat shock treatment, followed by a 1-h recovery period at 37°C before being stimulated with cytokines for varying periods of time. The cells then were harvested in Laemmli sample buffer, and the extent of IκBα phosphorylation was examined by Western blot (Fig. 2A). Within 5 min after cytokine stimulation, IκBα phosphorylation was observed in the control unheated cells. By 20 min, most of the phosphorylation IκBα could no longer be detected, most likely due to its known proteasome-dependent degradation. Indeed, inclusion of the proteasome inhibitor MG132 effectively blocked phosphorylated IκBα degradation. In cells subjected to heat shock treatment (43°C/60 min), followed by a 1-h recovery period at 37°C, cytokine-induced phosphorylation of IκBα no longer was observed (Fig. 2A). As a result, IκBα levels appeared unchanged over the course of the 20-min cytokine exposure. These observations are in concert with previous reports (14, 23, 24): heat shock treatment interferes with an early step in the NF-κB activation pathway, thereby resulting in a lack of IκBα phosphorylation, no IκBα degradation, and the subsequent failure to activate p50/p65 DNA binding.

Because IκBα phosphorylation was not observed in the heated cells following cytokine stimulation, we examined the status of upstream kinases involved in the cytokine-mediated phosphorylation of IκBα, the so-called IKK complex. Both control and heat shock-treated cells were stimulated with cytokines, and cell lysates were prepared by lysis using a buffer containing 0.1% nonionic detergent. After centrifugation at 16,000 × g, the resultant detergent-soluble fraction was used to immunoprecipitate IKK, and IKK activity was determined by presentation of its substrate, GST-IκBα, and [γ-32P]ATP. As is shown in Fig. 2B, the cytokine-stimulated control cells (i.e., no prior heat shock) exhibited significant IKK activity, as evidenced by the robust phosphorylation of the added IκBα (Fig. 2B, (+) HS). In contrast, no IKK activity was apparent in the detergent-soluble fraction prepared from the heat shock-treated cells that were exposed to cytokines (Fig. 2B, (−) HS).
HS). To determine whether such detergent insolubility after heat shock simply represented a general phenomenon, we examined the status of JNK, a component of the MAPK pathway that is activated in response to either cytokine stimulation or heat shock treatment. JNK immunoprecipitated from the detergent-soluble fraction of cells treated with either cytokines or following heat shock treatment exhibited significant kinase activity, as evidenced by increased phosphorylation of GST-cJun (Fig. 2B, lower).

Owing to our failure to detect IKK activity in the detergent-soluble extract following heat shock treatment, we examined the corresponding detergent-insoluble pellets for the presence of the IKK kinases. In the case of the control, unheated cells, the vast majority of IKKα and IKKβ were present within the detergent-soluble fraction (Fig. 2C). Note that cytokine addition resulted in a slight retardation in the gel migration of both IKKα and IKKβ, presumably indicative of their being phosphorylated in response to added cytokines. Heat shock treatment, as we suspected, resulted in the redistribution of both IKKα and IKKβ into the detergent-insoluble fraction (Fig. 2C). In contrast, heat shock treatment had no effect on the detergent solubility of JNK; all of this MAPK remained within the detergent-soluble fraction. Comparable results were obtained when the heat shock response was activated by a nonthermal stimulus. For example, exposure of A549 cell monolayers to sodium arsenite (0.25–4 mM for 2 h), a sublethal stress treatment, again resulted in most of the IKKβ to redistribute into the detergent-insoluble fraction (Fig. 2D).

Inhibition of the NF-κB pathway after heat shock treatment is reversible

Previous studies have reported that the activation of the heat shock response is accompanied by the reversible detergent insolubilization of two enzymes commonly used as reporter proteins, β-galactosidase and luciferase (18). Thus, in the next series of experiments, we examined whether the detergent insolubilization of the IKK complex after heat shock treatment was a reversible phenomenon. A549 cells were left untreated or subjected to a 43°C/60-min heat shock treatment. To some of the cells, we added the protein synthesis inhibitor, cycloheximide, to follow the fate of the already
toradiography.

