TRANSLATIONAL PHYSIOLOGY

Extracellular heat shock protein 72 is a marker of the stress protein response in acute lung injury

Michael T. Ganter,1 Lorraine B. Ware,2 Marybeth Howard,1 Jérémie Roux,1 Brandi Gartland,1 Michael A. Matthay,1 Monika Fleshner,3 and Jean-François Pittet1

1Departments of Anesthesia, Surgery, and Medicine and the Cardiovascular Research Institute, University of California, San Francisco, California; 2Division of Allergy, Pulmonary, and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee; and 3Department of Integrative Physiology and Center for Neuroscience, University of Colorado, Boulder, Colorado

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Ganter, Michael T., Lorraine B. Ware, Marybeth Howard, Jérémie Roux, Brandi Gartland, Michael A. Matthay, Monika Fleshner, and Jean-François Pittet. Extracellular heat shock protein 72 is a marker of the stress protein response in acute lung injury. Am J Physiol Lung Cell Mol Physiol 291: L354–L361, 2006. First published May 5, 2006; doi:10.1152/ajplung.00405.2005.—Previous studies have shown that heat shock protein 72 (Hsp72) is found in the extracellular space (eHsp72) and that eHsp72 has potent immunomodulatory effects. However, whether eHsp72 is present in the distal air spaces is unknown. We found that eHsp72 was present in the extracellular space of patients with ALI/ARDS. Patients with hydrostatic edema served as controls. The first objective was to determine whether Hsp72 is released in the extracellular space 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 whether activation of the stress protein response (SPR) caused the release of Hsp72 into the extracellular space in vivo and in vitro and to determine whether SPR activation and/or eHsp72 would prevent the release of Hsp72 into the extracellular space of patients with ALI.

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 air spaces that release high levels of oxidant species (46). Alveolar epithelial and endothelial injury leads to increased permeability, pulmonary edema, and acute respiratory failure. An important mechanism to prevent alveolar flooding is the maintenance or upregulation of 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 (Hsp) 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), Hsp are also 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) provides the cells and organs increased protection from insults that would otherwise 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 classic 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), and 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 such as sepsis and septic and hemorrhagic shock (10, 37, 40). Finally, SPR activation is known to be associated with the increased intracellular expression of inducible Hsp, 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 air spaces 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 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 (Univ. of California, San Francisco, CA) approved the study.

In Vivo Human Studies

Patients. Patients with ALI or acute respiratory distress syndrome, 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). The etiology of ALI was determined from clinical data. 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. Gram-negative bacteria were cultured from endotracheal tube aspirates for pathogenic bacteria. Aspiration of gastric contents was defined as a witnessed aspiration event. Sepsis was defined using published criteria (11).

Measurement of Hsp72 in lung tissue, BALF, 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), and SDS (1%), sonicated for 30 s, 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 g, 10 min), and supernatants were 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 Hsp72. eHsp72 levels were measured in triplicate from the initial samples collected immediately after intubation by a commercially available ELISA (Stressgen Biotechnologies, 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 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 × [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- to 8-week-old C57BL/6J mice (The Jackson Laboratory) were housed in air-filtered, temperature-controlled units (20 ± 2°C) with food and water ad libitum.

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 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 analog of geldanamycin, 17-AAG (InvivoGen, San Diego, CA; n = 4), 17-AAG (50 µg/kg) or its vehicle (DMSO) was injected once IP 48 h before the mice were euthanized.

Collection of plasma, lung tissue, and bronchoalveolar lavage fluid. After 30 (heat) or 48 h (17-AAG) of 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, and the lungs were lavaged with 30 ml/kg of 0.9% NaCl aspirated four times and quickly frozen at −70°C. Blood and bronchoalveolar lavage fluid (BALF) were centrifuged (3,000 g, 10 min) and stored at −70°C until further analysis.

Measurement of Hsp72 in lung tissue, BALF, 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), and SDS (1%), sonicated for 30 s, boiled for 10 min, and centrifuged at 14,000 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; 1:1,000) and a horseradish peroxidase-conjugated goat anti-mouse antibody (ICN, Costa Mesa, CA; 1:2,000). Proteins were visualized using chemiluminescence. eHsp72 in plasma and BALF was detected by ELISA (Stressgen Biotechnologies) as described above.

