Extracellular Heat Shock Protein 72 is a Marker of the Stress Protein Response in Acute Lung Injury

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Running title
Extracellular Hsp72 and Acute Lung Injury

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ABSTRACT

Previous studies have shown that Hsp72 is found in the extracellular space (eHsp72) and that eHsp72 has potent immunomodulatory effects. However, whether eHsp72 is present in the distal airspaces and whether eHsp72 could modulate the removal of alveolar edema is unknown. The first objective was to determine whether Hsp72 is released within the airspaces and whether Hsp72 levels in pulmonary edema fluid would correlate with the capacity of the alveolar epithelium to remove alveolar edema fluid in patients with ALI/ARDS. Patients with hydrostatic edema served as controls. The second objective was to determine if activation of the stress protein response (SPR) caused the release of Hsp72 into the extracellular space \textit{in vivo} and \textit{in vitro} and to determine whether SPR activation and/or eHsp72 itself would prevent the IL-1β mediated inhibition of the vectorial fluid transport across alveolar type II cells. We found that eHsp72 was present in plasma and pulmonary edema fluid of ALI patients and that eHsp72 was significantly higher in pulmonary edema fluid from patients with preserved alveolar epithelial fluid clearance. Furthermore, SPR activation \textit{in vivo} in mice and \textit{in vitro} in lung endothelial, epithelial and macrophage cells caused intracellular expression and extracellular release of Hsp72. Finally, SPR activation, but not eHsp72 itself, prevented the decrease in the alveolar epithelial ion transport induced by exposure to IL-1β. Thus, SPR may protect the alveolar epithelium against oxidative stress associated with experimental ALI, and eHsp72 may serve as marker of SPR activation in the distal airspaces of patients with ALI.
Keywords

INTRODUCTION

Acute lung injury (ALI) is a common cause of acute respiratory failure in critically ill patients. The early phase of ALI is characterized by the accumulation and activation of inflammatory cells (neutrophils and macrophages) within the distal airspaces that release high levels of oxidant species (46). Alveolar epithelial and lung endothelial injury leads to increased permeability, pulmonary edema and acute respiratory failure. An important mechanism to prevent alveolar flooding is to maintain or upregulate lung alveolar fluid clearance (AFC), requiring an intact alveolar epithelium (47). However, AFC has been shown to be impaired in the majority of ALI patients, and an impaired AFC was associated with worse clinical outcomes (47).

Heat shock or stress proteins (HSPs) are a family of highly conserved proteins found in cells of all organisms, from bacteria, plants and yeast to mammals. Although regarded typically as intracellular chaperone proteins (18), HSPs are also being released into the extracellular space exerting important immunomodulatory functions (13, 19). The 70 kDa Hsp (HSP70) family of proteins includes a constitutive 73 kDa protein (Hsc70) and a stress inducible 72 kDa protein (Hsp72), both typically located in the cytosol and nucleus (20). Other HSP70 family members are located in the endoplasmatic reticulum (BIP/Grp78; 78 kDa) and in the mitochondrion (Grp75; 75 kDa).

Activation of the stress protein response (SPR) provide the cells and organs increased protection from insults that otherwise would be lethal, e.g. viral and bacterial infections, oxidative stress, and ischemia-reperfusion injuries, a phenomenon referred to as thermotolerance or stress preconditioning (23, 26). Besides the classical activation of the SPR with heat or hyperthermia, also called heat shock (31, 33), SPR has been shown to be induced after various stimuli, e.g. corticosteroids (41, 42), catecholamines
(19), oxidative stress (24, 45), as well as after specific pharmacological treatments like 17-allylamino-17-demethoxy-geldanamycin (17-AAG) (38). Therefore, it is not surprising that SPR has been shown to be activated under certain clinical situations like sepsis, septic and hemorrhagic shock (10, 37, 40). Finally, SPR activation is known to be associated with the increased intracellular expression of inducible heat shock proteins, such as Hsp72. Whether Hsp72 is released in the extracellular space (eHsp72) of the lung after SPR activation and whether the presence of eHsp72 in pulmonary edema fluid has pathogenetic significance is still unknown.

Thus, the first objective of this study was to determine whether Hsp72 is released within the airspaces and whether eHsp72 levels in pulmonary edema fluid would correlate with the capacity of the alveolar epithelium to remove alveolar edema fluid in patients with acute lung injury (ALI). Patients with hydrostatic edema served as controls. The second objective was to test the hypothesis that SPR activation causes the release of Hsp72 into the extracellular space in vivo and in vitro and to determine whether SPR activation and/or eHsp72 itself would prevent the IL-1β mediated inhibition of the vectorial fluid transport across primary cultures of rat alveolar type II cells.
MATERIALS AND METHODS

The Committee for Human and Animal Research (University of California, San Francisco, CA) approved the study.