The extent of substrate phosphorylation was analyzed by SDS-PAGE and autoradiography. A mixture of proinflammatory cytokines for 5 or 10 min. Afterward, the cells were harvested in PBS supplemented with 0.1% Triton X-100, 5 mM sodium arsenite, a nonthermal activator of the stress response, also results in IKKα and IKKβ detergent insolubility. A549 cells were exposed to either 2.5 or 4 mM sodium arsenite for 2 h. The culture medium then was removed and the cells were washed with and further incubated in complete medium lacking sodium arsenite for 1 h. The cells then were harvested and subjected to detergent fractionation, as described in B. Aliquots of the total cell lysate (T), the detergent-insoluble material (I), and the detergent-soluble material (S) were analyzed for the presence of IKKα, IKKβ, and Jun kinase-1 by Western blotting. In each case, the same amount of total protein was applied to the gel. D, Exposure of cells to sodium arsenite, a nonthermal activator of the stress response, also results in IKKα and IKKβ detergent insolubility. A549 cells were exposed to either 2.5 or 4 mM sodium arsenite for 2 h. The culture medium then was removed and the cells were washed with and further incubated in complete medium lacking sodium arsenite for 1 h. The cells then were harvested and subjected to detergent fractionation, as described in B. Aliquots of the total cell lysate (T), the detergent-insoluble material (I), and the detergent-soluble material (S) were analyzed for the presence of IKKα and IKKβ by Western blotting (equal amounts of protein were analyzed in each case). For each panel, one representative experiment is shown for each experimental condition. Four additional experiments gave comparable results.

**Stress-dependent inhibition of the NF-κB pathway after heat shock treatment is reversible.** A549 cells were left untreated or treated with the protein synthesis inhibitor, cycloheximide (20 μg/ml), for 1 h. In the presence or absence of the drug, the cells were maintained at 37°C or subjected to a 43°C/60-min heat shock treatment (HS). After recovery back at 37°C for either 1 or 12 h, the cells were stimulated with a mixture of proinflammatory cytokines for 5 min, and the cells were then harvested and detergent fractionated, as described previously. Aliquots of the total cell lysate, the detergent-insoluble material, and the detergent-soluble material were analyzed for the presence of IKKα by Western blotting (equal amounts of protein were analyzed). In parallel, the relative amounts of IκBα and phospho-IκBα were determined via Western blotting using an anti-IκB Ab. One representative experiment is shown for each experimental condition. Four additional experiments gave comparable results.
IkBα revealed that heat shock treatment, followed by a 1-h recovery period, again blocked cytokine-induced IkBα phosphorylation and degradation (Fig. 4C). As in the A549 cells, failure to phosphorylate IkBα shortly after heat shock treatment of the type II cells in response to added cytokines correlated with a decrease in IKK activity, as evidenced by the absence of phosphorylation of the added IkBα (Fig. 4D). Cell fractionation studies revealed that both upstream kinases, IKKα and IKKβ, again partitioned into the detergent-insoluble fraction after heat shock treatment (Fig. 4E). Finally, cell viability, as measured by the Alamar blue assay, was not adversely affected by the heat shock treatment (Fig. 4F).

Prior treatment of cells with glycerol, known to protect proteins against heat shock-induced denaturation, prevents the stress-dependent inactivation of NF-κB signaling

Previous studies have reported that pretreatment of cells with glycerol, long known to help reduce the extent of thermal denaturation of proteins in vitro, also prevents heat shock-induced protein denaturation events in vivo and enhances overall cell survival (18, 25). Thus, in the next series of experiments, we examined whether heat shock-mediated inhibition of the NF-κB pathway, via detergent insolubilization of the IKK complex, might be prevented by...
pretreating the cell monolayers with glycerol. A549 cells were treated with glycerol (1 M) 1 h before being subjected to a 43°C/60-min heat shock treatment and then returned to 37°C for 1 h. Following a 5-min incubation with cytokines, the cells were subjected to detergent fractionation, as described earlier. As is shown in Fig. 5A, pretreatment of the cells with glycerol prevented the heat shock-dependent detergent insolubilization of IKK<sub>α</sub>. Comparable results were obtained for IKK<sub>β</sub> (data not shown). In addition, the glycerol-treated cells now exhibited rapid phosphorylation of IκBα upon stimulation with cytokines 1 h after heat shock (Fig. 5A, lower). Finally, in the glycerol-pretreated cells following heat shock, cytokine addition now resulted in the rapid movement of p65 into the nucleus (Fig. 5, B and C). These observations, taken all together, indicate that heat shock treatment most likely results in the partial denaturation of components of the IKK complex, thereby leading to their inclusion within the detergent-insoluble fraction. Addition of glycerol to the cells, a small molecule known to reduce the extent of thermally induced protein denaturation in vitro, effectively reduced the heat shock-dependent movement of the IKK complex into the detergent-insoluble fraction, thereby restoring the ability of the heated cells to now respond to added cytokines.

