Overexpression of inducible heat shock protein 70 and its mutants in astrocytes is associated with maintenance of mitochondrial physiology during glucose deprivation stress

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Abstract Wild-type inducible Hsp70 (WT) and 2 folding deficient mutants protect the brain against focal cerebral ischemia in vivo and brain cells from oxygen–glucose deprivation (OGD) in vitro, but the protective mechanisms remain unclear. Mitochondria are central to both normal physiological function and the regulation of cell death. We tested the effect of overexpressing Hsp70 and 2 mutants, Hsp70-K71E, an adenosine triphosphatase (ATPase)-deficient point mutant, and Hsp70-381–640, a deletion mutant lacking the ATPase domain on mitochondrial physiology under glucose deprivation (GD) stress in primary cultured astrocytes. Mitochondrial membrane potential was assessed using a potentiometric fluorescent dye tetramethylrhodamine ethyl ester (TMRE). By 5 hours of GD, the mitochondria in the LXSN control transfected astrocytes had markedly reduced membrane potential. However, in the Hsp70-WT, -K71E, and -381–640 groups, there was no apparent change in TMRE signal during 5 hours of GD. Oxygen consumption was measured to assess oxidative respiration. Overexpression of Hsp70-K71E and -381–640 prevented the decrease in state III respiration observed at 5 hours, and all 3 prevented the increase in state IV respiration found in LXSN controls after 5 hours of GD. Reactive oxygen species (ROS) production was assessed with hydroethidine. Hsp70 and its mutants all significantly reduced the increases in ROS accumulation during 5 hours of GD. The results demonstrate that the protective effect of the carboxyl-terminal half of Hsp70 and of the full-length molecule is associated with better maintained mitochondrial membrane potential, better maintained state IV respiration, and reduced ROS generation during GD.

INTRODUCTION

Astrocytes are dynamically involved in synaptic transmission, metabolic and ionic homeostasis, inflammatory response, antioxidant defense, trophic support of neurons, as well as the establishment and maintenance of the blood–brain barrier (Kettenmann and Ransom 2005). Astrocytes both respond to signals from and signal actively to neurons, endothelium, and microglia (for reviews, see Ransom et al 2003; Volterra and Meldolesi 2005). Astro-

cytes also play a central role in maintaining neuronal viability under stress conditions such as ischemia. Dysfunction or loss of astrocytes can lead to neuronal death or dysfunction (O'Malley et al 1992; Juurlink and Hertz 1993; Takeshima et al 1994; Chen and Swanson 2003). Therefore, study of the astrocytic response to stress is essential to understanding many types of brain pathology.

The neuroprotective effects of overproduction of heat shock protein 70 (Hsp70), the stress-inducible member of the 70-kDa family that is induced in the brain in response to several types of stress including cerebral ischemia, have been clearly demonstrated after ischemic injury both in vitro and in vivo (Giffard and Yenari 2004). Hsp70

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consists of an amino-terminal adenosine triphosphatase (ATPase) domain and a carboxyl-terminal domain that contains the substrate binding domain. Recent results from this laboratory demonstrate that the carboxyl-terminal half of Hsp70 is sufficient for protection from focal cerebral ischemia in rats and oxygen-glucose deprivation (OGD) in astrocytes, providing similar protection to that seen with wild-type Hsp70 (Hsp70-WT) (Sun et al 2005). The mechanisms underpinning this protection, however, are not fully understood. Furthermore, it is not yet established whether the mutants protect from the same range of stress as the wild-type protein.

