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Review

Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury

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Summary

Chaperones, especially the stress inducible Hsp70, have been studied for their potential to protect the brain from ischemic injury. While they protect from both global and focal ischemia *in vivo* and cell culture models of ischemia/reperfusion injury *in vitro*, the mechanism of protection is not well understood. Protein aggregation is part of the etiology of chronic neurodegenerative diseases such as Huntington's and Alzheimer's, and recent data demonstrate protein aggregates in animal models of stroke. We now demonstrate that overexpression of Hsp70 in hippocampal CA1 neurons reduces evidence of protein aggregation under conditions where neuronal survival is increased. We have also demonstrated protection by the cochaperone Hdj-2 *in vitro* and demonstrated that this is associated with reduced protein aggregation identified by ubiquitin immunostaining. Hdj-2 can prevent protein aggregate formation by itself, but can only facilitate protein folding in conjunction with Hsp70. Pharmacological induction of Hsp70 was found to reduce both apoptotic and necrotic astrocyte death induced by glucose deprivation or oxygen glucose deprivation. Protection from ischemia and ischemia-like injury by chaperones thus involves at least anti-apoptotic, anti-necrotic and anti-protein aggregation mechanisms.

Key words astrocyte, cell culture, global ischemia, HDJ-2, Hsp70, mouse, rat, CA-1, protein aggregation, apoptosis.

Introduction

Chaperones are a functionally related group of proteins that assist protein folding in bacteria, plant and animal cells under physiological and stress conditions. In addition to their role in protein folding, chaperones facilitate translocation of proteins across membranes, help assemble and disassemble protein complexes, help present substrates for degradation, and suppress protein aggregation (Hartl, 1996; Ohtsuka and Hata, 2000). An important subgroup of highly evolutionarily conserved chaperones is the ATP-dependent heat shock proteins (Hsps), which share the ability to recognize and bind nascent and unfolded proteins, thus preventing aggregation, and facilitating correct protein folding (Beissinger and Buchner, 1998; Frydman, 2001). Chaperones are currently being considered for the potential treatment of diseases involving protein aggregation and misfolding from neurodegenerative diseases (Bonini, 2002) to cancer (Scott and Frydman, 2003). The cell has a complex system for maintaining proper protein folding, which begins with facilitation of folding of nascent proteins, monitoring for the presence of unfolded proteins in different intracellular compartments, and targeting of misfolded or abnormal proteins for degradation. Many aspects of protein–protein interactions are also specifically regulated by chaperones. The accumulation of unfolded proteins in the endoplasmic reticulum lumen can trigger the unfolded protein response, which is implicated in the shutdown of protein synthesis that is a hallmark of the response to ischemia and other severe cellular stresses (Paschen, 2003). Thus regulation of the state of protein folding and protein association is a central aspect of normal cellular homeostatsis, which is severely perturbed by ischemia and reperfusion. Despite a large number of studies demonstrating neuroprotection by the chaperone Hsp70, in both animal stroke studies (Plumier et al., 1997; Rajdev et al., 2000; Yenari et al., 1998, 1999) and cell culture models of ischemia (Papadopoulos et al., 1996; Xu and Giffard, 1997), the mechanism, or more likely mechanisms, of protection are poorly understood.

Recent work has highlighted the ability of Hsp70 to suppress multiple types of cell death including necrotic death, classical apoptosis, and other programmed cell death pathways that are

independent of caspases and not blocked by Bcl-2 (Beere et al., 2000; Jaattela et al., 1998; Nylandsted et al., 2000; Ravagnan et al., 2001; Saleh et al., 2000). Since both apoptotic and necrotic cell death are involved in ischemic brain injury, the ability of Hsp70 to reduce both types of cell death makes it an appealing candidate for brain protection. Understanding the aspects of Hsp70 function that are required for protection will identify pathological processes that contribute to cell death and allow future work to target identified pathological mechanisms for brain protection.

