Localization of the Heat-Shock Protein Hsp70 to the Synapse Following Hyperthermic Stress in the Brain

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Abstract: Heat-shock proteins are induced in response to cellular stress. Although heat-shock proteins are known to function in repair and protective mechanisms, their relationship to critical neural processes, such as synaptic function, has received little attention. Here we investigate whether the major heat-shock protein Hsp70 localizes to the synapse following a physiologically relevant increase in temperature in the mammalian nervous system. Our results indicate that hyperthermia-induced Hsp70 is associated with pre- and postsynaptic elements, including the postsynaptic density. The positioning of Hsp70 at the synapse could facilitate the repair of stress-induced damage to synaptic proteins and also contribute to neuroprotective events at the synapse. Key Words: Heat-shock proteins—Synapse—Stress response—Hyperthermia—Electron microscopy. J. Neurochem. 74, 641–646 (2000).

In response to a range of stressful stimuli, the nervous system activates the highly conserved heat-shock or stress response in which ongoing gene expression is down-regulated and a set of heat-shock proteins (hsps) is induced (for reviews, see Mayer and Brown, 1994; Brown and Sharp, 1999). These up-regulated proteins are thought to be involved in cellular repair mechanisms. For example, they bind to proteins that have been distorted by stressful conditions and facilitate correct refolding (Kiang and Tsokos, 1998). In addition, prior heat shock, which is sufficient to induce hsps, protects neural cells from subsequent stress that would normally be damaging or lethal. This neuroprotective aspect of the heat-shock response has been observed in both in vitro and in vivo paradigms. A conditioning heat shock protects neural cells grown in culture from subsequent stress (Lowenstein et al., 1991; Rordorf et al., 1991; Mailhos et al., 1993). In the intact rat, prior whole-body hyperthermia protects retinal photoreceptors from degeneration induced by bright light (Barbe et al., 1988; Tytell et al., 1993, 1994). Experiments using transfected neural cells and transgenic animals suggest that Hsp70 plays a central role in neuroprotective phenomena (Fink et al., 1997; Plumier et al., 1997; Yenari et al., 1998).

A key question is whether protective features of the heat-shock response impact on critical neural processes, such as synaptic function. During stressful conditions, it is vital that the functionality of synapses be preserved to prevent a breakdown of neural communication. Can prior heat shock protect synaptic transmission from subsequent stress? Using a macropatch electrode to record synaptic activity at individual visualized synaptic boutons, our recent collaborative studies indicate that prior heat shock of Drosophila larvae protects synaptic performance at high test temperatures (Karunanithi et al., 1999). The time course of this synaptic protection parallels the rise and fall of induced Hsp70 levels in the larvae. In addition, an initial heat shock has been shown to protect synaptic activity involved in the locust flight system (Dawson-Scully and Robertson, 1998).

In the present investigation, we examine whether hsps localize to synapses of the mammalian nervous system following a physiologically relevant increase in body temperature. Using subcellular fractionation and electron microscopy immunocytochemistry, we demonstrated that stress-induced Hsp70 localizes to synapses in the rat brain. In addition, the subcellular distribution of constitutively expressed Hsc70, Hsp90, and Hsp60 were investigated. These constitutive hsps were detected in synaptic fractions derived from unstressed animals, suggesting that they play roles in normal synaptic function. Following hyperthermia, Hsp70 is strongly induced in the nervous system and is detected at synapses where it could facilitate the repair of distorted proteins.

EXPERIMENTAL PROCEDURES

Treatment of animals

The body temperature of male Wistar rats (30 days old) was raised 3.5°C above normal (38°C) using a dry air incubator set at 42°C. The rats were maintained at this temperature for 1 h.

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Abbreviations used: hsp, heat-shock protein; PBS, phosphate-buffered saline; PSD, postsynaptic density; SJ, synaptic junction; SM, synaptic membrane.
Following incubation, animals were placed at room temperature until they were killed.

