Effects of HSP70.1/3 gene knockout on acute respiratory distress syndrome and the inflammatory response following sepsis

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Singleton, Kristen D., and Paul E. Wischmeyer. Effects of HSP70.1/3 gene knockout on acute respiratory distress syndrome and the inflammatory response following sepsis. *Am J Physiol Lung Cell Mol Physiol* 290: L956–L961, 2006. First published December 16, 2005; doi:10.1152/ajplung.00466.2005.—Heat shock response has been implicated in attenuating NF-κB activation and inflammation following sepsis. Studies utilizing sublethal heat stress or chemical enhancers to induce in vivo HSP70 expression have demonstrated survival benefit after experimental sepsis. However, it is likely these methods of manipulating HSP70 expression have effects on other stress proteins. The aim of this study was to evaluate the role of specific deletion of HSP70.1/3 gene expression on ARDS, NF-κB activation, inflammatory cytokine expression, and survival following sepsis. To address this question, we induced sepsis in HSP70.1/3 KO and HSP70.1/3 WT mice via cecal ligation and puncture (CLP). We evaluated lung tissue NF-κB activation and TNF-α protein expression at 1 and 2 h, IL-6 protein expression at 1, 2, and 6, and lung histopathology 24 h after sepsis initiation. Survival was assessed for 5 days post-CLP. NF-κB activation in lung tissue was increased in HSP70.1/3 KO WT mice at all time points after sepsis initiation. Deletion of HSP70.1/3 prolonged NF-κB binding/activation in lung tissue. Peak expression of lung TNF-α at 1 and 2 h was also significantly increased in HSP70.1/3 KO mice. Expression of IL-6 was significantly increased at 2 and 6 h, and histopathology revealed a significant increase in lung injury in HSP70.1/3 KO mice. Last, deletion of the HSP70 gene led to increased mortality 5 days after sepsis initiation. These data reveal that absence of HSP70 alone can significantly increase ARDS, activation of NF-κB, and inflammatory cytokine response. The specific absence of HSP70 gene expression also leads to increased mortality after septic insult.

nuclear factor-κB; heat shock protein 70.1/3; cecal ligation and puncture

ONE OF THE MOST BASIC MECHANISMS of cellular protection involves the expression of a highly conserved family of proteins, known as heat shock or heat stress proteins (HSP). The 70-kDa heat shock protein (HSP70) family is a group of proteins that plays a crucial role in protein assembly, folding, and transport (4). The mouse is known to possess two inducible HSP70s, HSP70.1 and HSP70.3. These two genes are separated by 7 kb located on chromosome 17, show 99% homology, and are both responsible for the initiation of transcription to produce HSP70 protein (6, 13).

The expression of these proteins after injury can induce “stress tolerance” and protect against a stress that otherwise would be lethal. Previous data have shown that the induction of the heat stress response via HSP70 can provide significant protection against many forms of cellular injury, ischemia and reperfusion (14, 16, 19), lung injury (27), and sepsis and septic shock (11, 23, 28). Additionally, HSP expression has been shown to attenuate proinflammatory cytokine release in vitro and in vivo models of injury (2, 21), and this attenuation appears to correlate with improved survival from sepsis (2).

Specific to lung injury and sepsis, we have previously shown that enhanced expression of HSP has been shown to improve outcome after experimental sepsis and shock models that lead to acute respiratory distress syndrome (ARDS) (25). We have also shown that enhanced expression of HSP70 in the lung can decrease the proinflammatory response following sepsis (24).

To assess the mechanism of protection in these studies, quercetin, a nonspecific inhibitor of HSP70, was utilized. Although quercetin is an inhibitor of HSP expression, quercetin’s usefulness in the aforementioned experiments is limited by the fact that quercetin is not only a HSP70 inhibitor but also has inhibitory effects on other enzymes, such as phosphatidylinositol 3-kinase (15), phospholipase A2 (8), and phosphodiesterases (10). Given the plethora of effects ascribed to quercetin, it is not clear whether this benefit can be attributed to HSP expression without the specific gene knockout of HSP70.