Mitochondria are central to both apoptotic and necrotic brain cell death. Several prominent mitochondrial alterations occur during stress and can contribute to cell death. These include changes in mitochondrial respiratory function, production of reactive oxygen species (ROS), changes in mitochondrial membrane potential, and release of regulatory and signaling molecules from the intermembrane space. In liver mitochondria, heat shock suppresses the permeability transition (He and Lemasters 2003). Several reports (Tsuchiya et al 2003; Steel et al 2004; Stankiewicz et al 2005) have demonstrated reduced early mitochondrial cytochrome *c* release with Hsp70 overexpression, including after permanent focal ischemia (Tsuchiya et al 2003). Despite new information on the neuroprotective effects of Hsp70 proteins in the last few years, the extent to which cells protected by Hsp70 have improved or better sustained mitochondrial respiratory function has not been well studied. To further investigate the mechanism involved in the protective effect of Hsp70 and to determine whether folding activity is required and whether the carboxyl-terminal domain has similar effects to the full-length molecule, we examined the effect of overexpression of Hsp70-WT and 2 mutants on cell respiration, mitochondrial membrane potential, and ROS accumulation early after glucose deprivation (GD) in primary cultured mouse astrocytes.

MATERIALS AND METHODS

All chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise noted.

Astrocyte cultures

Primary astrocyte cultures were prepared from postnatal (days 1–3) Swiss Webster mice (Simonsen, Gilroy, CA, USA) in accordance with a protocol approved by the Stanford University animal care and use committee, in keeping with the National Institutes of Health guide. In brief, neocortices were dissected free of meninges, treated with 0.09% trypsin for 20 minutes, mechanically dissociated and plated as a single-cell suspension onto 22-mm

poly-D-lysine-coated glass coverslips at a density of 2 hemispheres/10 mL. Plating medium consisted of Eagle's Minimal Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 10% equine serum (Hyclone, Logan, UT, USA), 21 mM (final concentration) glucose, and 10 ng/mL epidermal growth factor. Cultures were maintained at 37°C in a 5% CO₂ incubator.

Overexpression of Hsp70 and its mutants

The Hsp70 clone we are using is the human inducible gene, also known as Hsp-72, Hsp70-1, HspA1, or hsp70-1a for the protein and HSPA1A for the locus. We will use Hsp-70 in this paper. Hsp70-WT, -K71E, or -381–640 were cloned downstream of the 5' long terminal repeat (LTR) promoter in the LXS_N backbone (Sun et al 2005). Retrovirus was produced using ψ -2 packaging cells (Xu and Giffard 1997). Astrocyte cultures from Swiss Webster mice were infected 36–48 hours after plating by exposure to medium from the packaging cells (after 0.2 μ m filtration) and containing 8 μ g/mL polybrene (Sigma) for 24 hours. Infections were repeated twice. Astrocytes expressing human Hsp70-WT, -K71E, -381–640, or control vector LXS_N (infection control) were selected in 400 μ g/mL G418 (Sigma) for 5 days. The infected astrocytes were grown for 2 weeks, during which time they were fed every 5 days with plating medium containing 100 μ g/mL G418.

Injury paradigms

Wash control cells had their medium changed to BSS_{5.5} containing the following (in mM): glucose, 5.5; NaCl, 116; CaCl₂, 1.8; MgSO₄, 0.8; KCl, 5.4; NaH₂PO₄, 1, and HEPES, 10 (pH 7.4). Transfected cells were subjected to GD by exchanging growth medium for BSS₀, which was identical to BSS_{5.5} but lacking glucose. The medium was replaced in each well 3 times (Papadopoulos et al 1998). Cell death was assessed 24 hours after beginning GD by assaying lactate dehydrogenase (LDH) release into the medium (Papadopoulos et al 1998).

Cell respiration

Cell respiration was measured following the method of Fiskum and colleagues (Moreadith and Fiskum 1984; Fiskum et al 2000) with some modification (Ouyang et al 1997a, 1997b). Astrocytes, either control uninfected, empty LXS_N infected, or Hsp70-WT, -K71E, or -381–640 overexpressing, in a 6-well plate were permeabilized by adding 0.01% (w/v) digitonin. The respiration medium contained 130 mM KCl, 2 mM KH₂PO₄, 1 mM MgCl₂, 5 mM HEPES, 5 mM malate, and 5 mM glutamate. State III res-

piration was initiated by adding 0.4 mM adenosine 5'-diphosphate (ADP). Oligomycin (2 μ g/mL) was used to initiate state IV respiration and the uncoupled rate was determined by addition of 0.1 μ M carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The oxygen consumption rate was measured using an oxygen electrode (Microelectrodes Inc, Bedford, NH, USA) connected to a digital multimeter, recorded using ScopeView software (Radio Shack, Fort Worth, TX, USA), and normalized to the protein content.