Measurement of 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 reinflated the solution three times, applied continuous positive airway pressure (8 cmH2O, 100% FIO2), and kept the animals at 37°C body core temperature. The instillate, an initial sample (after aspiration and reinflation), 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 air spaces 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 DMEM-H21 medium containing 10% FBS and 1% penicillin-streptomycin-amphotericin (Invitrogen Life Technologies). Bovine pulmonary arterial endothelial cells [BPAEC; American Type Culture Collection (ATCC) CCL-209] were cultured in DMEM/H21 medium with 20% FBS and 1% penicillin-streptomycin-amphotericin (Invitrogen Life Technologies).
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 intracellular and eHsp72, cells were plated in standard tissue-culture-treated polystyrene wells (Corning Life Sciences) 24 h before the experiment. To measure transcapillary current and α-epithelial Na⁺ channel (αENaC) mRNA, ATII cells were plated on polycarbonate Transwell dishes (0.4-µm pore size; Corning Life Sciences). Twenty-four-hour later, nonadherent epithelial cells were removed by being washed 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 transcapillary electrical resistance >1,500 Ω/cm² were used for experimentation.

**Activation of SPR in vitro.** Cell culture media were replaced with fresh media immediately before the experimental procedure. The activation of SPR was performed by incubating the cells at 43°C for 45 (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 were measured using the Alamar blue assay** (30).

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

**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 (1,000 g for 10 min). eHsp72 levels were measured in the supernatant by ELISA as described earlier. 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 and SPA-812; Stressgen Biotechnologies). Antibody-Hsp72 complexes were captured via the addition of protein G- and A-Sepharose beads (Roche Diagnostics) for 2 h and pelleted by centrifugation at 300 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 being blocked, the cells were washed three times and incubated with an antibody to Hsp72 (SPA-810, Stressgen Biotechnologies; 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 4,6-diamidino-2-phenylindole was added during the last wash. The cells were fixed with 3% paraformaldehyde and mounted.

**Measurement of transepithelial current.** The transepithelial resistance (IR, kΩ/cm²) and potential difference (PD; mV, apical side as reference) were measured using the Millicell-ERS Voltohmeter (Millipore). Transcapillary current (Iw; µA/cm²) was calculated from the relationship Iw = PD/R (Ohm’s law).

Polarized rat ATII monolayers were pretreated with dexamethasone for 12 h (100 nM) as previously described (34) and exposed to IL-1β for 6 h (10 ng/ml; R&D Systems, Minneapolis, MN) or its vehicle, and the bioelectric properties of ATII cell monolayers were evaluated. To determine whether prior SPR activation is protective in maintaining transcapillary ion transport, SPR was induced with heat followed by a recovery period of 1 h before 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 the presence or absence of low-endotoxin recombinant human or recombinant rat Hsp72 (600 ng/ml; ESP-555 or SPP-758, Stressgen Biotechnologies).

**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) at the 3’ end. Total RNA was extracted from rat ATII cells using the RNeasy mini kit (Qiagen). One microgram of total RNA was reverse transcribed using 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, Foster City, CA). Briefly, RT-PCR was carried out in a 25-µl reaction mixture containing 1X TaqMan Universal PCR Master Mix (PE-Biosystems), 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 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. Differences were considered significant at P < 0.05.

### Table 1. Sequences of primers and probes used

<table>
<thead>
<tr>
<th>Rat GAPDH</th>
<th>GenBank accession no.: AB017801</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong></td>
<td>5’-CTGCCAAAGTATGATGACATCAAAGAA-3’</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
<td>5’-ACCCAGAGGCCGCTTTAATGG-3’</td>
</tr>
<tr>
<td><strong>Probe</strong></td>
<td>5’-GGGCCCGTCGCTTTCAAGCA-3’</td>
</tr>
</tbody>
</table>