In vivo human studies

Patients. Patients with ALI or ARDS as defined by the North American European Consensus Conference definitions (4) were identified from adult intensive care units of Moffitt-Long Hospital San Francisco and San Francisco General Hospital. Additional inclusion criteria were acute respiratory failure requiring mechanical ventilation and aspirable pulmonary edema fluid within 1 h of endotracheal intubation. The initial edema-fluid to plasma protein ratio, a measure of alveolar-capillary barrier permeability, was required to be > 0.65, consistent with increased permeability pulmonary edema (12). Patients with severe hydrostatic pulmonary edema (HYDRO) served as controls. These patients had no evidence of ALI and were required to have an initial edema-fluid to plasma protein ratio of < 0.65 (44).

Clinical data collection. The etiology of ALI was determined from the clinical history. Sepsis was defined using published criteria (11). Pneumonia was defined as new radiographic infiltrates and positive cultures of endotracheal tube aspirates for pathogenic bacteria. Aspiration of gastric contents was defined as a witnessed aspiration event. Demographics, physiological data, respiratory parameters, medications, and multiorgan system function were recorded. The Simplified Acute Physiology Score II (SAPS II) (22) and Lung Injury Score (LIS) (28) were calculated for the 24 h period after initiation of mechanical ventilation.
**Collection of plasma and pulmonary edema fluid.** Undiluted pulmonary edema fluid and plasma were collected simultaneously immediately after intubation and 4 h later, as previously described (25). Briefly, a 14-Fr tracheal suction catheter was advanced through the endotracheal tube into a wedged position in a distal bronchus. Undiluted pulmonary edema fluid was aspirated by gentle suction. Pulmonary edema fluid and plasma samples were centrifuged (3,000 X g, 10 min) and supernatants stored at -70 °C until further analysis.

**Measurement of protein concentration.** The total protein concentration was measured by the biuret method (25). If the sample volume was insufficient (<1% of samples), then refractometry was used.

**Measurement of eHsp72.** eHsp72 levels were measured in triplicate from the initial samples collected immediately after intubation by a commercially available ELISA (Stressgen Biotechnologies Corp., Victoria, BC, Canada). The assay was performed according to the manufacturer’s protocol. The sensitivity of the assay was 500 pg/ml and the assay was specific for both natural source and recombinant inducible Hsp72 and did not cross-react with 100 ng/ml of Hsc70, recombinant hamster Grp78, *Escherichia coli* DnaK, or recombinant *Mycobacterium tuberculosis* Hsp71.

**Measurement of lung alveolar fluid clearance (AFC).** The net lung AFC rate was calculated from protein concentrations in serial samples of pulmonary edema fluid based on the observation that the rate of clearance of edema fluid from the alveolar space is much faster than the rate of protein removal (5): Percent lung AFC = 100 x [1 - (initial edema protein/final edema protein)]. This method has been validated in both clinical and experimental studies (47). Patients were further categorized into two groups of AFC on
the basis of extrapolations from studies in the *ex vivo* human lung and *in vivo* data on patients with ALI (5, 35, 44): preserved AFC, ≥ 3%/h and impaired AFC, < 3%/h.

**In vivo animal studies**

**Animals.** Male, 7-8 weeks old C57BL/6J mice (The Jackson Laboratory) were housed in air-filtered, temperature-controlled units (20±2 °C) with food and water *ad libitum*.

**Stress protein response (SPR) activation in vivo.** SPR was induced with whole body hyperthermia with heating lamps and heating pads in mice anesthetized with inhaled isoflurane (inspiratory concentration 1.5-3% in 100% O₂, *n*=8). Rectal temperature was continuously recorded and the animals were kept at 42±1 °C for 25 min. Then, the mice were cooled down to their normal body temperature. To prevent dehydration, 2 boluses of 0.5 ml of 0.9% NaCl were given intraperitoneally (i.p.) – immediately before and after hyperthermia. Control animals (*n*=4) were also anesthetized but kept at their normal body temperature. Furthermore, we also induced SPR pharmacologically with an analogue of geldanamycin, 17-AAG (17-Allylamino-17-demethoxygeldanamycin; InvivoGen, San Diego, CA; *n*=4). 50 µg/kg of 17-AAG or its vehicle (DMSO) was injected once i.p. 48 h before sacrificing the mice.

**Collection of plasma, lung tissue and bronchoalveolar lavage (BAL) fluid.** After 30 h (heat) or 48 h (17-AAG) recovery time, the mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90 and 10 mg/kg, respectively). Blood was collected in heparinized syringes from the inferior vena cava, the chest was opened, the lungs lavaged with 30 ml/kg 0.9% NaCl aspirated four times and the lungs quickly frozen.
at -70 °C. Blood and BAL fluid were centrifuged (3,000 X g, 10 min) and stored at -70 °C until further analysis.

