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Intracellular delivery of HSP70 using HIV-1 Tat protein transduction domain☆

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Abstract

Heat shock protein 70 (HSP70) is an intracellular stress protein that confers cytoprotection to a variety of cellular stressors. Several lines of evidence have suggested that augmentation of the heat shock response by increasing the expression of HSP70 represents a potential therapeutic strategy for the treatment of critically ill patients. The Tat protein of human immunodeficiency virus 1 (HIV-1) has been used previously to deliver functional cargo proteins intracellularly when added exogenously to cultured cells. We generated a Tat-HSP70 fusion protein using recombinant methods and treated HSF −/− cells with either Tat-HSP70 or recombinant HSP70 prior to exposure to hyperoxia or lethal heat shock. We showed that biologically active, exogenous HSP70 can be delivered into cells using the HIV-1 Tat protein, and that the Tat-mediated delivery of HSP70 confers cytoprotection against thermal stress and hyperoxia and may represent a novel approach to augmenting intracellular HSP70 levels.
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Keywords: Heat shock protein (hsp)-70; Tat; Heat shock response; Protein transduction; Hyperoxia; Heat shock factor

Virtually all cells possess certain endogenous cytoprotective molecules and mechanisms to maintain homeostasis and ensure survival in response to various forms of stress. The heat shock response is an example of one such cytoprotective defense mechanism that is characterized by the rapid expression of a specific group of proteins called heat shock proteins. While classically described as a response to thermal stress, a variety of non-thermal stressors, including sodium arsenite, heavy metals, and oxidants, have been shown to induce the heat shock response [1,2]. The increased expression of heat shock proteins confers protection against a broad array of otherwise lethal cellular injuries [3–7]. For example, in vitro experiments have demonstrated that the increased expression of heat shock protein-70 (HSP70) protects cultured human respiratory epithelial cells against hyperoxia-mediated cell injury and death [7]. In vivo studies have shown that induction of heat shock proteins confers protection in various models of organ injury, including sepsis, acute lung injury, and ischemia-reperfusion injury [8–15]. The ever-increasing amount of experimental data would therefore suggest that augmentation of heat shock proteins, either through pharmacological methods or gene therapy strategies, is a potential therapeutic strategy for many forms of cellular injury relevant to critical care medicine.

While an attractive strategy, the potential for the intracellular delivery of mature proteins for therapeutic purposes has been heretofore limited by the impermeable nature of the cell membrane. However, several small regions of proteins called protein transduction domains (PTDs) may hold promise for use in the delivery of exogenous proteins into living cells, including the HIV-1 Tat protein [16–21], the *Antennapedia* protein [22,23], and the VP22 protein from the herpes simplex virus [24,25]. The mechanisms by which these PTDs

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cross biologic membranes are not well understood, though it appears that transduction mediated by Tat does not occur through classical receptor-, transporter-, or endosome-mediated mechanisms [23].

In the current study, we cloned a human HSP70 cDNA gene and fused it with a gene fragment encoding the 11-amino acid transduction domain of the HIV-1 Tat protein. We then analyzed the efficiency of transduction of the resulting Tat-HSP70 fusion protein into a mouse cell line. Finally, we determined the ability of Tat-mediated transduction of the HSP70 protein to confer cytoprotection against a lethal heat stress or hyperoxia. The experimental data we report here suggest that the Tat-mediated delivery of HSP70 may represent a novel strategy of augmenting intracellular HSP70 levels.

Methods

Molecular cloning of human HSP70 and construction of expression vectors. The human HSP70 cDNA was generated by PCR using the pUCHsp70A vector (Stressgen) as a template. The sense primer was 5′-CAA AAA GGA TCC ATG GCC AAA GCC GCG GCG ATC-3′ and the antisense primer was 5′-ACA ACG AAT TCC TAA TCT ACC TCC TCA ATG G-3′. The PCR product was digested with *Eco*RI and *Kpn*I and subcloned into the *Eco*RI and *Kpn*I sites of the pTAT-HA bacterial expression vector (a gift from Dr. Stephen F. Dowdy, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO). The pTAT-HA bacterial expression vector contains an N′ terminal 6-histidine leader sequence, followed by the 11-amino acid transduction domain of the Tat protein, and a polylinker region [26]. The correct sequence and reading frame of the pTAT-HA-HSP70 vector were confirmed by DNA sequencing using the forward primer 5′-GTG TGT AAC CCC ATC ATC A-3′ and the reverse primer 5′-TTG GCG ATG ATC TCC ACC TTG CCG-3′.