Unfolded or misfolded proteins have exposed hydrophobic segments that render them prone to aggregation. Protein aggregates are thought to be toxic to the cell (Taylor et al., 2002), so to avoid aggregation, abnormal proteins are either kept soluble by molecular chaperones or quickly degraded by the ubiquitin/proteasome system (Hershko and Ciechanover, 1998). Under pathological conditions, the level of abnormal proteins may exceed the ability of the cell to maintain them in a soluble form or degrade them, allowing aggregation to proceed (Cohen, 1999; Zoghbi and Orr, 2000). Protein aggregates can inhibit function of the proteosome, thus further limiting the cell's ability to dispose of the protein aggregates and interfering with the normal processing of certain short-lived proteins (Bence et al., 2001). Protein aggregates commonly contain ubiquitin immunoreactivity, suggesting that proteins targeted for degradation that fail to be degraded may end up in aggregates (Alves-Rodrigues et al., 1998). Protein aggregates have been found in most chronic neurodegenerative diseases (Kakizuka, 1998; Taylor et al., 2002), in global and focal ischemia (Hu et al., 2001, 2000) as well as hypoglycemic coma (Ouyang and Hu, 2001). These earlier studies showed that ubiquitin immunoreactivity labeled protein aggregates associated with intracellular vesicles early after injury, and later associated with mitochondria, Golgi and certain regions of the plasmalemma (Hu et al., 2000).

The Hsp40 family constitutes a major group of Hsp70 cochaperone proteins. Hdj-2, a human DnaJ member of the Hsp40 family, is highly homologous to the bacterial DnaJ protein from *Escherichia coli*. Hdj-2 interacts with Hsp70 through its J domain (Gebauer et al., 1997; Minami et al., 1996; Tang et al., 1997), targeting Hsp70 to specific intracellular tasks and accelerating the Hsp70 ATPase activity. Hdj-2 has been shown to decrease injury in models of degenerative brain disease involving protein aggregation. Cotransfection of Hdj-2 with mutant ataxin-1 resulted in a significant reduction in aggregate formation (Stenoien et al., 1999). In a model of the polyglutamine disease Huntington's disease, overexpression of Hdj-2 suppressed aggregate formation, and was associated with decreased toxicity (Jana et al., 2000). We recently demonstrated that overexpression of Hdj-2 reduces ischemia-like injury *in vitro* (Qiao et al., 2003).

We present here new results on the ability of Hsp70 to reduce protein aggregation in a model of global ischemia and the ability of Hsp70 induction with geldanamycin to block apoptotic astrocyte death induced by glucose deprivation. We discuss additional results on the cochaperone Hdj-2's ability to reduce injury and protein aggregation and the association of Hsp70 overexpression with increased levels of the anti-apoptotic protein Bcl-2.

Materials and methods

Global cerebral ischemia

HSV vectors

The construction and production of amplicons pα22βgalα4hsp72 and pα22βgalα4s have been described previously (Fink et al., 1997; Ho, 1994; Lawrence et al., 1995). The amplicon plasmid pα22βgalα4hsp72 contains the rat inducible hsp72 gene (pHsp-8) (Longo et al., 1993) and the *Escherichia coli* lacZ gene under the control of the HSV α4 and α22 promoters, pα22βgalα4s, which lacks the hsp72 sequence, was used as the control vector. Titers were: $1.3–1.46\times10^6$ amplicons ml^{-1} and $8–9.75\times10^6$ helper virus titer ml^{-1}. For α22βgalα4s (control vector), titers were $1.4–4.35\times10^6$ amplicons ml^{-1} and $1.9–4.07\times10^6$ helper virus titer ml^{-1}, corresponding to amplicon: helper virus ratios of 1:2–7.

Ischemia model

All experimental protocols carried out on animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care and were in accordance with NIH guidelines. Surgical anesthesia was induced in male Sprague Dawley rats (body mass 350–450 g; Simonsen Laboratories, Inc., Gilroy, CA, USA) with isoflurane (5%) in a mixture of medical air and oxygen (700:300 ml min^{-1}). Anesthesia was maintained with isoflurane (2-3%) using a facemask. Coordinates for dorsal hippocampal CA1 injection from bregma were: anterior–posterior, –3.8 mm; medio-lateral, ±1.7 mm; dorso-ventral, –1.8 mm. The dorsal hippocampal CA1 region of each rat was infused with 3 μl of either Hsp72 or control vector. 17 h after vector delivery, rats were anesthetized again with isoflurane and subjected to 8 min global cerebral ischemia followed by 24 h reperfusion as previously described (Kelly et al., 2002). Physiological parameters including temperature were maintained within the normal range before, during, and after ischemia, with blood pressure intentionally lowered during the ischemic period by blood withdrawal. Sham-operated (control) rats underwent similar exposure to anesthesia and surgical manipulation, but the carotid arteries were not occluded and blood pressure was not altered. After surgery, all animals were closely monitored throughout the recovery period. At 24 h post ischemia animals were euthanized by halothane overdose and were transcardially perfused with 75 ml of normal saline followed by 75 ml of 3% paraformaldehyde (PFA). After removal brains were cryoprotected in 3% PFA/20% sucrose solution.