**Measurement of Hsp72 in lung tissue, BAL fluid and plasma.** Lung tissue samples were solubilized by homogenization in a TRIS buffer (50 mmol, pH 7.4) containing NaCl (20 mmol), KCl (10 mmol), DTT (0.1 mmol), EDTA (1 mmol), SDS (1%), sonicated for 30 s, boiled for 10 min and centrifuged at 14,000 X g for 10 min at 4 °C. Supernatants were then analyzed by Western blot analysis, as previously described (31). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA), and Hsp72 was detected using a specific antibody (Stressgen Biotechnologies Corp.; 1:1,000) and a HRP-conjugated goat anti-mouse antibody (ICN, Costa Mesa, CA; 1:2,000). Proteins were visualized using chemiluminescence. Extracellular Hsp72 in plasma and BAL fluid was detected by ELISA (Stressgen Biotechnologies Corp.), as described above.

**Measurement of alveolar fluid clearance (AFC) in mice.** AFC was determined in the absence of ventilation or blood flow by measuring the increase in protein tracer concentration (125I-labeled albumin, 0.1 μCi) in the lungs over a 30 min period using our previously described in situ model (14). For these experiments, we instilled 20 ml/kg of warmed, radioactive 5% albumin in 0.9% NaCl solution intratracheally, aspirated and re-instilled the solution three times, applied continuous positive airway pressure (8 cm H₂O, 100% FiO₂), and kept the animals at 37 °C body core temperature. The instillate, an initial sample (after aspiration and re-instillation) and a sample after 30 min were obtained and analyzed. The increase in protein concentration over 30 min has been shown to be a good estimate of the liquid volume removed from the distal airspaces of the lungs (14).
**In vitro studies**

**Cell culture.** Rat alveolar epithelial type II (ATII) cells were isolated as described previously (34), and cultured in Dulbecco's modified Eagle's medium/H21 medium containing 10% FBS and 1% penicillin/streptomycin/amphotericin (Invitrogen Life Technologies). Bovine pulmonary arterial endothelial cells (BPAEC; ATCC, CCL-209) were cultured in Dulbecco's modified Eagle's medium/H21 medium with 20% FBS and 1% penicillin/streptomycin/amphotericin (Invitrogen Life Technologies). MH-S cells (ATCC CRL-2019), a mouse alveolar macrophage cell line that shares many characteristics with primary alveolar macrophages, were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin/amphotericin (Invitrogen Life Technologies). All cells were kept at 37 °C in a humidified 95% air and 5% CO₂ environment.

To measure intra- and extracellular Hsp72, cells were plated in standard tissue culture treated polystyrene wells (Corning Life Sciences) 24 h before the experiment. To measure transepithelial current and αENaC mRNA, ATII cells were plated on polycarbonate Transwell dishes (0.4-µm pore size; Corning Life Sciences). 24 h later, non-adherent epithelial cells were removed by washing with PBS, and fresh medium was added to the lower compartments of the Transwell dishes, thus maintaining the ATII cell monolayers with an air-liquid interface on their apical side. After 72–96 h, cells that formed confluent monolayers reaching a transepithelial electrical resistance (TER) >1500 ohms/cm² were used for experimentation.

**Activation of SPR in vitro.** Cell culture media was replaced with fresh media immediately before the experimental procedure. The activation of SPR was performed by incubating the cells at 43 °C for 45 min (ATII cells) or 60 min (MH-S and BPAEC),
respectively. Control cells were kept at 37 °C. Additionally, we induced SPR pharmacologically with 17-AAG in a final concentration of 1 µM. Control cells were treated with its vehicle (DMSO) only.

Cell viability after exposure to different experimental conditions in control cells and cells after SPR was measured using the Alamar blue assay (30).

**Western blot analysis for intracellular Hsp72.** Western blot analysis for Hsp72 was performed, as described above.

**Measurement of Hsp72 in cell culture media.** Twenty-four hours after SPR induction, heat shock conditioned and control cell culture media were collected and centrifuged (1000 X g for 10 min). eHsp72 levels were measured in the supernatant by ELISA, as described above. As a control for the level of sensitivity and specificity of the ELISA, eHsp72 was removed from an aliquot of the supernatant by addition of two different Hsp72 antibodies (SPA-810, SPA-812; Stressgen Biotechnologies Corp.). Ab-Hsp72 complexes were captured via the addition of protein G- and A-Sepharose beads (Roche Biochemicals) for 2 h and pelleted by centrifugation at 300 X g for 1 min.

**Immunofluorescent staining of surface Hsp72.** After induction of SPR and recovery for 1, 6 and 14 h, cells were fast cooled to 4 °C in PBS and maintained at 4 °C for the remainder of the protocol. Cells were washed twice in PBS and then incubated in 3% BSA/PBS for 30 min. After blocking, the cells were washed three times and incubated with an antibody to Hsp72 (SPA-810, Stressgen Biotechnologies Corp.; 1:1,000) for 60 min. Again, the cells were washed three times in PBS and incubated with cy3-conjugated goat anti-mouse antibody (1:5,000) for 60 min. The cells were washed three times with PBS and DAPI added during the last wash. The cells were fixed with 3% paraformaldehyde and mounted.
Measurement of transepithelial current (TEC). The TER (kilo-ohms/cm²) and potential difference (PD; millivolts, apical side as reference) were measured using the Millicell-ERS Voltohmmeter (Millipore Corp., Bedford, MA). Transepithelial current (Isc; microamps/cm²) was calculated from the relationship Isc = PD/Rt (Ohm's law).