Live imaging

The imaging study was performed as previously described (Ouyang et al 2002). Briefly, mouse cortical astrocytes on 25-mm poly-D-lysine-coated round glass coverslips (Fisher Scientific, Houston, TX, USA) were washed into BSS_{5.5} for dye loading. After loading, cells were further washed with 3 changes of 2 mL of BSS_{5.5} for control or BSS₀ for GD. The loading dye was present in all washes. Coverslips were then mounted in a chamber adapter on an inverted microscope (Nikon Diaphot). Images were acquired every 15 minutes in GD experiment (5-hour time course). To analyze experiments, individual astrocytes were outlined and data were gathered on a Pentium III-based computer using Axon Imaging Workbench 4.0 from Axon Instruments (Foster City, CA).

Oxygen radical production was monitored using hydroethidine (HET). Cultures were loaded in the dark with 5 μ M HET in BSS_{5.5} (30 minutes, 37°C), and the same concentration of HET was maintained in the bath throughout each experiment. Cells were excited at 495 nm, and emission was monitored at 530 nm. Fluorescence measurements for each cell (F_x) were normalized to the initial fluorescence intensity (F_0) for that cell.

For assessment of changes in mitochondrial membrane potential, astrocytes were incubated for 30 minutes with tetramethylrhodamine ethylester (TMRE) (50 nM) at 37°C, and the same concentration of TMRE was maintained in all bathing solutions throughout the experiments. Cells were excited at 495 nm, and emission was monitored at 530 nm. Changes in fluorescence were quantified by selecting a cytoplasmic region of each cell that was strongly fluorescent at baseline (indicating that it was "mitochondria rich"), and normalizing subsequent fluorescence measurements to the basal fluorescence (F_0) for each cell at the start of the experiment.

Immunocytochemistry

Fluorescence immunocytochemistry was performed on cell cultures on coverslips as previously described (Ouyang and Giffard 2003). The coverslips were washed twice in phosphate-buffered saline (PBS) for 5 minutes at

room temperature (RT) and then in PBS containing 0.2% Triton X-100 (TX100) for 30 minutes. Nonspecific binding sites were blocked in 3% bovine serum albumin (BSA) in PBS/0.2% TX100 for 30 minutes. Antibody for inducible Hsp70 that does not recognize the constitutive Hsc70 (Hsp73) was from PharMingen (San Diego, CA, USA). The cells were incubated with Hsp70 primary antibody (1:500 dilution in PBS/0.1% TX100 and 1% BSA) overnight at 4°C, and then washed in PBS containing 0.1% TX100, 3 times at RT. The primary antibody was visualized with fluorescein-labeled anti-mouse secondary antibody, and propidium iodide was used to stain nuclei. Coverslips were washed several times in PBS/0.1% TX100, mounted on glass slides using Fluoromount-G (Southern Biotechnology Associates Inc, Birmingham, AL, USA) and observed with an epifluorescence microscope (Zeiss Axiover 200M, Carl Zeiss, Gottingen, Germany), and image was obtained on a Macintosh computer using Openlab from Improvision Inc (Lexington, MA, USA).

Statistics

All data reported represent at least 3 independent experiments. Data reported are means $\pm$ SD. Statistical difference was determined using analysis of variance (ANOVA) followed by Scheffé's (or Newman-Keuls) test with $P < 0.05$, the level considered significant.