Double label immunofluorescence and confocal microscopy

After postfixing in 3% PFA/20% sucrose solution for 1–2

days, 30 μm frozen sections in the coronal plane were taken at 100 μm increments 1 mm anterior and posterior to the needle track. Slices were stained with X-gal (5′-bromo-4-chloro-3indoly-β-D-galactopyranoside; Molecular Probes, Eugene, OR), a chromogenic substrate for β-galactosidase (β-gal), to identify neurons that had taken up the vector. Adjacent sections were then washed twice with 0.2% Triton X-100 (TX100) in phosphate-buffered saline (PBS), and nonspecific binding was blocked with 3% bovine serum albumin (BSA) in PBS/0.2% TX100 for 30 min followed by incubation with anti-ubiquitin monoclonal antibody at 1:400 dilution in PBS/0.1% TX100 overnight at 4°C. This monoclonal anti-ubiquitin antibody (MAB1510, Chemicon, Temecula, CA, USA) recognizes both free and bound ubiquitin and was previously characterized by western and immunocytochemistry (Morimoto et al., 1997). After three washes with PBS–0.1% TX100 the slices were incubated with fluorescein-labeled anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA) at 1:200 dilution. Sections were then labeled with a second primary antibody, a rabbit polyclonal antibody against β-galactosidase (ICN, Aurora, OH, USA, Catalog number 55976) followed by a rhodamine-conjugated, secondary anti-rabbit antibody, to identify vector targeted cells. Sections were washed three times then mounted on glass slides and coverslips fixed using Gelvatol. The slides were observed on a Bio-Rad (Hercules, CA, USA) MRC 1024 laser-scanning confocal microscope.

Primary astrocyte cultures

Cultures were prepared as previously described (Dugan et al., 1995). Briefly, newborn Swiss-Webster mice were anesthetized and then killed according to a protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care, in accordance with the NIH guide. In brief, brains were removed, freed of meninges, and the cortices minced and treated with 0.09% trypsin for 20 min at 37°C. After centrifugation at 400 ***g***, cells were resuspended in plating medium containing Eagle's minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% equine serum (Hyclone, Logan, UT, USA), 10% fetal bovine serum (Hyclone), 21 mmol l^{-1} glucose (Sigma, St Louis, MO, USA), 2 mmol l^{-1} glutamine (Gibco), 26.8 mmol l^{-1} $NaHCO_3$ and 10 ng ml^{-1} epidermal growth factor (Sigma) and triturated. The single cell suspension was plated in 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 2 hemispheres/10 ml, or on 25 mm coverslips precoated with poly-D-lysine (Sigma) (Dugan et al., 1995). Astrocyte cultures are ⩾95% glial fibrillary acidic protein immunoreactive cells, with the majority of remaining cells microglia, as identified by isolectin or CD11b staining; essentially no oligodendrocytes are present as determined by O4 antibody staining (Xu et al., 2001). Early or young cultures were subjected to injury after 5–7 days in culture while mature or older cultures were used after more than 25 days in culture.

Transfection to express chaperones

Ψ-2 or Phoenix packaging cells (a gift from Garry Nolan, Stanford University) were transfected with a retroviral plasmid containing inducible Hsp70 (Papadopoulos et al., 1996), or FLAG-hdj-2 (a gift from Don DeFranco; Qiao et al., 2003). Cells were infected with viral supernatant from the packaging cells as previously described (Papadopoulos et al., 1996). Uninfected astrocytes and astrocytes expressing the control gene β-galactosidase (lac-Z) were used as controls (Papadopoulos et al., 1996; Xu and Giffard, 1997).