**Preparation of synaptic fractions**

Synaptic membrane (SM) and synaptic junction (SJ) fractions were prepared using a method previously described (Gurd et al., 1992). In brief, brain tissue (14–18 rats per preparation) was homogenized in 0.32 M sucrose containing 1 mM MgCl₂ and centrifuged twice for 5 min at 1,000 g. The supernatant (S1) was centrifuged at 13,200 g for 15 min resulting in S2 and P2 fractions. Following lysis in 0.05 M (S1) was centrifuged at 13,200 g for 15 min resulting in S2 and P2 fractions. Following lysis in 0.05 mM CaCl₂, the P2 fraction was incubated with iodonitrotetrazolium and succinate for 30 min at 37°C. Membrane fractions were separated on a discontinuous sucrose gradient centrifuged at 100,000 g for 90 min, and SMs were harvested from the 1.0/1.2 M sucrose interface. SJs were isolated by the extraction of the SM fraction with Triton X-100 (0.5%, vol/vol).

Post-synaptic densities (PSDs) were extracted from the SM fraction described above using a two-phase system containing 1% n-octyl glucoside as previously described (Gurd et al., 1992). PSDs were harvested from the phase interface, washed with distilled H₂O, and resuspended in 0.32 M sucrose. To confirm the specific association of Hsp70 and Hsc70 with the PSD fraction, PSDs were extracted with high salt (1.0 M NaCl), high pH (0.2 M NaHCO₃, pH 11, 1.0 mM EGTA), or detergent (1% sarcosyl) as previously described (Gurd et al., 1992).

Synaptosomes were isolated using discontinuous Percoll gradients according to the method of Dunkley et al. (1988). Percoll-sucrose solutions (0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.4) were prepared at 23, 15, 10, and 3% (vol/vol) and layered, 2 ml each, in polycarbonate tubes. Brain tissue (three rats per preparation) was homogenized in sucrose buffer and centrifuged at 1,000 g for 10 min. Supernatant was layered 2 ml per gradient and centrifuged at 31,000 g for 5 min. Synaptosomes were harvested from the 15/23% interface and washed twice with Krebs buffer (Dunkley et al., 1988) before centrifugation at 15,300 g for 15 min.

**Immunoblotting**

Protein samples were solubilized by boiling in 2× dissociation buffer (8 M urea, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 20% glycerol) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% polyacrylamide gels with a 5% stacking gel (Laemmli, 1970). Protein transfer, nitrocellulose blots were stained with Ponceau S to ensure equal loading of protein samples. After blocking for 2 h with 5% Carnation milk powder, membranes were incubated 14–16 h with primary antibodies diluted 1:5,000 for Hsp90 (gift from A. C. Wikström), 1:100,000 for Hsc70 (StressGen, SPA-815), 1:5,000 for Hsp70 (StressGen, SPA-810), and 1:20,000 for Hsp60 (gift from R. Gupta, McMaster University, Hamilton, Ontario, Canada). Blots were incubated for 2 h with either anti-mouse IgG (Sigma) or anti-rat IgG (Sigma) diluted 1:5,000. Immunoreactivity was visualized using enhanced chemiluminescence western blotting detection reagents (Amersham, RPN 2106). Each of the subcellular fractions was collected from a minimum of three separate trials. Western blots shown are representative of repeat experiments on each set of fractions.

**Immunoelectron microscopy**

Rats were anesthetized with 1 ml of pentobarbital (65 mg/ml) and perfused intracardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 3.5% glutaraldehyde in PBS. Tissue was removed and fixed for an additional 2 h at 4°C. Following immersion in 0.1% sodium borohydride (30 min), 40–μm sections were cut using a Vibratome. Cerebellar sections were equilibrated to 30% sucrose and frozen in isopentane (−70°C) to increase antibody penetration. After blocking in 10% goat serum for 90 min, tissue sections were incubated with either the Hsp70- or Hsc70-specific antibody diluted 1:7,500 and 1:500, respectively, for 24–36 h at 4°C. Tissue was immersed in biotinylated anti-rabbit IgG at 1:400 for 2.5 h and then processed for 1 h with the Vectastain Elite ABC kit (Vector Laboratories). Immunoreaction was visualized using 3,3′-diaminobenzidine. Omission of either the primary or secondary antibody resulted in no disposition of reaction product. Sections were fixed in 1% OsO₄ in PBS for 30 min, dehydrated, and embedded in Epon 812 resin (EM Sciences). Ultrathin sections were collected and counterstained with uranyl acetate and lead citrate. Counterstaining was omitted on certain sections to verify localization of 3,3′-diaminobenzidine reaction product. Grids were examined and photographed using a Siemens Elmiskop102. Data presented for immunoelectron microscopy are representative of three animal trials for each condition.