RESULTS

Transfection of primary cultured astrocytes with the human Hsp70-WT, -K71E, or -381-640 genes using a retroviral vector resulted in markedly higher levels of expression of these proteins (Fig 1A-C) compared with the level of immunoreactivity in LXS_N-infected astrocytes (Fig 1D). Western blot demonstrates the high level of expression of Hsp70-WT and -K71E as well as the approximately 30-kDa band seen in the Hsp70-381-640 carboxyl-terminal domain-expressing cells (Fig 1E). Figure 1G shows a single Hsp70-expressing astrocyte at higher magnification. Astrocytes overexpressing Hsp70 or its mutants were significantly protected from 28-hour GD injury as assessed by LDH release (Fig 1F).

Overexpression of inducible Hsp70 and its mutants inhibit the mitochondrial membrane potential change induced by glucose deprivation

To determine the time course of changes in mitochondrial membrane potential, a potentiometric fluorescent dye, TMRE, which incorporates into mitochondria in a membrane potential-dependent manner was used. At 0 hour of GD, all of the groups showed similar levels of TMRE

Fig 1. Overexpression of Hsp70 and its mutants protect astrocytes from GD. Primary cortical astrocytes were infected with retrovirus encoding Hsp70-WT, -K71E, -381-640, or vector, selected for 5 days with G418 at a higher concentration followed by growth at a lower concentration for 2 weeks. Hsp70 immunostaining showed that Hsp70 proteins were expressed in essentially all of the astrocytes after selection (A and G) Hsp70-WT; (B) -K71E; (C) -381-640; (D) vector LXSX showed no immunoreactivity. Western blot demonstrates proteins of the expected molecular weights (E). Hsp70-overexpressing astrocytes and controls were subjected to GD for 28 hours. Cell death was significantly decreased in the Hsp70-WT, -K71E, and -381-640 groups compared to vector control as assessed by LDH release assay (F). * $P < 0.05$ compared with vector control (Vec), and # $P < 0.05$ compared with uninjured control (Ctr) by ANOVA and Newman-Keuls test. Data shown are mean values $\pm$ SD from representative experiments, performed in duplicate, which were repeated 3 times with similar results. Scale bars: A–C, 50 μ m; G, 20 μ m.

fluorescence intensity and with a similar punctate distribution of mitochondria around nuclei (Fig 2A, top row). At 1 hour of GD in the vector control group, although most of the cells are unchanged (Fig 2A, second row), mitochondria in some cells (indicated by an arrow) became brighter but blurry. At 3 hours (Fig 2A, third row), almost all of the mitochondria in the cells showed increased fluorescence. At 5 hours (Fig 2A, fourth row), the fluorescence intensity decreased significantly, consistent with our earlier findings (Ouyang et al 2002). In the Hsp70-overexpressing groups including -K71E and -381-640, no apparent mitochondrial membrane potential changes were seen through 5 hours of GD. Figure 2B shows the time course of TMRE mitochondrial fluorescence over 5 hours of GD in uninfected control, LXSX-infected control, as well as cultures overexpressing Hsp70 and its mutants. The vector-expressing cells (Ctrl, -G) show the pattern of fluorescence increase followed by a

Fig 2. Overexpressing Hsp70 and its mutants stabilize mitochondrial membrane potential with GD. Example of images of TMRE mitochondrial fluorescence (A). Red, TMRE; blue, Hoechst. Scale bar, 20 μ m. Time course of TMRE mitochondrial fluorescence changes (B). For each cell, the TMRE fluorescence at each time point was normalized to the starting fluorescence for that cell. All traces represent the mean (error bars omitted for clarity) of at least 50 astrocytes per condition, derived from at least 3 experiments. Bar graph (C) showing that at 5 hours of GD, Hsp70 and its mutants significantly prevent the decrease in mitochondrial membrane potential. * $P < 0.05$ compared with all other groups by ANOVA followed by Scheffé's test.

decrease in TMRE intensity as observed previously with GD (Ouyang et al 2002). About 5 hours of GD led to about 20% loss of mitochondrial membrane potential compared with the starting fluorescence. In Hsp70- and its mutant-expressing astrocytes, there was significantly less change in mitochondrial membrane potential until the end of the 5 hours of GD (Fig 2C).