Therefore, this study utilized mice deficient for both genes responsible for transcription and the production of HSP70 protein, HSP70.1 and HSP70.3. Hsp70.1/3 KO mice and their wild-type counterparts were used to verify whether the inducible HSP70 genes are beneficial or detrimental after a septic insult. We investigated the effects of HSP70 protein expression on survival, lung injury, and the induction of cytokines and the proinflammatory response following sepsis induced by cecal ligation and puncture (CLP).

**MATERIALS AND METHODS**

Animal preparation. The experiments described in this paper were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. Protocols were approved by the Animal Care and Use Committee of The University of Colorado Health Sciences Center. All experiments were conducted and the animals cared for in accordance with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

Animals and experimental protocol. Experiments were performed on genotyped male knockout mice bearing the null alleles and wild-type controls (25 g body wt). Targeted deletion of Hsp70.1/3 in embryonic stem cells and generation of Hsp70.1/3 KO mice was carried out as previously described (7). The mice were purchased from University of California at Davis Mutant Mouse Regional Resource Center (Davis, CA) and were maintained on a standard diet and water ad libitum. Animals were housed at constant temperature with 10 and 14 h of light and dark exposure, respectively. Animals underwent an
acclimatization period of at least 7 days before use in these experiments. Sepsis was induced by CLP. After anesthesia with intraperitoneal injection of ketamine (40 mg/kg) and xylazine (6 mg/kg), a 1-cm incision was made in the abdominal wall, and the cecum was carefully extruded. Approximately 25% of the cecum was then ligated just below the ileocecal valve to avoid bowel obstruction. The cecum was punctured twice using a sterile 22-gauge needle and squeezed to extrude fecal material into the peritoneal cavity. The muscle and skin layers of the abdomen were then closed. All of the above manipulations were performed by the same surgeon to ensure consistency. This length of cecum ligated was chosen after evaluation of multiple cecal length ligation distances (26). This distance was chosen as it consistently yielded ~70% mortality in control animals. Immediately after the procedure, the animals were given 5% body wt of normal saline for fluid resuscitation and were allowed to recover. A sham group of animals for each of the knockout and wild-type groups was also included; however, no cecal ligation or cecal puncture was performed, and the abdomen was closed.

Survival studies. Hsp70.1/3<sup>−/−</sup> (n = 20) and Hsp70.1/3<sup>+/+</sup> (n = 20) animals underwent CLP procedure as described above. Two groups of sham animals that received anesthesia had an abdominal incision created, the cecum manipulated (without ligation or puncture), and the abdomen closed, were also observed for survival (Hsp70.1/3<sup>−/−</sup> SHAM, n = 5 and Hsp70.1/3<sup>+/+</sup> SHAM, n = 5). Animals were returned to their cages and were allowed access to food and water ad libitum. Animals were observed at regular intervals for occurrence of mortality over the subsequent 5 days post-CLP. Moribund animals (defined as bradycardia to a heart rate <40, severe lethargy, and unresponsive to painful stimulation) were killed with a lethal dose of ketamine/xylazine as defined by the University of Colorado Animal Care Committee.

Tissue collection. In a separate set of animals (n = 4/time point), Hsp70.1/3<sup>−/−</sup> and Hsp70.1/3<sup>+/+</sup> mice underwent CLP, and lung tissue and plasma via cardiac puncture were collected at 1, 2, 6, and 24 h after CLP. Tissue and plasma were collected from Hsp70.1/3<sup>−/−</sup> SHAM, n = 4, and Hsp70.1/3<sup>+/+</sup> SHAM, n = 4, as well. All tissue and plasma were removed and immediately frozen in liquid nitrogen and stored at −80°C.