**Table 2. Clinical characteristics of patients with HYDRO and ALI/acute respiratory distress syndrome**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HYDRO (n = 20)</th>
<th>ALI (n = 35)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>9 (45%)</td>
<td>22 (63%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Caucasian race</td>
<td>12 (58%)</td>
<td>22 (63%)</td>
<td>0.63</td>
</tr>
<tr>
<td>Present smoker</td>
<td>4 (19%)</td>
<td>7 (21%)</td>
<td>0.49</td>
</tr>
<tr>
<td>Age, yr</td>
<td>57 (37–81)</td>
<td>39 (27–50)</td>
<td>0.01</td>
</tr>
<tr>
<td>Etiology of HYDRO and ALI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute myocardial ischemia</td>
<td>9 (45%)</td>
<td>4 (11%)</td>
<td></td>
</tr>
<tr>
<td>Volume overload/diastolic dysfunction</td>
<td>4 (20%)</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>2 (10%)</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td>Valvular dysfunction</td>
<td>2 (10%)</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td>Nonpulmonary sepsis</td>
<td>9 (26%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>10 (29%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspiration of gastric contents</td>
<td>4 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple transfusions</td>
<td>4 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma</td>
<td>4 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3 (15%)</td>
<td>6 (17%)</td>
<td></td>
</tr>
<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 (percentages of patients) or medians (interquartile ranges; n = nos. of patients). LIS, lung injury score; SAPS II, simplified acute physiology score; HYDRO, hydrostatic pulmonary edema; ALI, acute lung injury.
comparisons. Categorical data were compared by using \( \chi^2 \)-analysis. Nonparametric data were analyzed by using the Mann-Whitney \( U \)-test. Statistical significance was defined as \( P < 0.05 \). Parametric data are presented as means \( \pm \) SE and nonparametric data as medians and interquartile ranges.

**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 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 (Fig. 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 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 (Fig. 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 with patients with HYDRO (Hsp72 in pulmonary edema fluid: 160 \( \pm \) 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 \( \pm \) 153 ng/ml, \( P = 0.004 \), and 430 \( \pm \) 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 \( \pm \) 90 and 150 \( \pm \) 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 levels in their pulmonary edema fluid than patients with impaired AFC (Fig. 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 with patients with HYDRO (Hsp72 in pulmonary edema fluid: 160 \( \pm \) 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 \( \pm \) 153 ng/ml, \( P = 0.004 \), and 430 \( \pm \) 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 \( \pm \) 90 and 150 \( \pm \) 72 ng/ml, respectively).

**Table 3. Extracellular release of Hsp72 after stress protein response activation with heat and 17-AAG**

<table>
<thead>
<tr>
<th>eHsp72 After Heat Shock</th>
<th>eHsp72 After 17-AAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage fluid (mouse)</td>
<td>7.4 ( \pm ) 2.2</td>
</tr>
<tr>
<td>Cell culture media (ATII cells)</td>
<td>9.5 ( \pm ) 0.1</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE (in fold increase over controls). Stress protein response was activated in mice with whole body hyperthermia (heat shock, 42 \( \pm \) 1°C for 25 min) or 17-allylamino-17-demethoxy-geldanamycin (17-AAG; 50 \( \mu \)g/kg ip, 1 dose), and the animals recovered for 30 or 48 h, respectively. Rat alveolar epithelial type II (ATII) cells were incubated at 43°C for 45 min or exposed to 1 \( \mu \)M 17-AAG, and the cell culture media was collected 24 h later. eHsp72, extracellular heat shock protein 72.
expression in lung tissue (Fig. 3A). Furthermore, levels of Hsp72 increased 7.4-fold in BALF after SPR activation, and Hsp72 was only detected in plasma of mice that underwent SPR activation (Fig. 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 air spaces of the lung (Hsp72 levels increased by 38% in BALF of 17-AAG-treated animals compared with controls, Table 3).