Expression and purification of Tat-HSP70 fusion protein. BL21 (DE3)LysS *Escherichia coli* (Invitrogen) transformed with plasmids encoding the Tat-HSP70 fusion protein were grown for 6 h at 37 °C in LB broth supplemented with 100 mg/ml ampicillin. These cultures were diluted 50-fold with fresh LB media and cultured at 37 °C overnight while shaking at 250 rpm. Protein expression was induced by the addition of 1 M IPTG to a final concentration of 1.0 mM for 5 h while shaking at 37 °C. The Tat-HSP70 fusion proteins were then isolated using a urea-denaturing protein purification protocol [26]. In order to prepare the denatured fusion protein, the induced cells were harvested and lysed by sonication in binding buffer (8 M urea, 100 mM NaCl, and 20 mM Hepes, pH 8.0) plus 5 mM imidazole. Cell debris was removed by centrifugation and the cell extracts were then loaded onto a Ni^{2+}-IDA column. The column was first prepared by washing sequentially with the binding buffer, binding buffer plus 10 mM imidazole, and binding buffer plus 100 mM imidazole. The proteins were then eluted by the stepwise addition of binding buffer containing increasing concentrations of imidazole (100–1000 mM). In order to treat tissue culture cells with the Tat-HSP70 fusion protein, it was necessary to first remove the urea denaturant. The protein fractions were diluted 1:1 vol/vol with binding buffer without NaCl (8 M urea, 20 mM Hepes, and pH 8.0) and loaded onto a Mono Q ionic exchange column which was pre-equilibrated with low-salt buffer (50 mM NaCl, 20 mM Hepes, and pH 8.0) plus 8 M urea. The column was washed with the low-salt buffer and the proteins were eluted with a high-salt buffer (1 M NaCl, 20 mM Hepes, and pH 8.0). The proteins were next loaded onto a PD10 Sephadex size exclusion column in order to rapidly desalt the Tat-HSP70 fusion protein product. The protein concentrations in each fraction were quantified using the Bradford assay (BioRad, Hercules, CA), using bovine serum albumin (BSA) as the standard. The purity of the Tat-HSP70 fusion protein was assessed using a Coomassie stain following separation by SDS–PAGE. The purified Tat-HSP70 fusion proteins were flash frozen in a dry ice ethanol bath and stored at −80 °C.

Cell culture. In all of the experiments, we used an embryonic fibroblast cell line derived from heat shock factor-1 (HSF-1) null mutant (HSF −/−) mice (a gift from Dr. Ivor Benjamin, University of Texas Southwestern Medical Center, Dallas, TX), which therefore have a highly limited ability to produce endogenous HSP70 in response to stress [27–29]. Cells were maintained in a room air/5% CO_2 incubator at 37 °C using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 55 μM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acid solution, 0.29 mg/ml L-glutamine, and 10 ml per liter of antibiotic–antimycotic solution containing penicillin G 10,000 U/ml, streptomycin sulfate 10,000 μg/ml, and amphotericin B 25 μg/ml (Gibco-BRL, Rockville, MD).

Transduction of fusion proteins into mammalian cells. HSF −/− cells were treated with increasing concentrations of either the Tat-HSP70 fusion protein or a recombinant, wild-type HSP70 protein for periods ranging from 1 to 4 h. Cells were harvested for the preparation of cell extracts to perform Western blot analysis.

Western blot analysis. Treated cells were washed once in PBS and lysed in ice-cold lysis buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, and PMSF (100 μg/ml). Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). Whole cell lysates containing 50 μg protein were boiled in equal volumes of loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% of 2-β-mercaptoethanol). Proteins were separated electrophoretically on 8–16% Tris–glycine gradient gels (Novex, San Diego, CA) and subsequently transferred to nitrocellulose membranes (Novex) using the Novex Xcell Mini-Gel system. For immunoblotting, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) for 1 h. Primary antibody against the HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:2500 dilutions for 1 h. After washing twice with TBS containing 0.05% Tween 20 (TTBS), secondary antibody (peroxidase-conjugated goat anti-rabbit immunoglobulin G; Stressgen, Victoria, British Columbia) was applied at 1:2500 dilution for 1 h. Blots were washed in TTBS twice for 10 min, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham, Buckinghamshire, England), and exposed to photographic film.