Polarized rat ATII monolayers were pretreated with dexamethasone for 12 h (100 nM) as previously described (34), exposed to interleukin-1β for 6 h (IL-1β, 10 ng/ml; R&D Systems, Minneapolis, MN) or its vehicle and the bioelectric properties of ATII cell monolayers evaluated. To determine if prior SPR activation is protective in maintaining transepithelial ion transport, SPR was induced with heat followed by a recovery period of 1 h prior to administration of IL-1β or its vehicle in some experiments. Furthermore, to detect a potential direct effect of eHsp72 on transepithelial current, further measurements were done exposing ATII cells to IL-1β in presence or absence of low-endotoxin recombinant human or recombinant rat Hsp72 (600 ng/ml; #ESP-555 or #SPP-758, Stressgen Biotechnologies Corp.).

Quantitative real-time RT-PCR for αENaC mRNA. Primers and probes used are described in Table 1. The TaqMan probe was labeled with a fluorophore reporter dye (6-carboxyfluorescein) at the 5'-end and a Black Hole Quencher dye (Biosearch Technologies, Inc.) at the 3'-end. Total RNA was extracted from rat ATII cells using the RNeasy mini kit (Qiagen Inc.). One microgram of total RNA was reverse-transcribed using the Superscript first-strand synthesis system (Invitrogen). RT-PCRs were performed, and the results were analyzed using the ABI PRISM 7700 sequence detection system (PE-Applied Biosystems). Briefly, RT-PCR was carried out in a 25-µl reaction mixture containing 1x TaqMan Universal PCR Master Mix (PE-Biosystems, Foster City, CA), 10 pmol of primers, 5 pmol of TaqMan probe, and an equivalent of 100
ng of total RNA for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The number of cycles to threshold of fluorescence detection was normalized to the number of cycles to threshold of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample tested. Results are expressed as a percentage of cDNA abundance compared with the control.

**Statistical analysis**

Continuous variables were compared by Student's *t* test or by analysis of variance with the Student-Newman-Keuls test for multiple comparisons. Categorical data were compared by using $\chi^2$ analysis. Non-parametric data were analyzed by using the Mann-Whitney *U* test. Statistical significance was defined as $P < 0.05$. Parametric data are presented as mean ± standard error of the mean (SEM), non parametric data as median and inter-quartile range (IQR).
RESULTS

Clinical Studies

Patients. There were 35 patients with ALI included in this study. To test whether the alterations in eHsp72 levels were specific for ALI, a comparison population of 20 patients with hydrostatic pulmonary edema (HYDRO) served as a control group. As per definition, the edema-fluid to plasma protein ratio was significantly different between the groups. Detailed patient characteristics are shown in Table 2.

eHsp72 levels in patient samples. Hsp72 could be detected in the plasma and pulmonary edema fluid from both ALI and HYDRO patients. Plasma levels of Hsp72 did not differ between ALI and HYDRO groups. However, Hsp72 levels were significantly higher in the pulmonary edema fluid from ALI patients compared with the values measured in plasma (Figure 1).

To determine whether levels of Hsp72 in pulmonary edema fluid in ALI had potential pathogenetic significance, we compared levels of Hsp72 from the initial samples of pulmonary edema fluid to the rate of lung alveolar fluid clearance (AFC) in patients with ALI. The ALI patients with preserved AFC had significantly higher levels of Hsp72 in their pulmonary edema fluid than patients with impaired AFC (Figure 2). However, the initial edema-fluid to plasma protein ratio did not differ between patients with impaired or preserved AFC (P = 0.27), excluding a potential false increase in the initial concentration of eHsp72 as a result of better AFC. There was no difference in plasma levels of Hsp72 between ALI patients with impaired and preserved AFC (data not shown). Compared to patients with hydrostatic pulmonary edema (Hsp72 in pulmonary edema fluid 160 ± 51 ng/ml), ALI patients with sepsis (n = 13) and shock (n = 21) had significantly higher
levels of Hsp72 in their pulmonary edema fluid (603.0 ± 153 ng/ml, \( P = 0.004 \) and 430 ± 100 ng/ml, \( P = 0.04 \), respectively). Hsp72 in pulmonary edema fluid from ALI patients without sepsis (\( n = 22 \)) or without shock (\( n = 14 \)) did not differ from HYDRO patients (224 ± 90 ng/ml and 150 ± 72 ng/ml, respectively).