**A549 cells first made thermotolerant now exhibit cytokine-mediated IκB kinase activity, p50/p65 DNA binding, and NF-κB-dependent transcription following heat shock treatment**

Previous studies have shown that cells subjected to a nonlethal heat shock treatment and then allowed to recover for 12–36 h now are capable of surviving a subsequent and what would otherwise be a lethal heat shock challenge (reviewed in Refs. 1 and 2). This phenomenon, referred to as thermotolerance or preconditioning, appears to be dependent, at least in part, upon the increased expression and accumulation of the different Hsp. Having shown that heat shock treatment blocks activation of the NF-κB pathway, we examined whether cells that were first rendered thermotolerant might now be capable of responding to added cytokines following a 43°C/60-min heat shock treatment. A549 cells were made thermotolerant via a 43°C/60-min heat shock treatment and a subsequent recovery period at 37°C for 12 h. These now preconditioned cells were left at 37°C or subjected to another 43°C/60-min heat shock treatment and 1 h later analyzed for their response to cytokines. The preconditioned cells, in the absence of a second heat shock treatment, exhibited normal levels of both IKK<sub>α</sub> and IKK<sub>β</sub>, and both kinases fractionated within the detergent-soluble fraction. Moreover, and in contrast to nontolerant cells, the vast majority of IKK<sub>α</sub> and IKK<sub>β</sub> remained within the detergent-soluble fraction when the thermotolerant cells were subjected to a second heat shock treatment (Fig. 6A). Thus, rendering the cells thermotolerant now prevented the heat shock-dependent movement of the IKK complex into the detergent-insoluble fraction. To confirm that the IKK complex was biologically active, cell lysates from the heat shock-treated preconditioned cells were examined for their ability to mediate cytokine-dependent phosphorylation of IκBα. After only 1 h following heat shock treatment, the preconditioned cells now showed robust phosphorylation of IκBα in response to added cytokines (Fig. 6, B and C). Similarly, p50/p65 DNA binding, as shown by EMSA analysis, was observed in the heat shock-treated preconditioned cells (Fig. 6D) upon stimulation with cytokines. Comparable results were observed when using the NF-κB reporter system (Fig. 6E). Taken together, these results indicate that in cells first rendered thermotolerant, severe heat shock treatment no longer resulted in the detergent insolubilization of the IκB kinase complex, nor inhibited the activation of the NF-κB signaling pathway in response to proinflammatory cytokines.

**Treatment of cells with geldanamycin, an Hsp90 inhibitor, results in IKK<sub>α</sub> and IKK<sub>β</sub> detergent insolubility and their subsequent degradation via the proteasome**