Immunocytochemistry. For indirect immunofluorescence, HSF −/− cells were grown on glass coverslips and treated with 50 nM tat-HSP70 or recombinant, wild-type HSP70. Following incubation for 2 h at 37 °C, the cells were washed twice with PBS and then fixed with 2% paraformaldehyde in 0.5 mL PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in 0.5% triton/PBS. The cells were then blocked in 10% goat serum/PBS at room temperature and then incubated for 1 h at room temperature with rabbit polyclonal HSP70 antibody as the primary antibody. The cells were rewashed four times for 10 min with PBS before incubation with the secondary antibody (FITC-conjugated goat anti-rabbit antibody) for 1 h at room temperature. Transduced cells were analyzed by immunofluorescence microscopy.

Experimental conditions. HSF −/− cells were treated with 300 nM Tat-HSP70 or recombinant HSP70 (wt-HSP70) for 2 h prior to a lethal heat stress at 43 °C for 2 h. Cells were allowed to recover at 37 °C overnight. In separate experiments, HSF −/− cells were treated with 300 nM Tat-HSP70 or wt-HSP70 for 2 h prior to exposure to hyperoxia (95% oxygen, 5% carbon dioxide) for 24 h. Cells were placed in sealed modular chambers (Billups-Rothenberg, Del Mar, CA) and flushed with 95% O_2 and 5% CO_2 for 30 min, following which the entry and exit ports were clamped and the chambers were placed in a 37 °C incubator for the duration of the hyperoxia treatment.

Cell viability. Cell viability after 24 h exposure to hyperoxia was determined by measuring the remaining mass of attached cells as previously described [6]. Briefly, cells were washed once in PBS to remove dead or detached cells. The remaining attached cells were lysed in 0.5 M NaOH and the DNA concentration was measured by spectrophotometry. Percent viability was calculated as the absorbance of treated cells/absorbance of control cells × 100.

Statistical analysis. Differences in cell viability between the experimental groups were evaluated by one-way analysis of variance and Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

Results

Generation of a pure Tat-HSP70 fusion protein

Fig. 1 shows a Coomassie stain of an SDS–PAGE blot, demonstrating that our Tat-HSP70 fusion protein migrates with a pattern similar to native, recombinant HSP70, and that there is minimal to no contamination by other proteins.

Transduction of Tat-HSP70 fusion protein

Transduction of the Tat-HSP70 fusion protein was verified using both Western blot analysis and immunocytochemistry. In the first group of experiments, HSF −/− cells were treated with either the Tat-HSP70 fusion protein or a recombinant, wild-type HSP70 protein for periods ranging from 1 to 4 h. As shown in Fig. 2, Western blot analysis demonstrated the presence of intracellular HSP70 in HSF −/− cells that were treated with the Tat-HSP70 fusion protein. HSF −/− cells have a highly impaired ability to induce expression of HSP70 when subjected to stress [27–29]. Therefore, we were assured that the HSP70 that was detected was due, in fact, to intracellular delivery of our Tat-HSP70 fusion protein and not endogenous expression of HSP70. In another set of experiments, we demonstrated that

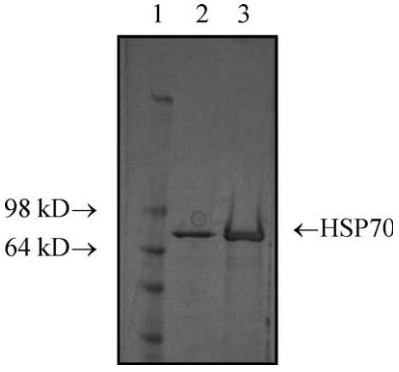

Fig. 1. Coomassie stain of a SDS–PAGE demonstrating the purity of the isolated Tat-HSP70 fusion protein. The Tat-HSP70 fusion protein migrates with a band at approximately 70 kDa, similar to the recombinant, wild-type HSP70 protein.

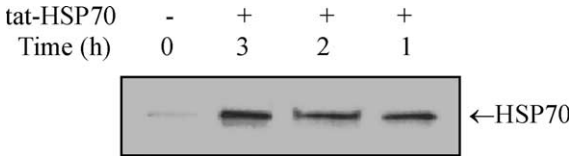

Fig. 2. Western blot analysis demonstrating transduction of the Tat-HSP70 fusion protein. The Tat-HSP70 fusion protein is detected intracellularly as early as 1 h and as late as 4 h following exogenous addition to culture media.

treatment with recombinant wild-type HSP70 did not result in the intracellular accumulation of HSP70 as detected by Western blot analysis (data not shown).