Injury paradigms

Once confluent, transfected cells were subjected to glucose deprivation (GD) by exchanging growth medium for balanced salt solution lacking glucose (BSS_0); wash control cells had their medium changed to $BSS_{5.5}$ (balanced salt solution with 5.5 mmol l^{-1} glucose). The medium was replaced in each well three times (Papadopoulos et al., 1998). Cell death was assessed 24 h after beginning GD. For ubiquitin staining, 8 h GD was followed by 16 h recovery in the presence of glucose prior to staining. Combined oxygen and glucose deprivation (OGD) was performed by placing transfected and control cells in an anoxic chamber in an atmosphere of 85% N_2 10% H_2 and 5% CO_2, with triple exchange of the medium with deoxygenated BSS_0 (bubbled with N_2). Oxygen and 5.5 mmol l^{-1} glucose were present in controls. After incubation at 37°C in the anoxic chamber, glucose and oxygen were restored to the medium. Cell survival was determined after 24 h recovery in the 37°C 5% CO_2/room air incubator. Injury was quantified either by assaying lactate dehydrogenase (LDH) release into the medium, or by Trypan Blue or propidium iodide (PI) vital staining and cell counting (Papadopoulos et al., 1998; Qiao et al., 2003). Apoptotic *vs* necrotic cell death was quantitated based on nuclear morphology by counting cells after staining with Hoechst 33258 dye and PI to identify cells that had lost membrane integrity. Cells showing clear apoptotic nuclear morphology consisting of apoptotic bodies or bright condensed nuclei were counted as apoptotic. Cells with diffusely PI-stained normal sized nuclei were counted as necrotic and normal Hoechst-stained nuclei as viable cells (Xu et al., 2003).

Immunocytochemistry

Fluorescence immunocytochemistry for ubiquitin was performed on cell cultures on coverslips as previously described (Ouyang and Giffard, 2003; Qiao et al., 2003) using the same Chemicon antibody as above. Cells on coverslips were fixed with 4% paraformaldehyde for 1 h, washed twice in PBS for 5 min at room temperature and then in PBS containing 0.2% TX100 for 30 min. Non-specific binding sites were blocked with 3% BSA in PBS/0.2% TX100 for 30 min. The cells were incubated with primary antibody (1:500 dilution in PBS/0.1% TX100 and 1% BSA) overnight at 4°C, then washed in PBS/0.1% TX100, 3 times. The primary antibody was visualized with fluorescein-labeled anti-mouse secondary antibody. Coverslips were washed several times in PBS/0.1%

TX100, mounted on glass slides using Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL 35226, USA) and observed using an epifluorescence microscope (Nikon Diaphot, Nikon Corporation, Tokyo, Japan).

Results

Previous work demonstrated that expression of inducible Hsp70 using Herpes viral vectors reduces injury and loss of CA1 hippocampal neurons (Kelly et al., 2002) following global ischemia. We have now repeated the same injury paradigm and stained brain sections with ubiquitin to demonstrate the change in immunolabeling pattern in CA1 neurons after 8 min global ischemia and 24 h reperfusion. We observe a change from diffuse uniform labeling (Fig. 1A) to a pattern in which the nuclear region is depleted of immunoreactivity and the neuronal processes show patchy immunoreactivity (Fig. 1B), consistent with prior findings (Hu et al., 2000). We then examined the ubiquitin staining pattern in animals injected with Herpes amplicon vector to express inducible Hsp70 or the control vector that only expresses β-galactosidase in the hippocampal CA1 region prior to the episode of global ischemia. To identify the neurons that had taken up vector the sections were colabeled for β-galactosidase immunoreactivity and ubiquitin immunoreactivity. The previously described change in ubiquitin staining observed when the vector only contained β-galactosidase (Fig. 1C) was largely prevented, or had already been reversed, by 24 h reperfusion (Fig. 1D) if the vector contained Hsp70.