A recent study has shown that Hsp90 and Cdc37 are components of the IKK complex (26). Thus, we examined whether treatment of...
cells with geldanamycin, known to bind tightly to Hsp90 and to cause the activation of the heat shock response, might also lead to the rapid movement of IKK complex into the detergent-insoluble fraction. Rat A/TII cells were treated with geldanamycin (0.5 μM) for varying times (1–8 h), and the cells were detergent fractionated, as previously described. Cell viability, as measured by the Alamar blue assay, was not adversely affected by the geldanamycin treatment (data not shown). Treatment with geldanamycin caused a time-dependent degradation of IKKα. Note as well that geldanamycin elicited activation of the heat shock response, as evidenced by the induction of Hsp72 (Fig. 7A). Comparable results were obtained for IKKβ (data not shown). Treatment of the cells with cycloheximide, to inhibit new
protein synthesis, demonstrated that the induction of Hsp72 protein expression was not required for the degradation of the IKK complex after exposure to geldanamycin (Fig. 7A). Furthermore, cotreatment of the cell monolayers with MG132, a proteasome inhibitor, revealed that exposure to geldanamycin initially resulted in IKKα detergent insolubility most likely followed by its degradation via the proteasome (Fig. 7B). Exposure of the cells to geldanamycin eventually resulted in an inhibition of cytokine-mediated phosphorylation of the p65 subunit of NF-κB (Fig. 7C). This inhibitory effect on the activation of NF-κB correlated with the geldanamycin-mediated degradation of the IKK complex (Fig. 7C). Specifically after 8 h of treatment with geldanamycin, IKKα and IKKβ levels were significantly reduced, and now p65 phosphorylation in response to added cytokines no longer was observed.

![FIGURE 7](image) Treatment of cells with geldanamycin, an inhibitor of Hsp90, results in IKKα detergent insolubility and degradation via the proteasome. A. Treatment of ATII cells with geldanamycin results in the degradation of the IKKα protein independent of the activation of the heat shock response. Rat ATII cells were treated with geldanamycin (Geld) (0.5 μM), cycloheximide (CHX) (20 μg/ml), or the combination of the two for 1–8 h. After 1, 2, 4, or 8 h, the cells were lysed in PBS containing 0.1% nonionic detergent, and aliquots of the total cell lysate were analyzed for their content of IKKα and Hsp72 via Western blotting. Equal amounts of total protein were applied to the gel for each sample. B. Treatment of ATII cells with geldanamycin causes detergent insolubility and proteasome-dependent degradation of the IKKα protein. MG132 (4 μM) was added to ATII cell monolayers treated with or without geldanamycin for 1, 2, 4, and 8 h. Aliquots of the total cell lysate, the detergent-insoluble material, and the detergent-soluble material were analyzed for their content of IKKα and Hsp72 via Western blotting. C. Treatment of ATII cells with geldanamycin results in the inhibition of the phosphorylation of IκBα and p65 subunit upon cytokine stimulation. In an experiment similar to A, rat ATII cells were treated with geldanamycin for 1–8 h. Proinflammatory cytokines were then added at a concentration of 10 ng/ml per cytokine for 10 min. The cells were lysed and analyzed for their content of IKKα, IκBα, and phospho-p65 via Western blotting.

![FIGURE 8](image) Prior heat shock treatment or exposure to geldanamycin causes a dissociation of the complex formed between IκB kinase and Hsp90. A. IKKα levels are significantly reduced in those lysates first cleared of Hsp90. Rat ATII cell monolayers, lysed in a 0.1% Nonidet P-40-containing solution, were vortexed gently and clarified at 16,000 × g for 10 min. A total of 10 μg/ml anti-IKKα or anti-Hsp90 was then added to the supernatant and allowed to rotate overnight at 4°C. The samples were cleared of IKKα or Hsp90 by the addition of protein G-Sepharose beads and the subsequent removal of the bead-Ab complexes through centrifugation. The preclarified lysates were visualized via Western blotting for IKKα and Hsp90. B. The amount of IKKα coprecipitating with Hsp90 is reduced in ATII cells subjected to exposure to geldanamycin. Rat alveolar type II cells were treated with geldanamycin for 8 h. The cells were then lysed in a buffer containing 0.1% Nonidet P-40. Following centrifugation, the resultant supernatants were used for immunoprecipitation reactions using an Ab specific for Hsp90. The resultant immunoprecipitates were then examined via Western blotting for IKKα and Hsp90. C. The amount of IKKα coprecipitating with Hsp90 is reduced in ATII cells early after heat shock treatment, but is comparable to controls in thermotolerant cells (12 h after heat shock). Rat alveolar type II cells either were maintained at 37°C, or subjected to a 43°C/30-min heat shock treatment and then returned to 37°C for 1 or 12 h. The cells were then lysed in a buffer containing 0.1% Nonidet P-40. Following centrifugation, the resultant supernatants were used for immunoprecipitation reactions using an Ab specific for Hsp90. The resultant immunoprecipitates then were examined via Western blotting for IKKα and Hsp90.
Hsp90 was greatly reduced. Finally, lysates were prepared from the rat ATII cells that had been incubated under control conditions or subjected to heat shock treatment and allowed to recover for 1 or 12 h. In the control cells, IKK<sub>B</sub> was observed to coprecipitate with Hsp90. The amount of IKK<sub>α</sub> coprecipitating with Hsp90 was reduced in ATII cells early after heat shock treatment. After 12 h of recovery from the heat shock treatment, Hsp90 and IKK<sub>B</sub> again were found to coprecipitate with one another (Fig. 8C).