In the second set of experiments, HSF −/− cells were treated with either Tat-HSP70 or recombinant, wild-type HSP70 for 2 h prior to indirect immunocytochemistry. Immunocytochemistry demonstrated significant accumulation of intracellular HSP70 in nearly 100% of cells and no intracellular accumulation of recombinant HSP70. As shown in Fig. 3A, there was a basal level of both intranuclear and intracytoplasmic HSP70 immunofluorescence in the untreated, control cells. Following treatment with the recombinant HSP70 protein, there was no significant change in the immunofluorescence (Fig. 3B). Cells treated with the Tat-HSP70 fusion protein, on the other hand, showed a significant increase in immunofluorescence (Fig. 3C), again verifying

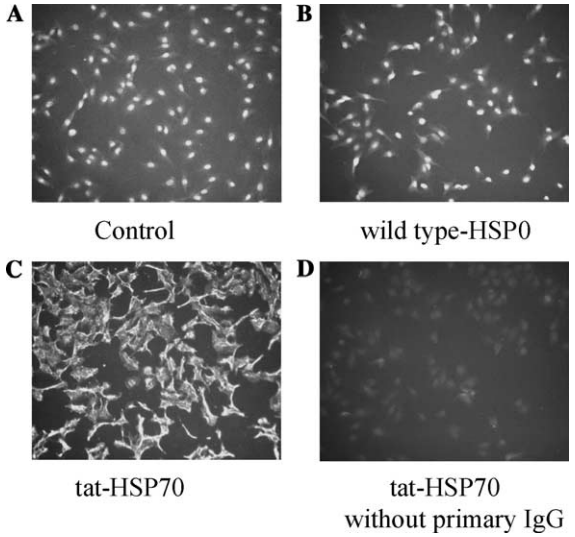

Fig. 3. Immunocytochemistry photomicrograph demonstrating high efficiency transduction of Tat-HSP70 fusion protein in HSF −/− cells. Control cells which were treated with vehicle alone demonstrate a low-level of HSP70 (A). Cells treated with 50 nM recombinant "wild-type" HSP70 did not show a significant change in HSP70 staining compared to controls (B). In contrast, cells treated with 50 nM Tat-HSP70 demonstrated a significant increase in both intranuclear and intracytoplasmic staining of HSP70 (C). Panel D shows a control, in which cells were treated with Tat-HSP70 and immunocytochemistry was performed in the absence of the primary antibody.

transduction of the Tat-HSP70 fusion protein. As a further control, cells were treated with Tat-HSP70 and were stained without the addition of the primary antibody, demonstrating that the increased immunofluorescence following treatment with Tat-HSP70 was indeed due to transduction and not artifactual (Fig. 3D). Collectively, these data demonstrate that we can efficiently deliver high levels of HSP70 into HSF −/− cells using Tat-HSP70.

Transduction of Tat-HSP70 fusion protein confers cytoprotection in HSF −/− cells

Having demonstrated intracellular delivery of mature HSP70, using Tat-HSP70, we next determined if the transduced protein is functional. To this end we determined if treatment with Tat-HSP70 confers thermotolerance, one of the better known functional consequences of increased intracellular levels of HSP70. HSF −/− cells were treated with 300 nM Tat-HSP70 or recombinant HSP70 (wt-HSP70) for 2 h prior to a lethal heat stress at 43 °C for 2 h. Cells were then allowed to recover at 37 °C overnight. Cell viability was determined by measuring the remaining mass of attached cells as previously described [6]. As shown in Fig. 4, treatment with Tat-HSP70 increased cell viability after lethal hyperthermia by $67 \pm 14\%$, as compared to treatment with wild type HSP70 which increased cell viability after lethal hyperthermia by $5 \pm 5\%$. To further test the functionality of Tat-HSP70 we also determined if treatment with Tat-HSP70 increases resistance to hyperoxia, since we previously demonstrated that increased intracellular levels of HSP70 protected respiratory epithelium against hyperoxia [7]. HSF −/− cells were treated with 300 nM Tat-HSP70 or wt-HSP70 for 2 h prior to exposure to hyperoxia (95% oxygen, 5% carbon dioxide) for 24 h. As shown in Fig. 5, treatment with Tat-HSP70 increased cell viability in hyperoxia by $24 \pm 14\%$, as compared to