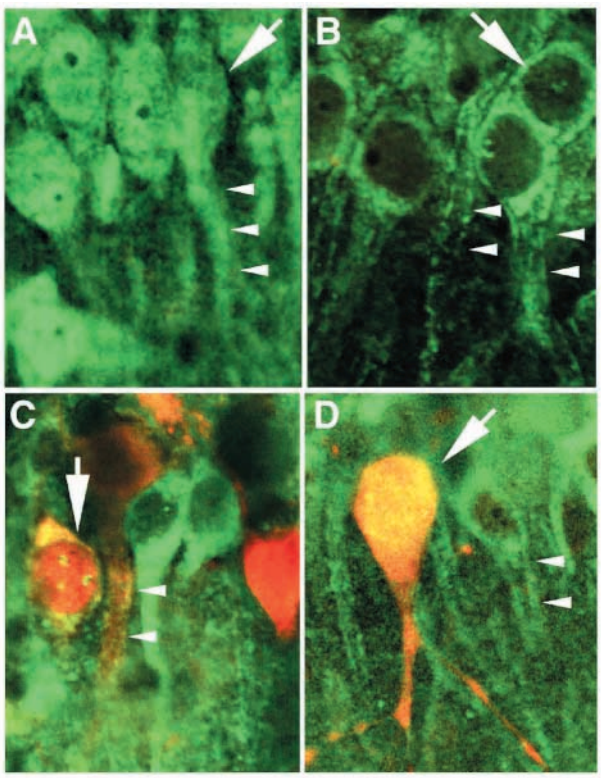

Fig. 1. Ubiquitin staining patterns in CA1 neurons, 24 h following 8 min of dense forebrain ischemia. Ubiquitin staining is color-coded green, β-gal staining is color-coded red. (A) A sham control animal not subjected to ischemia was examined 24 h later. A diffuse pattern in the processes (arrowheads) with strong nuclear staining (arrow) is observed. (B) In an animal subjected to dense forebrain ischemia, the pattern has changed to a patchy pattern in processes (arrowheads) with little nuclear staining (arrow). (C) An animal subjected to ischemia following injection with Herpes vector encoding only β-gal and then colabeled for β-gal to identify a vector targeted cell shows the same pattern in the targeted neuron as in B and in neighboring untargeted neurons with loss of nuclear staining (arrow). (D) An animal injected with Hsp-72 vector shows a relatively maintained pattern of ubiquitin staining in the neuron that is overexpressing Hsp72; note colocalization of ubiquitin and β-gal staining in the nucleus (arrow) identified by the yellow color.

To pursue the issue of protein aggregation in astrocyte cell culture injury where the effects of different genes can be more rapidly assessed, we developed a ubiquitin staining method that allowed us to visualize protein aggregates following 8 h glucose deprivation and 16 h recovery. We observed diffuse labeling of control cells with greater immunoreactivity in the nucleus (Fig. 2A,B) which changed dramatically after injury to show reduced nuclear staining, increased cytoplasmic staining and the occurrence of fine to coarse clumps in the cytoplasm (Fig. 2C–I). Sometimes a reticular pattern was observed in the cytoplasm (Fig. 2F). These patterns observed in astrocytes resembled those mentioned above with respect to CA1 neurons following ischemia. We have previously tested the ability of Hdj-2 to protect astrocytes and found it reduced both GD and OGD injury (Qiao et al., 2003). Protection from GD was associated with reduced redistribution of ubiquitin staining, suggesting reduced protein aggregation (Fig. 3). Overexpression of Hdj-2 was associated with a two thirds reduction in GD-induced cell death and a similar reduction in the number of cells showing aggregates revealed by ubiquitin immunohistochemistry (Qiao et al., 2003).

The last aspect of Hsp70 protection that we considered was the ability to block apoptotic cell death compared to necrotic cell death. Earlier work in a variety of cells has demonstrated that Hsp70 can block apoptosis. We directly compared the ability of Hsp70 to protect astrocytes from apoptosis or necrosis induced by two ischemia-like insults, OGD and GD. We took advantage of the observation that early cultures, less than 8 days in culture, underwent largely apoptotic death in response to stress while cells allowed to mature in culture for more than 20 days showed essentially only necrotic death (Xu et al., 2003, 2004). Increased levels of Hsp70 were induced pharmacologically using geldanamycin (GA) at 0.1 $\mu g\ ml^{-1}$ (Xu et al., 2003). GA has previously been shown to induce increased Hsp70 expression and to protect from focal cerebral ischemia *in vivo* (Lu et al., 2002). We observed that both early and mature cultures were less injured after GD (Fig. 4) when pretreated with GA to induce Hsp70 or when transfected with a retroviral vector to overexpress Hsp70 (Papadapoulos et al., 1996; Xu et al., 2003). GA reduced apoptosis of young cultures by about half (Fig. 4E). We previously showed that retroviral overexpression of Hsp70

reduced GD-induced necrotic astrocyte death by about 50% (Xu and Giffard, 1997), and GA treatment reduced death of older cultures by about 30–40% (Fig. 4F).