**Mouse Studies**

*SPR activation induces intracellular Hsp72 expression as well as a release of Hsp72 into the extracellular space.* Induction of SPR with heat caused a significant increase of Hsp72 expression in lung tissue (Figure 3A). Furthermore, levels of Hsp72 increased 7.4 fold in BAL fluid after SPR activation and Hsp72 was only detected in plasma of mice that underwent SPR activation (Figure 3B). To test that the release of Hsp72 in the extracellular space was not a nonspecific effect of heat, we also pharmacologically induced SPR with 17-AAG. The results indicate that 17-AAG caused a significant increase in the expression of Hsp72 in lung tissue (data not shown) and its release in the distal airspaces of the lung (Hsp72 levels increased by 38% in BAL fluid of 17-AAG treated animals compared to controls, Table 3).

*SPR activation does not alter baseline alveolar fluid clearance (AFC) in vivo.* In order to determine if the function of the alveolar epithelium is not altered by SPR activation with heat, we measured AFC in mice. There was no difference in AFC between control mice (AFC = 13.0 ± 0.8 % of instilled volume / 30 min) and mice after SPR activation (AFC = 12.9 ± 0.7 % of instilled volume / 30 min), confirming our previously published results in rats (31).
In vitro Studies

Hsp72 is expressed intracellularly and actively released by all major cell types in the distal lung after SPR activation. Our in vivo results showed that Hsp72 levels were markedly higher in BAL fluid in mice that underwent SPR activation compared to controls (Figure 2B), suggesting that Hsp72 could be released locally within the distal airspaces after SPR activation. The next series of studies was designed to determine whether SPR activation would be associated with the release of Hsp72 in the major cell types present within the distal airspaces (alveolar epithelial, pulmonary endothelial cells, and alveolar macrophages). The results indicate that all three cell types expressed Hsp72 intracellularly (Figures 4A, 4C, 5A) after SPR activation with heat. Furthermore, Hsp72 was also released into the cell culture media after SPR activation (heat shock conditioned media, Figures 4B, 4D, 5B). In addition, significant levels of Hsp72 were present on the cell surface of MH-S cells 1 and 6 h after SPR activation, (Figure 5C, panels C-2, C-3) as compared to control cells (Figure 5C, panel C-1). By 14 hrs post SPR activation, surface Hsp72 expression was decreased (Figure 5C, panel C-4). Importantly, SPR activation did not cause a decrease in cell viability over time in all cell lines, as measured by the Alamar blue assay (data not shown). Pharmacological activation of SPR with 17-AAG showed comparable results compared to SPR activation with heat in rat ATII cells. Indeed, exposure to 17-AAG caused intracellular expression of Hsp72 (data not shown) as well as its release in cell culture media (12-fold increase over controls, Table 3).

Prior SPR activation prevents the decrease in transepithelial ion transport by IL-1β across rat ATII cell monolayers. Preserved alveolar fluid clearance requires a normal function of the alveolar epithelium to prevent flooding. We have previously
reported that exposure to IL-1β for 6 h, one of the most biologically active cytokines in the pulmonary edema fluid from patients with early ALI (32), causes a decrease in transepithelial current across ATII cell monolayers (34). The results of the next experiments indicate that prior SPR activation in ATII cell monolayers using heat prevented the IL-1β mediated decrease in transepithelial current (Figure 6A) by maintaining the expression of epithelial sodium channels (αENaC; Figures 6B).

**eHsp72 itself has no effect on IL-1β mediated decrease in transepithelial current.** Finally, we found that the exposure of rat ATII cell monolayers to recombinant rat and human Hsp72 at a concentration comparable to that measured in the pulmonary edema fluid of our ALI patients with preserved AFC (600 ng/ml) did not increase the transepithelial current nor inhibit the IL-1β mediated decrease in transepithelial current across the ATII cell monolayers (Figure 7).
DISCUSSION

The major findings of this study can be summarized as follows: (a) eHsp72 is present in the plasma and pulmonary edema fluid of patients with ALI and levels of eHsp72 are highest in the pulmonary edema fluid from ALI patients with preserved alveolar epithelial fluid clearance; (b) SPR activation in lung endothelial, epithelial and macrophage cells as well as in mice causes not only intracellular expression but also extracellular release of Hsp72; (c) SPR activation, but not eHsp72 itself, prevents the decrease in alveolar epithelial fluid transport induced by exposure to IL-1β. These results indicate that eHsp72 may serve as marker of SPR activation in the distal airspaces of patients with ALI and that SPR activation may protect the alveolar epithelium against oxidative stress associated with acute lung injury.

The first important result of these studies is that Hsp72 is detectable in the pulmonary edema fluid of patients with ALI and that eHsp72 pulmonary edema fluid levels are the highest in ALI patients with sepsis and/or shock. Two major mechanisms may explain the release of Hsp72 into the extracellular space. It was originally suggested that Hsp72 is only released as the result of necrotic/lytic cell death (3, 6, 15, 36). However, it is now recognized that Hsp72 may be found in the extracellular space in absence of cell death. For example, glial cells (17), B cells (9), tumor cells (16) and human peripheral mononuclear cells (21) have all been shown to release Hsp72. The mechanism of Hsp72 secretion into the extracellular space has been linked to the presence of Hsp72 in exosomes, membranous vesicles that form with multivesicular bodies and are secreted from the cells (16).