TNF-α and IL-6 detection. TNF-α and IL-6 concentrations from lung tissue were measured using an ELISA. Lung tissue samples were homogenized in homogenization buffer containing a protease inhibitor cocktail tablet (Roche Molecular, Indianapolis, IN). Lung homogenate was then centrifuged for 15 min at 10,000 revolutions/min, and supernatant was removed and frozen at −80°C. The supernatant was then analyzed utilizing an ELISA kit for TNF-α from Endogen (Woburn, MA) and an ELISA kit for IL-6 from Roche (Mannheim, Germany). Results were determined spectrophotometrically using a microplate reader (Opsys MR; Thermo Lab Systems, Chantilly, VA).

Nuclear and cytoplasmic protein extraction. All nuclear and cytoplasmic protein extractions were performed on ice with ice-cold reagents. Protease inhibitors were added to reagents before use, and the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL) was utilized to obtain nuclear fractions. The fractions were then stored at −80°C and used for Western blot analysis.

Western blot analysis. Lung tissue was removed, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. Western blotting was performed as previously described (32). For HSP70 detection, the membranes were incubated with the specific mouse monoclonal antibody C92 (cat. no. SPA-810; Stressgen, Victoria, Canada). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (cat. no. 610094; Santa Cruz Biotechnology, Santa Cruz, CA). For IκBα detection, cytoplasmic extracts were prepared as stated above. Membranes were incubated overnight with rabbit polyclonal antibody specific to IκBα (cat. no. sc-847, Santa Cruz Biotechnology). The membranes were washed and incubated with secondary donkey anti-rabbit antibody HRP (cat. no. sc-2305; Santa Cruz Biotechnology). All Western blots were normalized against β-actin to control for protein loading (data not shown). For β-actin measurements, the aforementioned Western blot technique was applied using a specific mouse monoclonal antibody to β-actin (cat. no. A5441; Sigma-Aldrich, St. Louis, MO).

NF-κB detection. A kit detecting activation of NF-κB and binding of the p65 subunit was utilized (Pierce Biotechnology). Nuclear extracts were prepared as described above. Samples were assayed in duplicate in a 96-well plate coated with the consensus NF-κB binding element. After incubation with anti-p65 antibody, followed by a secondary antibody linked to HRP, plates were developed with a chemiluminescent substrate and read in the UVP Chemiluminescent Darkroom System (UVP, Upland, CA). An internal positive control of TNF-α-activated nuclear extract was used as a reference point for maximal signal. “Cold competition” for this assay was performed by adding wild-type NF-κB competitor consensus binding element to the wells. This reduced the signal level to near zero. A mutated NF-κB binding sequence had no effect on signal, ensuring signal specificity.

Lung histology. In a separate set of animals (n = 4/group), the aforementioned groups (Hsp70.1/3<sup>−/−</sup>, Hsp70.1/3<sup>+/+</sup>, Hsp70.1/3<sup>−/−</sup> SHAM, and Hsp70.1/3<sup>+/+</sup> SHAM) underwent CLP, and 24 h post-CLP, both lungs were harvested for assessment of lung pathology. The chest cavity was opened, and mainstem bronchi were loosely ligated. Tracheotomy was performed, and a 22-gauge cannula was inserted into the trachea. A second 22-gauge cannula was inserted into the left ventricle, and 10% buffered formalin was injected into the lung to inflate the lung. As a result, residual blood was removed from the lung. All lungs were fixed under standardized levels of inflation (20 cmH₂O pressure). Once the lungs were fully filled with formalin, the mainstem bronchi were ligated. Lungs were then removed and immediately fixed in 10% PBS-buffered formaldehyde for 24 h, rinsed in PBS for 1 h, and stored in 70% ethanol. Lungs were embedded in paraffin and sectioned at 5 μm. For histology, the sections were deparaffinized to water and stained with hematoxylin and eosin. Digital images were captured in a blinded manner with a Qimaging Ratiga 1300 color digital camera and Olympus BX51 microscope (Olympus America, Melville, NY). All histological images were processed and evaluated blindly by a pathologist using a systematic scoring system (3).