SPR activation does not alter baseline AFC in vivo. To determine whether 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 BALF in mice that underwent SPR activation compared with controls (Fig. 3B), suggesting that Hsp72 could be released locally within the distal air spaces 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 air spaces (alveolar epithelial and pulmonary endothelial cells and alveolar macrophages). The results indicate that all three cell types expressed Hsp72 intracellularly (Figs. 4, A and C, and 5A) after SPR activation with heat. Furthermore, Hsp72 was also released into the cell culture media after SPR activation (heat shock-conditioned media, Figs. 4, B and D, and 5B). In addition, significant levels of Hsp72 were present on the cell surface after SPR activation.

Fig. 4. Hsp72 is expressed intracellularly and released into cell culture media of alveolar epithelial (A and B) and lung endothelial cells (C and D) after activation of the SPR. Cells were plated at 1 × 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 cells at 43°C for 60 min; control cells were kept at 37°C. A and C: intracellular Hsp72 expression was measured by Western blot analysis 12 h after SPR activation. B and D: 24 h after SPR activation, cell culture media from control and SPR-activated cells (heat shock-conditioned media (HS-cond media)) were collected and centrifuged and the supernatant analyzed. eHsp72 was measured by ELISA. *P < 0.05 compared with controls.

Fig. 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 SPR. MH-S cells were plated at 1 × 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 and centrifuged, and the supernatant was analyzed. Removal of eHsp72 by depletion with Hsp72 antibodies (abs) was performed in some of the cell culture media. eHsp72 was measured by ELISA. C: nonpermeabilized cells were immunofluorescently stained for surface Hsp72 after SPR activation. C-1: non-heat-treated cells. C-2: 1 h after SPR. C-3: 6 h after SPR. C-4: 14 h after SPR. Blue = 4,6-diamidino-2-phenylindole; red = Cy3-stained Hsp72. Data are presented as means ± SE. *P < 0.05 compared with all other groups.
surface of MH-S cells 1 and 6 h after SPR activation (Fig. 5C, C-2 and C-3) compared with control cells (Fig. 5C, C-1). By 14 h post-SPR activation, surface Hsp72 expression was decreased (Fig. 5C, 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 with 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 decrease in transepithelial ion transport by IL-1β across rat ATII cell monolayers. Preserved AFC 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 (Fig. 6A) by maintaining the expression of αENaC (Fig. 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 human and mouse 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 or inhibit the IL-1β-mediated decrease in transepithelial current across the ATII cell monolayers (Fig. 7).

DISCUSSION

The major findings of this study can be summarized as follows: 1) 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 AFC; 2) 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; and 3) 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 a marker of SPR activation in the distal air spaces of patients with ALI and that SPR activation may protect the alveolar epithelium against oxidative stress associated with ALI.

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 the 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 cell types present in the distal air spaces of the lung, alveolar macrophages, lung epithelial, and endothelial cells, are able to release Hsp72 extracellularly and that SPR activation is associated with a severalfold 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 air spaces 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 nonspecific 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 BALF than the one observed with heat-induced SPR activation. In the present
studies, we did not perform a dose response with 17-AAG 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 ip) 48 h before euthanizing the mice. This could explain that this dose of 17-AAG caused a smaller SPR activation than the one observed with heat. Finally, the fact that the mice that underwent SPR activation with heat had normal AFC 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? Hsp are typically induced in cells exposed to sublethal heat shock. However, since the first report about the detection of Hsp after a temperature shock in 1962 by Ritossa (33), there is considerable evidence that Hsp can also be induced by a variety of other stressful stimuli, including corticosteroids (41, 42), catecholamines (19), and oxidative stress (24, 45) and 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 ALI patients with sepsis and/or shock had the highest levels of Hsp72 in their pulmonary edema fluid (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 ALI patients with sepsis and/or shock had the highest levels of Hsp72 in their pulmonary edema fluid (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. 

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 AFC [a measure of alveolar epithelial integrity (47)]. We have previously reported that SPR activation preserved a normal AFC in an experimental model of ALI, induced by hemorrhage and fluid resuscitation in rats (31). It is also shown that eHsp72 exerts important biological functions, for example, by modulating the immune response. In the acute phase of inflammation, eHsp72 is proinflammatory 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 proinflammatory 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 trans-epithelial 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 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 AFC. 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 a marker of SPR activation in the distal air spaces of patients with ALI.

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REFERENCES

In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. 