Discussion

Overexpression of Hsp70 by either viral or transgenic overexpression (Plumier et al., 1997; Yenari et al., 1998) before ischemia *in vivo* protects neurons from injury. The protection may be mediated by one or more of the many activities ascribed to Hsp70, including refolding denatured proteins and preventing unfolded and damaged proteins from aggregating, or by a direct anti-apoptotic mechanism. We have presented several pieces of evidence for a correlation between decreased protein aggregation and protection from ischemia. Wild-type inducible Hsp70 protects CA1 neurons from global ischemic injury and reduces evidence of protein aggregation in these cells. *In vitro* overexpression of the cochaperone Hdj-2 was associated with protection from ischemia-like insults and reduction of protein aggregation to a similar extent. Isolated overexpression of Hsp70 was also associated with reduced cell death and reduced protein aggregation. More work needs to be done to see if there are situations in which reduced aggregation can be separated from protection.

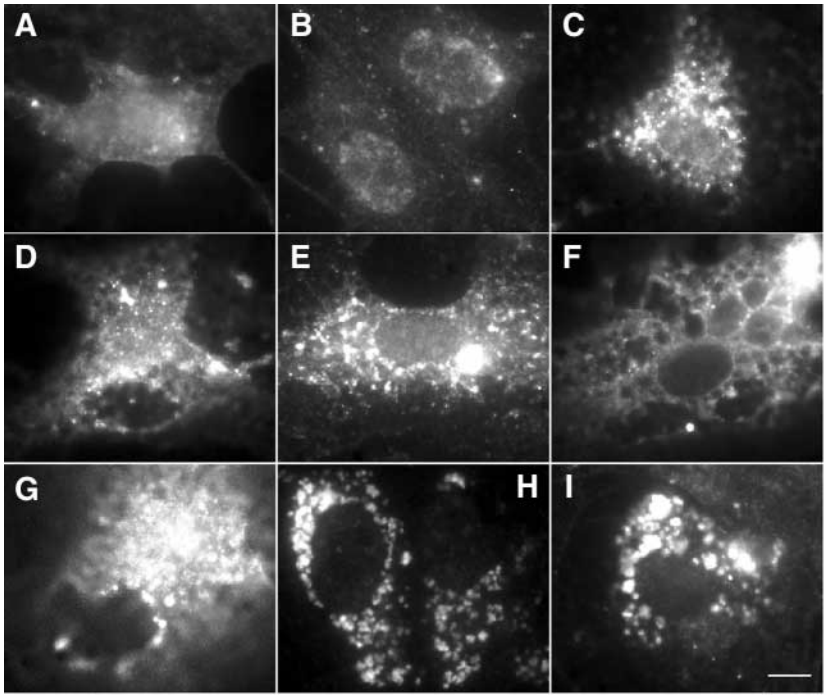

Fig. 2. The pattern of ubiquitin immunostaining in astrocytes changes after glucose deprivation (GD). Astrocyte cultures were subjected to sham wash (A,B) or allowed to recover 16 h after an 8 h GD insult (C–I). Uninjured cells show diffuse immunoreactivity with greater nuclear staining compared to cytoplasmic (A,B). After GD there is loss of nuclear staining and the appearance of aggregates throughout the cytoplasm, varying from coarse to fine. Scale bar, 10 μm.

We have shown that both induction of Hsp70 with geldanamycin and by retroviral overexpression can result in protection of astrocytes from ischemia-like injury. Protection by retroviral expression is somewhat greater in some paradigms. This may in part reflect the added stress of reduced Hsp90 levels caused by geldanamycin. Geldanamycin is known to bind the amino-terminal ATP binding site of Hsp90, disrupting its function and activating heat shock factor 1, leading to increased levels of Hsp70 and Hsp25 (Zou et al., 1998).

The J domain of Hdj-2 is the region of the protein responsible for mediating the binding of Hdj-2 to Hsp70 (Hartl, 1996). Hdj-2 binds to and stimulates the ATPase activity of Hsp70, thereby enhancing the chaperone function of Hsp70 (Hartl, 1996). The fact that Hdj-2 is still able to suppress aggregation when the J domain is deleted suggests that this suppression is independent of interactions with Hsp70 (Chai et al., 1999). It has been suggested that Hdj-2 alone can bind misfolded proteins, suppress aggregation, and facilitate delivery of misfolded polypeptides to the cellular machinery for proteolytic degradation (Stenoien et al., 1999). It has been speculated that the chaperone function of Hdj-2 in the brain may be one of the factors responsible for the relative resistance of brain cells to damage (Jana et al., 2000; Miller et al., 1990). Since Hsp70 levels are not increased in parallel with Hdj-2 in our astrocytes (Qiao et al., 2003), the protection observed in this study is likely to reflect the direct effects of Hdj-2. However, we cannot rule out a contribution from modulation of the function of Hsp70 that is present in the cells. It will be interesting to test deletion mutants of Hdj-2 in future experiments to identify the domains required for protection from ischemia-like injury.