Statistical analysis. Results are presented as an average ± SD. Lung tissue cytokines, Western blotting, NF-κB activation, and histology were compared using the Student’s t-test or ANOVA followed by Student-Newman-Keuls test where applicable. Fisher’s exact test was utilized to compare survival data. Results were considered significant at a P value <0.05.

RESULTS

Deletion of the HSP70.1 and HSP70.3 genes suppresses production of HSP70 in the lung. To ensure that the production of HSP70 protein was suppressed in the knockout Hsp70.1/3<sup>−/−</sup> mice, a Western blot was conducted to detect protein synthesis (Fig. 1). All Hsp70.1/3<sup>−/−</sup> mice (including SHAM) expressed little to no HSP70, whereas the wild-type Hsp70.1/3<sup>−/−</sup> mice expressed increased levels of HSP70 (P < 0.001 vs. Hsp70.1/3<sup>−/−</sup>). The Hsp70.1/3<sup>+/+</sup> SHAM group expressed a basal amount of HSP70, with an increase of expression after CLP-induced sepsis (P < 0.05 vs. Hsp70.1/3<sup>−/−</sup>). All results are representative of experiments carried out in triplicate.

Reduced activation of pulmonary NF-κB and degradation of IκBα after sepsis in mice containing the HSP70.1/3 gene. To determine whether the ability to make HSP70 has an effect on NF-κB activation, nuclear extracts from Hsp70.1/3<sup>−/−</sup>,
Hsp70.1/3+/+, Hsp70.1/3−/− SHAM, and Hsp70.1/3+/+ SHAM (n = 4/group) lung tissues were assayed for specific nuclear p65 binding activity at 1 and 2 h postsepsis by using a transcription factor assay kit as previously described. Figure 2 shows that sepsis-induced activation of NF-κB signaling was decreased significantly at 1 and 2 h postsepsis in the Hsp70.1/3−/− mice (P < 0.01 vs. Hsp70.1/3+/+). Similar to EMSA, addition of wild-type NF-κB competitor duplex highly suppressed detection of signal. Incubation with a mutated NF-κB p65 probe showed no effect, validating the signal specificity of these experiments. An internal positive control of TNF-α-activated nuclear extract was used as a reference point for maximal signal.

Activation of IkB kinase leads to phosphorylation, ubiquitination, and degradation of IkBα, which allows NF-κB to translate to the nucleus and induce transcription (34). Western blot analysis shows that Hsp70.1/3−/− mouse lung cytosolic extracts exhibited a degradation of IkBα, whereas the wild-type mouse lung tissue did not portray this degradation in the cytosol (Fig. 3).

**Reduced proinflammatory cytokine release following sepsis in mice possessing the HSP70.1/3 gene.** TNF-α and IL-6 expression is attenuated in Hsp70.1/3+/+ mice in response to sepsis. To examine whether the HSP70.1/3 gene could modulate proinflammatory cytokine release after CLP-induced polymicrobial sepsis, we studied four groups of animals: Hsp70.1/3−/−, Hsp70.1/3+/+, Hsp70.1/3−/− SHAM, and Hsp70.1/3+/+ SHAM (n = 4/group) at 1, 2, and 6 h post-CLP procedure. Expression of the HSP70.1/3 gene significantly decreased release of TNF-α at 1 and 2 h and IL-6 at 2 and 6 h post-CLP in Hsp70.1/3+/+ vs. Hsp70.1/3−/− (P < 0.01) for both TNF-α and IL-6 vs. Hsp70.1/3−/− (Fig. 4, A and B). Wild-type mice had an average lung IL-6 level at 6 h of 56 ± 11 pg/ml (means ± SD) and HSP70 knockout mice had an average IL-6 level of 156 ± 26 pg/ml (means ± SD).