Overexpression of inducible Hsp70 and its mutants decreases ROS formation induced by glucose deprivation

ROS accumulation following GD was monitored using the ROS-sensitive fluorescent dye HET (Fig 3). Basal levels of ROS accumulation were not significantly different between the different types of astrocytes. Upon exposure to GD, LXSX-expressing astrocytes showed an immediate and rapid increase in ROS accumulation (Fig 3A), reaching approximately 8 times the initial fluorescence level after 5 hours of GD (Fig 3B). All of the Hsp70 and its mutant overexpressing astrocytes showed more modest elevations, reaching about half of the level seen in the injury control group after 5 hours of GD. When compared with the noninjury group (control with glucose), there are still significant increases in HET fluorescence in all of the Hsp70 groups at 5 hours of GD.

Fig 3. Overexpressing Hsp70 and its mutants reduce ROS production with GD. Time course of ROS accumulation with GD in astrocytes (A). For each cell, the H₂O₂ fluorescence at each time point was normalized to the starting fluorescence for that cell. All traces represent the mean (error bars omitted for clarity) of at least 50 astrocytes per condition, derived from at least 3 experiments. Bar graph (B) showing that, at 5 hours of GD, Hsp70 and its mutants significantly moderate the increase in ROS production. * $P < 0.05$ compared with Ctrl.-G; ** $P < 0.05$ compared with Ctrl.-G, by ANOVA followed by Scheffé's test.

The effect of Hsp70-WT on state III respiration differs from that of Hsp70-K71E or -381-640 after glucose deprivation

The oxygen consumption rate was measured using an oxygen electrode. Rates of state III (phosphorylating) respiration are measured in the presence of oxidizable substrates and ADP, state IV (resting) in the absence of ADP or in the presence of the ATP synthase inhibitor oligomycin, and uncoupled respiration by addition of the uncoupler CCCP. For control cells subjected to GD, state III respiration decreased significantly as early as 3 hours after removal of glucose and declined somewhat further at 5 hours of GD (Fig 4A). State IV respiration did not change at 3 hours of GD but increased significantly at 5 hours of GD (Fig 4B). Uncoupled respiration did not change much compared with 0 hour of GD at any time (Fig 4C). Overexpression of Hsp70 and its mutants prevented the increase in state IV respiration at 5 hours of GD (Fig 4B). However, Hsp70-WT overexpression did not prevent the decrease in state III respiration at 3 or 5 hours of GD (Fig 4A). Interestingly, both Hsp70-K71E and -381-

Fig 4. Effect of overexpressing Hsp70 and its mutants on mitochondrial respiration after GD. State III (A), state IV (B), and uncoupled (C) respiration were measured after 0, 3, and 5 hours of glucose deprivation using 0.01% digitonin-permeabilized astrocytes. * $P < 0.05$ compared with the vector control (Ctrl) at the same time; # $P < 0.05$ compared with 0 hour same condition by ANOVA followed by Scheffé's test. CCCP, Carbonylcyanide *m*-chlorophenylthiohydrazone.

640 significantly moderated the state III decrease seen after 5 hours of GD (Fig 4A).

DISCUSSION

Heat shock proteins, including Hsp70, play an essential role in assisting the correct folding of nascent and stress-accumulated misfolded proteins and preventing their aggregation. In this report, we studied Hsp70-K71E, a point mutation that abrogates ATP binding and renders the protein deficient in folding ability, and Hsp70-381-640, a deletion mutant lacking the first 380 amino acids. Both mutants can bind denatured proteins and maintain their solubility, but are unable to fold them. The present study

demonstrates that retroviral-mediated overexpression of Hsp70-WT or its mutants lacking folding activity protect astrocytes to a similar extent from glucose deprivation injury. The mechanism of protection includes maintaining mitochondrial membrane potential, decreasing ROS accumulation, and moderating the increase in state IV respiration.