**RESULTS**

**Time-course analysis of the neural hsp following hyperthermia**

Following elevation of body temperature by 3.5°C, levels of constitutively expressed Hsp90, Hsc70, and Hsp60 did not change in the forebrain and cerebellum at 1–24 h post-hyperthermia in total tissue homogenates (Fig. 1). In contrast, a pronounced induction of Hsp70 was observed in these brain regions, which was still apparent 24 h post-hyperthermia.

**Association of hsp90 with synaptic fractions**

Synaptosomes, which are composed of both pre- and postsynaptic components, showed signals for constitutively expressed Hsp90, Hsc70, and Hsp60 in both forebrain and cerebellum of control animals with no major changes following hyperthermia (Fig. 2). Basal expression of Hsp70 was not detectable in cerebellar synaptosomes from control animals; however, following hyperthermia, this stress-inducible hsp was apparent at 5 h post-hyperthermia and was maintained at 15 and 24 h.
Forebrain synaptosomes demonstrated a weaker accumulation of Hsp70.

SM, SJ, and PSD fractions, which have been well characterized by biochemical and electron microscope analysis, were isolated (Gurd, 1997; Clark et al., 1998). As shown in Fig. 3, Hsp90 and Hsp60, which were detected in the SM fraction of the unstressed animals, were not observed as purification proceeded to the SJ and PSD fractions. This suggests a preferential localization of these constitutive hsps to the presynaptic elements of the synapse. Hsp70 was detected in all three synaptic fractions. Basal expression of Hsp70 was detected in the forebrain SJ fraction of the unstressed animal and enriched in the PSD component, suggesting a role for Hsp70 in postsynaptic structures. Following elevation of body temperature, an accumulation of stress-inducible Hsp70 was detected in SM and SJ fractions (Fig. 4A) and the PSD (Fig. 4B), whereas levels of Hsc70 remained constant.

To determine whether Hsp70 and Hsc70 were integral components of the PSD, isolated PSDs were subjected to salt, pH, and sarcosol extraction followed by high-speed centrifugation. The particulate matter, containing PSDs, was collected as a pellet and solubilized material was recovered in the supernatant. As seen in Fig. 4C, the extractions did not remove Hsp70 or Hsc70 from the PSDs, suggesting that they are integral synaptic components. The fraction used for these extraction assays was isolated from 15 h post-hyperthermic forebrain.

Visualization of hsps at the synapse

Immunoelectron microscopy provided visual confirmation of the localization of stress-induced Hsp70 to postsynaptic and presynaptic structures in the cerebellum following hyperthermia (Fig. 5A and B). Hsp70 immunoreactivity was not observed at synapses in control animals (Fig. 5C). Signal reflecting constitutively expressed Hsc70 protein was evident in postsynaptic structures of unstressed animals (Fig. 5D and E) and also in presynaptic structures (Fig. 5F). This Hsc70 pattern did not change following hyperthermia, in agreement with western blot data shown in Fig. 4A and B.