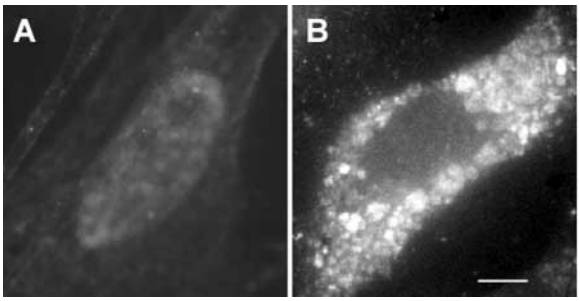

Fig. 3. Hdj-2 reduces aggregation caused by GD. Cultures were subjected to 8 h GD and 16 h recovery. (A) A cell overexpressing Hdj-2. It retains a diffuse staining pattern with darker nuclear staining even after GD. (B) A cell expressing β-galactosidase as a control. There are densely staining patches throughout the cytoplasm. Scale bar, 10 μm.

Using retroviral vectors, we previously showed that Hsp70 overexpression protects astrocytes from glucose deprivation,

combined oxygen-glucose deprivation, and H_2O_2 exposure (Papadopoulos et al., 1996; Xu and Giffard, 1997). Overexpression of Hsp70 in cultured neurons is also associated with protection (Amin et al., 1996; Beaucamp et al., 1998; Fink et al., 1997; Uney et al., 1993), and overexpression of Hsp70 in astrocytes was found to protect cocultured wild type neurons (Xu et al., 1999). These *in vitro* injury models mimic some of the aspects of injury involved in ischemic damage during stroke and suggest several ways in which Hsp70 could provide protection. Astrocytes protected from injury by Hsp70 had higher levels of glutathione than did control cells subjected to the same stress (Xu and Giffard, 1997). The ability to refold proteins or prevent aggregation may allow the cell to conserve glutathione and possibly ATP. Neurons overexpressing Hsp70 were also found to overexpress the anti-apoptotic protein bcl-2, both *in vitro* and *in vivo* (Kelly et al., 2002). The ability of astrocytes to better protect neurons when only the astrocytes overexpress Hsp70 raises several intriguing issues about the ways in which astrocytes interact with neurons during and following ischemia. Protection may be due to better antioxidant support of neurons and/or even direct provision of Hsp70 from astrocytes to neurons (Guzhova et al., 2001).