There are few studies of the effect of cytosolic inducible Hsp70 on mitochondrial respiration. Whereas state III reflects the maximal rate of coupled respiration, that is, when electron transport is coupled to ATP synthesis, state IV (resting) respiration reflects the rate of leakage of protons back across the inner mitochondrial membrane into the matrix. In this study, the observation that both Hsp70-K71E and -381-640 mutants prevented the state III respiration decrease whereas Hsp70-WT did not, yet all 3 proteins reduce cell death from prolonged GD to a similar extent, shows that this early change in state III respiration is not highly correlated with cell survival. This difference, however, implies that the mechanisms of protection may differ in some ways between wild-type and folding-incompetent forms of Hsp70. The changes in state IV respiration representing the leakiness of the inner mitochondrial membrane may be more closely related to cell death as all of the proteins had similar effects on it.

How can inducible Hsp70, a cytosolic protein, affect mitochondrial function? One likely possibility is the chaperoning function of Hsp70 for mitochondrial proteins, including respiratory chain enzymes that are nuclear encoded and need to be imported from the cytosol. Recent data from Young et al (2003) demonstrated that Hsp90 and Hsp70 interact with the mitochondrial protein import receptor Tom70 at the outer membrane and are required for translocation of mitochondrial proteins from the cytosol into mitochondria. Because Tom70 is expected to interact with the carboxyl terminus of Hsp70, it is possible that the deletion mutant may still be able to bind preproteins and deliver them to mitochondria. This possibility remains to be tested directly. The cardiac mitochondrial complex activity is enhanced by Hsp70, which may be achieved primarily through facilitation of nuclear-encoded protein import and assembly in the mitochondrial matrix (Sammut and Harrison 2003). Studies in *Saccharomyces cerevisiae* (Becker et al 1996) have shown that translocation of the β -subunit of the F1F0 ATPase (complex V) into mitochondria is dependent on cytosolic Hsp70.

A further aspect of protein import into mitochondria is the requirement for the mitochondrial transmembrane potential for import to occur. Earlier work demonstrated that it was not the mitochondrial ATP content but rather the mitochondrial membrane potential that was required for protein import (Schleyer et al 1982). Our observation that both WT and Hsp70 mutants maintain mitochondrial

membrane potential suggests that they may also contribute to maintaining mitochondrial function via this mechanism.

Several authors (Simpkins et al 1993; Samali and Orrenius 1998) have postulated an involvement of Hsp70 in preventing electron leak between complexes III and IV by binding and consequently reducing cytochrome *c* loss from mitochondrial membranes, thereby averting an increase in state IV respiration rates and induction of cytochrome *c*-linked apoptosis. This possibility is consistent with our observation of prevention of increases in state IV respiration in stressed astrocytes. By enhancing the activities of other mitochondrial antioxidant enzymes such as manganese superoxide dismutase (Suzuki et al 2002), overexpressed Hsp70 might also reduce ROS formation after ischemia-like stress.

Another aspect of Hsp70 protection is its interaction with various components of the tightly regulated programmed cell death machinery upstream (Steel et al 2004; Stankiewicz et al 2005) or downstream (Ravagnan et al 2001) of mitochondrial events. The mitochondrial pathway of cell death is initiated by the release into the cytosol of apoptogenic molecules that include cytochrome *c*, which participates in caspase activation and apoptosis-inducing factor (AIF), which directly translocates to the nucleus and triggers caspase-independent nuclear changes (Susin et al 1999; Daugas et al 2000; Joza et al 2001). Results from others (Ravagnan et al 2001) for full-length Hsp70, and our group for Hsp70-WT, -K71E, and -381-640 (Sun et al 2005) demonstrate inhibition of AIF translocation to the nucleus, which in the latter report was associated with reduced ischemic cell death (Sun et al 2005). Hsp70 has also been shown to decrease cytochrome *c* release (Tsuchiya et al 2003).

In conclusion, we have demonstrated that both WT and 2 folding-deficient mutants of Hsp70 can protect astrocytes from GD injury and improve mitochondrial function in terms of membrane potential change, respiration, and accumulation of free radicals. Preserving mitochondrial physiology likely contributes to the Hsp70-mediated neuroprotective effect observed against ischemia.

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