What is the mechanism that explains the presence of Hsp72 in the pulmonary edema fluid of patients with ALI? Our in vitro results indicate that the three major cells types
present in the distal airspaces of the lung, alveolar macrophages, lung epithelial and endothelial cells, are able to release Hsp72 extracellularly, and that SPR activation is associated with a several-fold increase in Hsp72 extracellular release without significant cell death. Furthermore, our in vivo studies in mice confirm that Hsp72 can be detected in the distal airspaces of the lung and that the extracellular secretion of Hsp72 is significantly increased in mice that underwent SPR activation with heat. Further experiments indicate that these results are not explained by a non-specific effect of heat because comparable data were obtained when rat ATII cells were treated with a pharmacological inducer of SPR, 17-AAG (Table 3). However, SPR activation in mice with 17-AAG resulted in a smaller increase in eHsp72 concentration in BAL fluid than the one observed with heat-induced SPR activation. In the present studies, we did not perform a dose-response with 17-AAG in order to determine a SPR activation with this compound comparable to the one observed with heat. We only used one dose of 17-AAG (50 µg/kg) i.p. 48 h before sacrificing the mice. This could explain that this dose of 17-AAG caused a smaller SPR activation that the one observed with heat. Finally, since the mice that underwent SPR activation with heat had normal alveolar fluid clearance provides some evidence against the possibility that the increased release of Hsp72 into the alveolar space after heat shock was due to alveolar epithelial cell injury.

Is there any evidence that some of our patients with ALI did undergo SPR activation? Heat shock proteins (HSPs) are typically induced in cells exposed to sublethal heat shock. However, since the first report about the detection of HSPs after a temperature shock in 1962 by Ritossa (33), there is considerable evidence that HSPs can also be induced by a variety of other stressful stimuli, including corticosteroids (41, 42), catecholamines (19), oxidative stress (24, 45), as well as after specific pharmacological
treatments (38). Furthermore, increased lung expression of Hsp72 indicative of SPR activation has been reported after onset of experimental septic and hemorrhagic shock (10, 37, 40). Thus, the fact that ALI patients with sepsis and/or shock had the highest levels of Hsp72 in their pulmonary edema fluid suggests that these patients may have undergone an SPR response. This assumption is further supported by the fact that (a) ALI patients had significantly higher values of Hsp72 in the pulmonary edema fluid compared to plasma, strongly suggesting an intrapulmonary source for eHsp72; (b) it is very unlikely that the high levels of Hsp72 measured in patients with preserved AFC were the consequence of cell injury in the distal airspaces because the presence of alveolar epithelial injury is associated with impaired AFC in humans.

The second important result of these studies is that levels of eHsp72 are significantly higher in the pulmonary edema fluid from ALI patients with preserved alveolar epithelial fluid clearance (a measure of alveolar epithelial integrity (47)). We have previously reported that SPR activation preserved a normal alveolar epithelial fluid clearance in an experimental model of ALI, induced by hemorrhage and fluid resuscitation in rats (31). It is also known that eHsp72 exerts important biological functions, for example, by modulating the immune response. In the acute phase of inflammation, eHsp72 is pro-inflammatory and may serve as a danger signal to the immune system (1, 2, 7, 8, 27, 29, 39, 43). Subsequently, eHsp72 may promote a switch from a pro-inflammatory cytokine-secretion profile of T cells to a regulatory cytokine-secretion profile inducing immunotolerance (43). However, whether eHsp72 itself could also preserve the vectorial alveolar epithelial fluid transport in these patients with ALI is still unknown. Thus, to answer this question, we examined the effect of SPR activation or of eHsp72 itself on the IL-1β mediated decrease in transepithelial current and αENaC gene expression in
rat ATII cell monolayers. IL-1β was chosen for these experiments because we have previously shown that it is a strong inhibitor of the vectorial alveolar epithelial fluid transport (34) and one of the most biologically active cytokines in the pulmonary fluid of patients with ALI (32). The results indicate that prior SPR activation prevented the IL-1β mediated decrease in transepithelial current by maintaining the expression of epithelial sodium channel αENaC. In contrast, exposure of rat ATII cell monolayers to recombinant rat and human Hsp72 at a concentration comparable to that measured in the pulmonary edema fluid of our ALI patients with preserved AFC (600 ng/ml) had no effect.