**DISCUSSION**

Hsps are composed of constitutively expressed members that are present in unstressed cells and inducible...
members that are expressed in response to stressful stimuli. The present studies indicate that constitutively expressed Hsc70, Hsp90, and Hsp60 are associated with synaptic elements in the unstressed mammalian brain, suggesting that they play roles in normal synaptic function. Hsc70 was observed at presynaptic and postsynaptic loci, including the PSD. Constitutive members of the hsp70 multigene family have been reported to be associated with endocytotic steps of synaptic vesicle recycling (Ungewickell, 1985; Chappell et al., 1986; Green and Liem, 1989; Zhang et al., 1994; Ungewickell et al., 1995). They are also components of the synaptic vesicle fusing and docking complex (Söllner et al., 1993). These reports are consistent with our present observations on the association of Hsc70 with presynaptic elements; however, our study indicates additional Hsc70 roles at the postsynaptic elements, including the PSD. Hsc70 is likely to be involved in the modification and turnover of synaptic proteins, a role that is supported by the presence of the DnaJ-like proteins, cysteine-string protein and Hsp40 at synaptic sites (Kohan et al., 1995; Suzuki et al., 1999). Both cysteine-string protein and Hsp40 have been shown to modulate the chaperoning activity of Hsc70/Hsp70 (Minami et al., 1996; Chamberlain and Burgoyne, 1997).

In response to temperature increases and other stresses, cells exhibit an induction of a set of genes encoding hsps, which contribute to cellular repair and protective mechanisms (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993; Kiang and Tsokos, 1998). It is well established that this classic heat-shock response protects the nervous system from subsequent stress (Mayer and Brown, 1994; Brown and Sharp, 1999; Sharp et al., 1999). Prior heat shock protects neural cells grown in tissue culture from cell death induced by stressful stimuli (Lowenstein et al., 1991; Rordorf et al., 1991; Mailhos et al., 1993). In addition, prior heat shock protects rat embryos from thermally induced neural defects (Walsh et al., 1987, 1991), and prior whole-body hyperthermia shields photoreceptors in the rat retina from degeneration induced by bright light (Barbe et al., 1988; Tytell et al., 1993). Hsp70 appears to play a role in neuroprotection phenomena because intracocular injection of purified Hsp70 confers protection in the retinal system (Tytell et al., 1993) and neurons in transgenic

**FIG. 5.** Localization of Hsp70 and Hsc70 immunoreactivity to synapses by electron microscopy. A–C: Hsp70 immunoreactivity. Stress-induced Hsp70 was localized to presynaptic (A) and postsynaptic (B) elements in the molecular layer of the cerebellum at 15 h post-hyperthermia. Immunoreaction product was not detected in synapses in control animals (C). D–F: Hsc70 immunoreactivity. In unstressed animals, constitutively expressed Hsc70 was detected at postsynaptic (D and E) and presynaptic (F) structures in the molecular layer of the cerebellum. Arrows point to PSDs and arrowheads to synaptic vesicles. Po, postsynaptic element; Pr, presynaptic element. Bar = 160 nm.
animals overexpressing Hsp70 are protected from stressful stimuli (Plumier et al., 1997).

The induction of hsp7s in the nervous system following hyperthermia and other stresses has been widely studied (Mayer and Brown, 1994; Brown and Sharp, 1999); however, little is known on whether the heat-shock response impacts on critical neural processes, such as synaptic function. During stressful conditions, it is important that the functionality of synapses be preserved to prevent the breakdown of neural communication systems. A connection between the heat-shock response and preservation of synaptic transmission under stressful conditions has been established because prior heat shock of Drosophila larvae, sufficient to induce Hsp70, protects synaptic transmission from high test temperatures (Karunanithi et al., 1999).

To explore further the intriguing dialogue between the heat-shock response and the synapse, we have addressed the question of whether stress-induced Hsp70 localizes to synapses in the mammalian nervous system. Hsp70 is a known “conformational repair agent” that can distinguish between folded and unfolded forms of the same protein and selectively bind to the perturbed form to facilitate correct refolding (Kiang and Tsokos, 1998). Our findings indicate that hyperthermia-induced Hsp70 localizes to synaptic elements in the rat brain, including the PSD. This raises the possibility that Hsp70, positioned at the synapse, could correct stress-induced damage to synaptic proteins and could underlie the neuroprotective effects of prior heat shock at the synapse.

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