**Reduced occurrence of lung injury after sepsis in mice containing the HSP70.1/3 gene.** Pulmonary changes consistent with lung injury and ARDS, including neutrophil accumulation, septal thickening, and hyaline membrane formation (30) (Fig. 5) were seen in knockout Hsp70.1/3−/− mice. There were no pathological changes in lung tissue from sham-operated animals in either wild-type or knockout groups. As scored by a blinded pathologist, changes in pulmonary pathology seen in the knockout Hsp70.1/3−/− mice were attenuated in their wild-type counterparts. Low-power sections (×10) demonstrated the aforementioned changes consistent with ARDS and lung injury after CLP in the HSP70 knockout mice, but a significant decrease in lung injury was demonstrated in the wild-type mice (P < 0.05 vs. Hsp70.1/3+/+). A higher resolution (×40) demonstrated the lack of lung consolidation, decreased cellularity, reduced septal edema, and absence of proteinaceous exudate associated with the wild-type mice.

**Reduced occurrence of 5-day mortality after CLP-induced sepsis in mice possessing the HSP70.1/3 gene.** Animals were followed for 5 days for occurrence of mortality. All mortality occurred within the first 72 h after CLP. Figure 6 depicts survival from CLP in knockout Hsp70.1/3−/− (n = 20), wild-type Hsp70.1/3+/+ (n = 20), and sham Hsp70.1/3−/− SHAM, Hsp70.1/3+/+ SHAM (n = 4/group) mice. Of 20 Hsp70.1/3−/− mice, 14 (70%) died by 3 days. In wild-type mice, only 7 of 20 (35%) died within 3 days (P < 0.01). No mortalities occurred in either of the SHAM groups.
DISCUSSION

These data demonstrate that the ability to make and express HSP70 can significantly attenuate the expression of proinflammatory cytokines and systemic inflammation after a septic insult independently of other heat stress proteins. We have shown that the genes responsible for inducing HSP70 expression can affect the lethality and the occurrence of ARDS as measured through end organ histological damage. The findings of this study define a potentially vital mechanism by which heat stress proteins can protect against septic injury.

Because the suppression of NF-κB is associated with an anti-inflammatory effect, we hypothesized that the expression of HSP70 could mediate the attenuation of proinflammatory cytokine release though the suppression of NF-κB. Activation of NF-κB is dependent on the phosphorylation and degradation of IκBα, an endogenous inhibitory molecule that binds to NF-κB in the cytoplasm (34). In this study, we demonstrated that the expression of inducible HSP70 was able to suppress NF-κB transcriptional activation, translocation to the nucleus, and significantly inhibit IκBα phosphorylation and degradation in lung cytosolic tissue. However, when the gene for HSP70 is deleted, these effects are reversed.

These results suggest that protection from injury that is conferred by inducible HSP70 is acting at least partly through the NF-κB pathway. Recent data have shown that heat-shocked human monocytes can release HSP70 into cell culture media. Subsequently, when the monocytes are incubated with the HSP70-conditioned media and exposed to endotoxin, the activation and DNA binding of NF-κB is inhibited. When anti-HSP70 is added to the media, this reverses the inhibitory effect of the HSP70-conditioned media on the endotoxin-induced activation of NF-κB (1). Multiple recent studies have also shown that constitutive activation of NF-κB is prevented by HSP70 induction through maintenance in IκBα synthesis (9, 12).

Fig. 3. Effect of HSP70 gene on cytosolic IκBα expression. Representative Western blot of degradation of IκBα in Hsp70.1/3−/− mice in response to CLP (upper). IκB protein levels were assessed from cytosolic extracts prepared 1, 2, 6, and 24 h after the induction of sepsis or sham operation. Results are representative of 3 separate experiments. All data are expressed as means ± SE. *P < 0.01 vs. Hsp70.1/3−/− at corresponding time point.