In summary, the results of this study indicate that eHsp72 is present in the plasma and pulmonary edema fluid of patients with ALI and that levels of eHsp72 are significantly higher in the pulmonary edema fluid from ALI patients with preserved alveolar epithelial fluid clearance. Furthermore, SPR activation in vitro in lung endothelial, epithelial and macrophage cells as well as in vivo in mice caused intracellular expression as well as extracellular release of Hsp72. SPR activation, not eHsp72 itself, prevented the decrease in the alveolar epithelial fluid transport induced by exposure to IL-1β. Thus, eHsp72 may serve as marker of SPR activation in the distal airspaces of patients with ALI.
GRANTS, FUNDING

NIH GM 62188 (JFP)

NIH HL 70521 and HL 081332 (LBW)

NIH HL 51854 and HL 51856 (MAM)

DISCLOSURES

None
REFERENCES


Table 1. Sequences of Primers and Probes used.

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Table 2. Clinical characteristics of patients with hydrostatic pulmonary edema (HYDRO) and acute lung injury/acute respiratory distress syndrome (ALI).

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Etiology of HYDRO and ALI

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<th>HYDRO</th>
<th>ALI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute myocardial ischemia</td>
<td>9 (45%)</td>
<td></td>
</tr>
<tr>
<td>Volume overload/diastolic dysfunction</td>
<td>4 (20%)</td>
<td></td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>2 (10%)</td>
<td></td>
</tr>
<tr>
<td>Valvular dysfunction</td>
<td>2 (10%)</td>
<td></td>
</tr>
<tr>
<td>Non-pulmonary sepsis</td>
<td></td>
<td>9 (26%)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td></td>
<td>10 (29%)</td>
</tr>
<tr>
<td>Aspiration of gastric contents</td>
<td></td>
<td>4 (11%)</td>
</tr>
<tr>
<td>Multiple transfusions</td>
<td></td>
<td>4 (11%)</td>
</tr>
<tr>
<td>Trauma</td>
<td></td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (15%)</td>
<td>6 (17%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HYDRO (range)</th>
<th>ALI (range)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema-fluid to plasma protein ratio</td>
<td>0.4 (0.3-0.6)</td>
<td>0.9 (0.7-1.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SAPS II</td>
<td>45 (35-51)</td>
<td>49 (36-64)</td>
<td>0.29</td>
</tr>
<tr>
<td>LIS</td>
<td>2.5 (2.0-3.0)</td>
<td>3.3 (2.7-3.3)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are presented as numbers (percent of patients) or median (inter-quartile range).

LIS, Lung Injury Score; SAPS II, Simplified Acute Physiology Score.
Table 3. Extracellular release of Hsp72 after stress protein response (SPR) activation with heat and 17-AAG.

<table>
<thead>
<tr>
<th></th>
<th>eHsp72 after Heat Shock (fold increase over control)</th>
<th>eHsp72 after 17-AAG (fold increase over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mouse)</td>
<td>7.4 ± 2.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td><strong>Cell culture media</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ATII cells)</td>
<td>9.5 ± 0.1</td>
<td>12.0 ± 0.2</td>
</tr>
</tbody>
</table>

BAL, bronchoalveolar lavage fluid; ATII cells, rat epithelial type II cells; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

SPR was activated in mice with whole body hyperthermia (Heat Shock, 42±1 °C for 25 min) or 17-AAG (50 µg/kg i.p. one dose) and the animals recovered for 30 h or 48 h, respectively. Rat ATII cells were incubated at 43 °C for 45 min or exposed to 1 µM 17-AAG and the cell culture media was collected 24 h later.
FIGURE LEGENDS

Figure 1. Patients with acute lung injury (ALI) have significantly higher levels of extracellular Hsp72 (eHsp72) in their pulmonary edema fluid compared to plasma. Hsp72 was measured in plasma and pulmonary edema fluid immediately after endotracheal intubation and initiation of mechanical ventilation. Patients with hydrostatic pulmonary edema (HYDRO) served as controls. Data are presented as mean ± SEM. *P < 0.05 compared with plasma levels of eHsp72.

Figure 2. High levels of extracellular Hsp72 (eHsp72) in pulmonary edema fluid are associated with a preserved alveolar fluid clearance (AFC) in patients with acute lung injury (ALI). Hsp72 and total protein concentrations were measured in pulmonary edema fluid immediately after endotracheal intubation and initiation of mechanical ventilation. AFC was calculated using sequential measurements of alveolar protein concentration in pulmonary edema fluid. Impaired AFC was defined as AFC < 3%/h (n = 10); preserved AFC as AFC ≥ 3%/h (n = 16). Data are presented as mean ± SEM. *P < 0.05 for AFC ≥ 3%/h compared to AFC < 3%/h.