**Discussion**

Activation of the stress response is thought to result from the accumulation of abnormally folded proteins (27). Cytosolic and nuclear proteins, partially or fully denatured as a consequence of a thermal stress and/or the depletion of intracellular glutathione levels, now become targets for molecular chaperones, many of which themselves are heat shock-induced proteins. As more of the molecular chaperones are called into action to deal with the increasing problem of unfolded proteins, the cell promptly responds by activating the stress response. Transcriptional and translational events in the cell are then redirected toward the production of more molecular chaperones. As a consequence of all these events, the cells oftentimes become unresponsive to other external cues. Cell cycle progression as well as responses to growth factors or cytokines are generally suppressed. Only after a period of time (usually 8–12 h following a nonlethal stress and commensurate with the completion of stress protein synthesis) do the cells begin to resume their normal lifestyle (reviewed in Ref. 28).

As we showed in this work, both A549 cells as well as primary ATII cells subjected to heat shock treatment or exposure to sodium arsenite became refractory to the effects of added cytokines. One hour after a nonlethal heat shock treatment, cells no longer responded to added cytokines, as evidenced by the significant reduction in p50/p65 DNA binding, and suppression of NF-κB-driven reporter enzyme activity. As was shown in this study and previously by others (3, 8, 29, 30), cells after stress failed to phosphorylate and subsequently degrade IkB<sub>α</sub>, the result being a failure of p50/p65 to translocate to the nucleus and bind to and stimulate the expression of genes normally activated following cytokine stimulation.

Our biochemical studies have revealed a novel mechanism by which the NF-κB pathway is blocked in cells after metabolic stress. Detergent fractionation of the cells shortly after heat shock or sodium arsenite exposure revealed that the kinases upstream of IkB<sub>α</sub> were rendered insoluble. Specifically, components of the IKK complex, namely IKKα and IKKβ, were found predominantly within the nonionic detergent-insoluble complex following heat shock, or exposure of the cells to a nonthermal stressor, sodium arsenite. As a consequence, IkB<sub>α</sub> was neither phosphorylated nor degraded following stimulation of the cells with cytokines. Over time and as the cells recovered from the stress event, components of the IKK complex were found to return to the detergent-soluble fraction and now again were competent to facilitate cytokine-stimulated phosphorylation of IkB<sub>α</sub>. Thus, components of the IKK complex, like certain other cellular proteins, are transiently rendered detergent insoluble in cells subjected to nonlethal heat shock treatments, thereby explaining, at least in part, how cells become refractory to cytokine stimulation following stress. Interestingly, another important protein involved in signal transduction events, the JNK phosphatase M3/6, has also been shown to be rendered detergent insoluble after heat shock, and this may explain the sustained activation of the JNK pathway in cells subjected to stress (31).

Our results now pinpoint the IKK complex as being an important stress-sensitive component, and therefore responsible for the failure of NF-κB activation early after heat shock. We suspect the kinase complex may be partially denatured after heat shock, but over time is restored to a conformation that allows for its return to the detergent-soluble fraction and restoration of biological activity. Support for this idea were our observations showing that pretreatment of the cells with glycerol, known to reduce the extent of stress-induced protein denaturation events, resulted in the maintenance of IKK detergent solubility after stress. Similarly, in cells first rendered thermostolerant, again known to reduce the adverse effects of severe stress (reviewed in Ref. 16), a subsequent heat shock treatment no longer caused IKK insolubility nor blocked cytokine activation of the NF-κB pathway. Thus, all of our results indicate that heat shock treatment as well as nonthermal activators of the stress response (e.g., sodium arsenite or MG132, a proteasome inhibitor) lead to transient alterations in the IKK complex, resulting in its detergent insolubility and thereby rendering the cells refractory to cytokine stimulation.