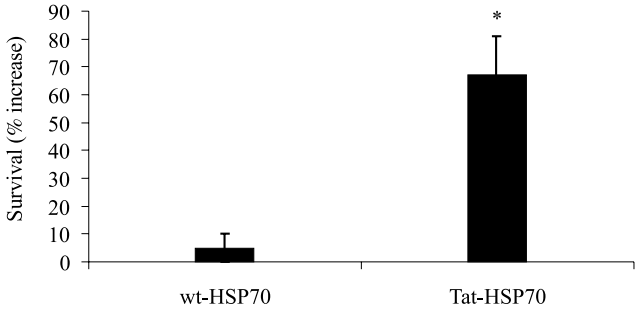

Fig. 4. HSF −/− cells were treated with either 300 nM wt-HSP70 or Tat-HSP70 for 2 h prior to a subsequent 2-h exposure to lethal heat stress at 43 °C. Treatment with Tat-HSP70 increased cell viability after lethal hyperthermia by $67 \pm 14\%$, as compared to treatment with wild type HSP70 which increased cell viability after lethal hyperthermia by $5 \pm 5\%$ ($p < 0.05$, Tat-HSP70 vs wild-type HSP70).

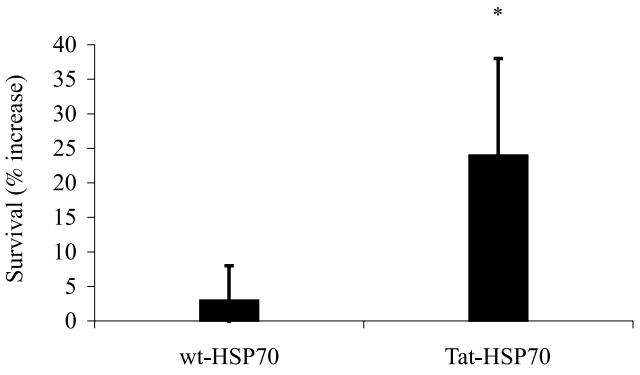

Fig. 5. HSF −/− cells were treated with either 300 nM wt-HSP70 or Tat-HSP70 for 2 h prior to a subsequent 24-h exposure to hyperoxia (95% N_2/5% CO_2). Treatment with Tat-HSP70 increased cell viability in hyperoxia by $24 \pm 14\%$, as compared to treatment with wild type HSP70 which increased viability by $3 \pm 5\%$ ($p < 0.05$, Tat-HSP70 vs wild-type HSP70).

treatment with wild type HSP70 which increased viability by $3 \pm 5\%$. Collectively, these data demonstrate that treatment with Tat-HSP70 leads to functional increases of intracellular HSP70 inasmuch as treated cells have increased resistance to lethal hyperthermia (thermotolerance) and hyperoxia.

Discussion

We have shown that biologically active, exogenous HSP70 can be delivered into cells using the 11-amino acid transduction domain of the HIV-1 Tat protein. The Tat-mediated delivery of HSP70 confers cytoprotection against both thermal stress and hyperoxia in HSF −/− cells. As the HSF −/− cell line has a profoundly reduced ability to mount an endogenous stress response [27–29] we are assured that the effects demonstrated are in fact due to transduction of the Tat-HSP70 cargo protein and not to an endogenous stress response. Several lines of evidence suggest that augmentation of the heat shock response by increasing the expression of HSP70 represents a potential therapeutic strategy for the treatment of critically ill patients. Further studies are necessary, however, before this novel strategy will assume a role in the clinical armamentarium.

Previous to this report, there have been three different methods of augmenting intracellular levels of HSP70, both in vitro and in vivo, described in the literature. A commonly employed strategy is to apply a sublethal heat stress, thereby augmenting intracellular levels of HSP70 through the classic heat shock response. While this technique is easy to perform in vitro and is theoretically possible in vivo, it may not represent the most optimal strategy for use in the clinical setting. Another potential strategy is to augment intracellular HSP70 levels through pharmacological means. There are several compounds

that are known to readily induce HSP70, though the toxicity of many of these agents may limit their use in the clinical setting, and the search continues for the ''perfect'' pharmacologic agent to induce HSP70 [1,2]. Finally, several authors have augmented intracellular levels of HSP70 using gene therapy [30–34].

Herein we report for the first time the direct intracellular delivery of biologically active, mature HSP70 protein. While the direct delivery of proteins for therapeutic purposes has been an attractive strategy, such a method has been heretofore limited by the impermeable nature of the cell membrane. We have taken advantage of one member of a class of unique proteins containing short segments called protein transduction domains (PTDs) in order to augment intracellular levels of HSP70. These proteins, which include the HIV-1 Tat protein [16–21], the *Antennapedia* protein [22,23], and the VP22 protein from the herpes simplex virus [24,25], may hold promise for use in the delivery of exogenous proteins into living cells. The experimental data we report here suggest that the Tat-mediated delivery of HSP70 may represent a novel strategy of augmenting intracellular HSP70 levels.

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