Figure 3. Activation of the stress protein response (SPR) in mice induces the intracellular expression of Hsp72 in lung tissue and the release of Hsp72 into the alveolar space and plasma. SPR was activated with whole body hyperthermia (heat shock, 42±1 °C for 25 min) and the animals recovered for 30 h. Control animals were kept at their normal body temperature. A. Hsp72 was detected in lung homogenates by Western blot analysis. B. eHsp72 was detected in plasma and BAL fluid by ELISA. *P < 0.05 for SPR activated animals compared to control animals.
Figure 4: Hsp72 is expressed intracellularly and released into cell culture media of alveolar epithelial and lung endothelial cells after activation of the stress protein response (SPR). Cells were plated at $1 \times 10^6$ cells/well. SPR was activated by incubating primary rat alveolar type II (ATII) cells at 43 °C for 45 min and bovine pulmonary artery endothelial (BPAEC) at 43 °C for 60 min, control cells were kept at 37 °C. **A, C:** Intracellular Hsp72 expression was measured by Western blot analysis 12 h after SPR activation. **B, D:** 24 h after SPR activation, cell culture media from control and SPR activated cells (HS-cond media) were collected, centrifuged and the supernatant analyzed. eHsp72 was measured by ELISA. *$P < 0.05$ compared to controls.

Figure 5. Hsp72 is expressed intracellularly, on the cell surface and released into cell culture media by alveolar macrophages (MH-S cells) after activation of the stress protein response (SPR). MH-S cells were plated at $1 \times 10^6$ cells/well. SPR was activated by incubating MH-S cells at 43 °C for 60 min, control cells were kept at 37 °C. **A:** Intracellular Hsp72 expression was measured by Western blot analysis 6 h after SPR activation. **B:** 24 h after SPR activation, cell culture media from control and SPR activated cells (HS-cond media) were collected, centrifuged and the supernatant analyzed. Removal of eHsp72 by depletion with Hsp72 antibodies was performed in some of the cell culture media. eHsp72 was measured by ELISA. **C:** Non-permeabilized cells were immunofluorescently stained for surface Hsp72 after SPR activation. Panel C-1, non-heat treated cells; Panel C-2, 1 h after SPR; Panel C-3, 6 hrs after SPR; Panel C-4, 14 hrs after SPR. Blue = DAPI, red = cy3 stained Hsp72. Data are presented as mean ± SEM. *$P < 0.05$ compared to all other groups.

Figure 6. Prior stress protein response (SPR) activation prevents the IL-1β–mediated decrease in transepithelial current (TEC) and αENaC mRNA expression.
in primary rat alveolar epithelial (ATII) cell monolayers. Primary rat ATII cells were cultured in an air-liquid interface for 4 days. SPR was activated by incubating the cells at 43 °C for 45 min, control cells were kept at 37 °C. After a recovery period of 1 h, cells were treated with IL-1β (10 ng/ml) or its vehicle. A: 6 h after IL-β treatment, transepithelial resistance and potential difference were measured across the cell monolayer and TEC calculated. B: 6 h after IL-β treatment, mRNA for ßENaC was detected by real-time RT-PCR.

Figure 7. Extracellular Hsp72 (eHsp72) has no effect on IL-1β mediated decrease in transepithelial current (TEC) across primary rat alveolar epithelial (ATII) cell monolayers. Primary rat ATII cells were cultured in an air-liquid interface for 4 days. ATII cells were then treated with IL-1β (10 ng/ml) alone or in presence of recombinant human or rat Hsp72 (600 ng/ml). After 6 h, transepithelial resistance and potential difference was measured across the cell monolayers and TEC calculated.
Figure 1

eHsp72 [ng/ml]

Plasma  Edema Fluid

HYDRO  ALI
Figure 2

eHsp72 in pulmonary edema fluid [ng/ml]

AFC < 3%/h

AFC ≥ 3%/h
Figure 3

A

70 kDa -

Control + -
Heat Shock - +

B

eHsp72 [ng/ml]

Control Heat Shock

Plasma BAL *

0 10 20 30
Figure 4

**Alveolar epithelial cells**

A

70 kDa -
Control + -
Heat Shock - +

B

<table>
<thead>
<tr>
<th>eHsp72 [ng/ml]</th>
<th>Control</th>
<th>HS-cond media</th>
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</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>-</td>
<td>+</td>
<td>-</td>
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**Pulmonary artery endothelial cells**

C

70 kDa -
Control + -
Heat Shock - +

D

<table>
<thead>
<tr>
<th>eHsp72 [ng/ml]</th>
<th>Control</th>
<th>HS-cond media</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>-</td>
<td>+</td>
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</tbody>
</table>
Figure 5

Alveolar macrophages

A

70 kDa -
Control + -
Heat Shock - +

B

\[
\begin{array}{c|c|c|c|c|c|c}
& \text{Control media} & \text{HS-cond media} & \text{Depletion with Hsp72 abs} \\
\hline
\text{eHsp72 [ng/ml]} & + & + & - & - & + & + \\
\end{array}
\]

C

C-1

C-2

C-3

C-4
Figure 6

A

Transcellular Current (% of Control)

IL-1β - + - +
Heat Shock - - + +

B

αENaC mRNA (% of Control)

IL-1β - + - +
Heat Shock - - + +
Figure 7

Transepithelial Current (% of Control)

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>human Hsp72</th>
<th>rat Hsp72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
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