What is the mechanism by which the IKK complex is rendered detergent insoluble in the cell experiencing stress? Two recent studies have shown that Hsp90 and Cdc37 are components of the IKK complex (26, 32). Therefore, we examined whether treatment of cells with geldanamycin, known to bind tightly to Hsp90 and cause release of its client proteins, might also lead to the rapid movement of IKK complex into the detergent-insoluble fraction. Treatment of lung epithelial cells with geldanamycin caused a time-dependent degradation of IKK<sub>α</sub> and IKKβ. Furthermore, co-treatment of the cell monolayers with MG132, a proteasome inhibitor, revealed that exposure to geldanamycin initially resulted in IKK detergent insolubility. Not surprisingly, geldanamycin treatment eventually resulted in an inhibition of cytokine-mediated phosphorylation of the p65 subunit of NF-κB. Prompted by these observations, we examined the interaction of Hsp90 with IKK in cells subjected to either heat shock treatment or exposure to geldanamycin. Although IKKα coprecipitated with Hsp90 in control cells, coprecipitation of the two proteins was greatly reduced in those cells early after heat shock or treatment with geldanamycin. After 12 h of recovery from the heat shock treatment, Hsp90 and IKKα were again found to coprecipitate. Thus, continued interaction of components of the IKK complex with Hsp90 (and its cochaperones) may be necessary for the maintenance of IKK solubility and therefore its function. During the course of review/revision of our studies, Broemer et al. (33) similarly concluded a requirement for Hsp90 and IKK function/stability. Specifically, treatment of cells with geldanamycin resulted in a rapid inhibition of IKK activity and a somewhat slower turnover of both IKKα and IKKβ. Pulse-chase radiolabeling in cells exposed to geldanamycin revealed a rapid turnover of both newly synthesized IKKα and IKKβ. In contrast, the mature forms of both IKKα and IKKβ were largely unaffected in the drug-treated cells. As we have shown in this work, however, the mature forms of IKKα and IKKβ are affected via exposure of the cells to geldanamycin, with both kinases being rapidly redistributed into the detergent-insoluble fraction, and then over time targeted to the proteasome for their eventual degradation. Thus, our results and those reported by Broemer et al. demonstrate that Hsp90 most likely serves a critical function in facilitating both the orderly synthesis of components of the IKK complex, as well as maintaining the mature forms of the kinase complex in a conformation that allows for its eventual biochemical activation.

The present observations do not exclude that the activation of the heat shock response may also impact the NF-κB pathway at other points (upstream or downstream) distinct of the IKK complex. For example, other proteins, which are involved in the NF-κB pathway, also have been reported to require Hsp90 for their
correct function. Both the LPS receptor (CD14) as well as receptor-interacting protein (another upstream kinase component of the NF-κB pathway) appear also to form a complex with Hsp90 (34, 35). Finally, Hsp other than Hsp90 may also be important for IKK complex activities. Specifically, the low m.w. Hsp27 protein recently was reported to bind to IKK and thereby affect TNF-mediated NF-κB activation (36).

Our work presented in this study helps to explain how cells under stress redirect important signaling pathways. As mentioned earlier, cells subjected to heat shock treatments oftentimes exhibit cell cycle arrest and/or become refractive to other signaling cues. Presumably, the down-regulation of such pathways along with the activation of the stress response together are necessary for the cell to survive the insult at hand. Interestingly, it is one of the Hsp, Hsp90, that appears to serve a role in coordinating these activities. As is shown in this study, stress-induced dissociation of Hsp90 with components of the IKK complex leads to a transient kinase insolvency, thereby preventing activation of the NF-κB pathway. On the other side, stress-induced release of Hsp90 from another of its client proteins, the monomeric form of the HSF, leads to HSF trimerization and activation, resulting in the increased expression of genes encoding the Hsp. Thus, these coordinated responses of the cell, mediated at least in part by Hsp90, help to explain how cells under stress transiently attenuate their activation of the NF-κB-dependent inflammatory pathway.

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References