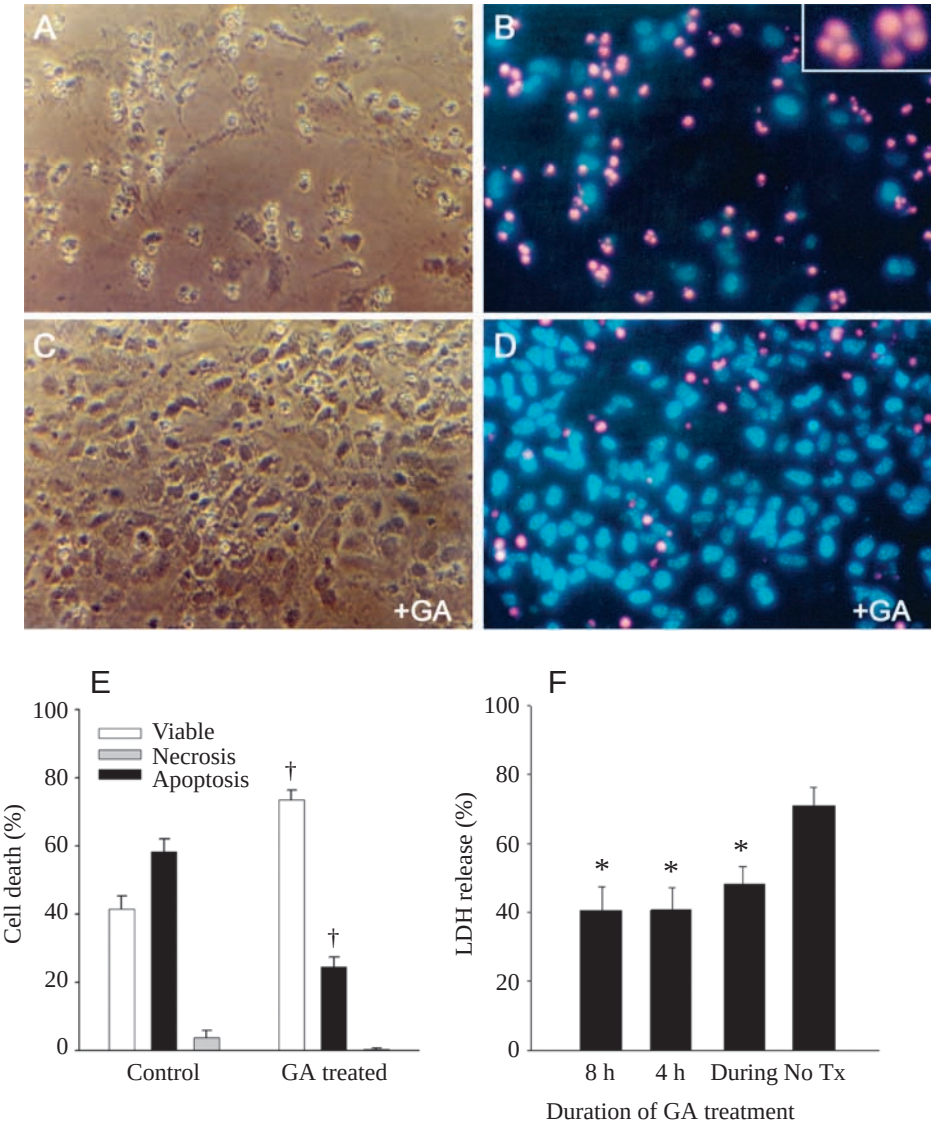

Fig. 4. Geldanamycin (GA) blocks astrocyte death induced by glucose deprivation (GD). Sister early cultures of astrocytes subjected to 24 h GD without (A,B) or after pretreatment with GA (C,D). The same microscope field of each culture after 24 h GD was photographed using phase contrast optics (A,C) or by fluorescence microscopy (B,D) after staining with propidium iodide (PI) and Hoechst dye. The insert in B shows two of the apoptotic PI-staining nuclei from the lower part of the field, at higher magnification so the apoptotic bodies can be easily seen. (E) The extent of apoptotic and necrotic cell death in early cultures was determined by nuclear morphology after GD and PI plus Hoechst staining. At least 100 cells per culture were counted, with 4–8 cultures per condition. Values are means ± S.E.M. $^{\dagger}P<0.05$ compared to GD without GA treatment (Control). (F) Quantitation of cell death in mature cultures after 24 h GD by lactate dehydrogenase (LDH) release. GA was present beginning 8 or 4 h prior to GD or first added at the beginning of GD (During); No Tx indicates GD without GA treatment. Pretreatment for 8 or 4 h was most effective, but treatment only during GD was still able to reduce injury in older cultures. $^{*}P<0.05$ compared to GD with GA (No Tx) condition. N=8–12 cultures per condition.

Recently studies from several laboratories have begun to define the ways in which Hsp70 can inhibit the apoptosis signal transduction pathway. Studies performed in cell lines and immune cells have shown that Hsp70 can block apoptosis at both early (Gabai et al., 1998) and late (Jaattela et al., 1998) steps in the cascade. Apoptosis is one of the ways neurons and astrocytes die after ischemia (Graham and Chen, 2001). A functional analysis of the role of the different Hsp70 subdomains in brain cells subjected to ischemia-like injury will begin to elucidate how Hsp70 protein modulates apoptosis in this setting, and could lead to new therapeutic approaches. Because Hsp70 can block both apoptotic and necrotic cell deaths, it is an especially interesting target for anti-ischemic therapy. Future work with mutants of Hsp70 will help define those protein domains and activities necessary to inhibit apoptosis in brain cells stressed by ischemia and reperfusion.

In conclusion, chaperones and cochaperones are interesting candidates for brain protection from ischemia–reperfusion injury since they can block multiple modes of cell death.

Identifying those actions of the chaperones that are most important for blocking injury will likely lead to the development of novel approaches to reduce damage from both chronic and acute neurodegeneration. Reduction of protein aggregation is one likely direction. Structure–function studies of chaperones will identify which activities and which protein–protein interactions are most relevant to different injuries and are most broadly protective.

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