Fig. 4. Effect of HSP70 gene on lung concentrations of proinflammatory cytokines after CLP. Hsp70.1/3−/− (n = 4) and Hsp70.1/3+/+ (n = 4) mice underwent CLP, and lung tissue was collected at 1, 2, and 6 h. Lung tissue from 2 groups of sham-operated mice were also analyzed [Hsp70.1/3−/− SHAM and Hsp70.1/3+/+ SHAM (n = 4/group)]. Lung tissue was homogenized, and TNF-α was assayed at 1 and 6 h (A) and IL-6 at 2 and 6 h (B) by ELISA as described in MATERIALS AND METHODS. All data are expressed as means ± SE. *P < 0.01 vs. KO at 1 and 2 h (A) and vs. KO at 2 and 6 h (B). #P < 0.01 vs. sham KO and sham WT in A and B.
The most stress-inducible type of HSP belongs to the HSP70 family, which has a molecular mass of 70 kDa (31). After stress, acute elevations of inducible HSP70 occur (17). A constitutively expressed form of HSP70, Hsc70 (also referred to as HSP73), exists as well. This protein does not change in response to stress but has been shown to participate in various stages of protein maturation (17). The mice utilized in this study have a specific deletion of the genes for the inducible form of HSP70 (7).

Together, these results indicate that the expression of inducible HSP70 is vital to protect against the proinflammatory response and lung injury associated with sepsis. These data are particularly important as the significance of the HSP70.1 and HSP70.3 genes has never been previously investigated in an experimental model of sepsis. This study is clinically relevant because there are a multitude of previous experimental studies that show dramatic benefits of enhanced HSP expression in the lung and other organs after sepsis (22, 29). These results reveal that the expression of inducible HSP70 may have a therapeutic potential for diseases mediated by the release of proinflammatory cytokines. The effect HSP70 on systemic inflammation could be applied not only for the treatment of sepsis but to other inflammatory diseases as well.

To further investigate the significance of inducible HSP70, we intend to use glutamine (GLN), a potent enhancer of HSP70, to examine the effects of enhanced expression of this protein. GLN has been shown to improve the outcomes in various states of critical illness, injury, and surgical intervention (5, 18, 33); however, the mechanism of this protection has yet to be elucidated (20). It is our intention to further investigate this mechanism through the use of the HSP70.1/3 knockout mice and the data obtained from this novel study.

**Fig. 5.** HSP70 protects lung tissue from damage after CLP. Sections of lung from Hsp70.1/3−/− and Hsp70.1/3+/− or sham-operated animals [Hsp70.1/3−/− SHAM and Hsp70.1/3+/− SHAM (n = 4/group)] were harvested 24 h after CLP. Tissue sections were stained with hematoxylin and eosin. Magnifications are indicated. Pathologist injury scores were 0.0 ± 0.0 for all SHAM animals. For ×10 magnification, pathologist injury scores were Hsp70.1/3−/− (3.0 ± 0.5, 773) vs. Hsp70.1/3+/+ (1.2 ± 0.3, 557). For ×40 magnification, pathologist injury scores were Hsp70.1/3−/− (2.14 ± 0.3, 779) vs. Hsp70.1/3+/+ (1.0 ± 0.287). *P < 0.05 vs. Hsp70.1/3−/− alone via Student’s t-test.

**Fig. 6.** Effect of HSP70 gene on mortality after CLP. Hsp70.1/3−/− (n = 20) and Hsp70.1/3+/+ (n = 20) or sham operated [Hsp70.1/3−/− SHAM and Hsp70.1/3+/− SHAM (n = 6/group)] underwent CLP and were monitored for survival for 5 days. Of 20 Hsp70.1/3−/− mice, 14 (70%) died by 3 days. In WT mice, only 7 of 20 (35%) died within 3 days (*P < 0.01 vs. Hsp70.1/3−/− via Fisher’s exact test at days 3–5). No mortalities occurred in either of the SHAM groups.