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Heat shock response in the central nervous system

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Abstract. The heat shock response is induced in nervous tissue in a variety of clinically significant experimental models including ischemic brain injury (stroke), trauma, thermal stress and status epilepticus. Excessive excitatory neurotransmission or the inability to metabolically support normal levels of excitatory neurotransmission may contribute to neuronal death in the nervous system in many of the same pathophysiologic circumstances. We demonstrated that in vitro glutamate-neurotransmitter induced excitotoxicity is attenuated by the prior induction of the heat shock response. A short thermal stress induced a pattern of protein synthesis characteristic of the highly conserved heat shock response and increased the expression of heat shock protein (HSP) mRNA. Protein synthesis was necessary for the neuroprotective effect. The study of the mechanisms of heat shock mediated protection may lead to important clues as to the basic mechanisms underlying the molecular actions of the HSP and the factors important for excitotoxic neuronal injury. The clinical relevance of these findings in vitro is suggested by experiments performed by others in vivo demonstrating that pretreatment of animals with a submaximal thermal or ischemic stress confers protection from a subsequent ischemic insult.
Key words. Excitotoxicity; stroke; stress response; neuro protection; ischemia; brain; neuron.

Excitotoxicity and heat shock protein induced neuroprotection

An important focus of neurologic research is the understanding of the mechanisms which underly the process of neuronal death that occurs after specific insults to the brain. Exposure of nervous tissue to excitotoxins, in vivo and in vitro, has become a useful experimental approach for the examination of some of the cellular events which make neurons highly susceptible to energy deficiency states. A destructive cascade triggered by excessive glutamate-receptor activation, amplified by conditions of metabolic compromise, has been identified as a potential contributor to neuronal death in disorders such as stroke and hypoglycemia, Alzheimers disease, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease[3,8]. When cells are stressed they initiate a stereotypic response involving activation of genetic programs, some of which are probably designed to confer protection against further stress[26]. The gene families expressed as part of the phylogenetically conserved heat shock response[1] are induced by a variety of noxious stimuli in a large number of cell types. In some cases individual stress proteins have been shown to mediate tolerance to cellular insults[17,26,45,49]. The stress response has been shown to occur in the central nervous system in a number of clinically relevant situations in which excitotoxicity may play a role[13,21,33,38,43,50,54,56,58,60,66].

Glutamate is the primary excitatory transmitter in the central nervous system. It is present in metabolic pools in the neurons and glia and is released by tissue injury. It is normally released from synaptic vesicles during excitatory neurotransmission by a calcium-mediated release process activated by depolarization of the nerve terminal. Glutamate diffuses across the synaptic cleft to activate specific postsynaptic receptors[16]. Its physiological action is terminated by glutamate uptake by transporters into surrounding cells. Glutamate binds to a variety of receptor families. Glutamate binding to the N-methyl-d-aspartate (NMDA) receptors opens relatively large membrane ion channels and sodium and calcium flow directly into neurons. Sodium is the major cation that enters neurons as a result of glutamate binding to non-NMDA ionotropic receptors. However, this sodium entry causes depolarization of the cell membrane and secondary opening of voltage-sensitive calcium channels with resultant calcium entry. The metabotropic type glutamate receptor, is linked by G-proteins to phospholipase C.

The NMDA receptor is blocked by physiological concentrations of magnesium except during periods of substantial membrane depolarization. In pathological situations, neurons may not be able to repolarize due to lack of energy to pump ions across chemical gradients. Prolonged depolarization leads to excessive opening of NMDA channels. Many experiments have demonstrated that robust Ca^{++} flow through NMDA and non-NMDA channels is toxic, presumably by activation of calcium-activated enzymes, including one or more of the following: proteases, calcium-calmodulin

dependent nitric oxide synthase, phospholipases, and endonucleases. These enzymatic processes have been implicated as potential mediators of ischemic brain injury[8].

We have studied the neuroprotective properties of the heat shock response in a model of calcium-dependent excitotoxicity[50]. Thermal stress was used to trigger the heat shock response in 2-week-old cultures grown from the cortex of post-natal day 1 rat pups. The cultures contained both glia and neurons. Cultures were heated to 42.2 °C for 20 minutes. Since the stress response is usually associated with the increased synthesis of multiple families of HSP as well as decreased synthesis of proteins which are not members of the HSP families, we analyzed the pattern of protein synthesis after heat shock. ^{35}S methionine labeling of protein from the heat shocked cultures showed increased levels of proteins of approximately 72 kDa and 85 kDa but decreased total protein synthesis[50]. In some experiments, increased synthesis of proteins of lower molecular weights was also seen. Consistent with the heat shock response, general protein synthesis was depressed at 3 hours after the thermal stress in all cases. In addition, using a deoxynucleotide probe for the inducible form of hsp70, we demonstrated that hsp70 mRNA was markedly increased by our heat shock protocol[50]. Four hours after thermal stress, Western blot analysis also showed expression of hsp70 in the cortical cultures.

The neuroprotective effect of the heat shock response was investigated. We utilized a toxicity model that minimized early cell swelling and displayed dose-dependent, calcium-dependent neuronal death over 24 hours[7]. Neuronal cell death was caused by exposing cultures to 20 minutes of various doses of glutamate. The effects of prior heat shock on the dose-response curve of glutamate-induced cell death was determined. Thermal stress, 42.2 °C for 20 minutes, applied 3 hours prior to glutamate exposure conferred neuroprotection. This effect was most apparent when the glutamate dose was lethal to only a fraction of the cells (fig. 1). Consistent with data in other cell systems regarding the turnover rate of HSP, neuroprotection in cortical cultures persisted for at least 24 hours after the temperature of the cultures was returned to 37 °C (fig. 2). Lowenstein and colleagues examined HSP-induced protection in experiments in cerebellar granule cultures where hsp72 expression was seen on immunoblots from cultures treated with a calcium ionophore, arsenite, and thermal stress but not glutamate[33]. Pretreatment with thermal stress also conferred neuroprotection in subsequent excitotoxicity experiments.

In our experiments we did not identify the cellular site of the heat shock response, glia or neurons. Others have reported that hsp70 induction by thermal stress in vitro occurs preferentially in glial cells. Lowenstein et al.[33], found hsp70 immunoreactivity in neurons from glial poor cerebellear cultures exposed to thermal stress. Using the polymerase chain reaction, we have also found that an increase in HSP mRNA occurs in pure glial

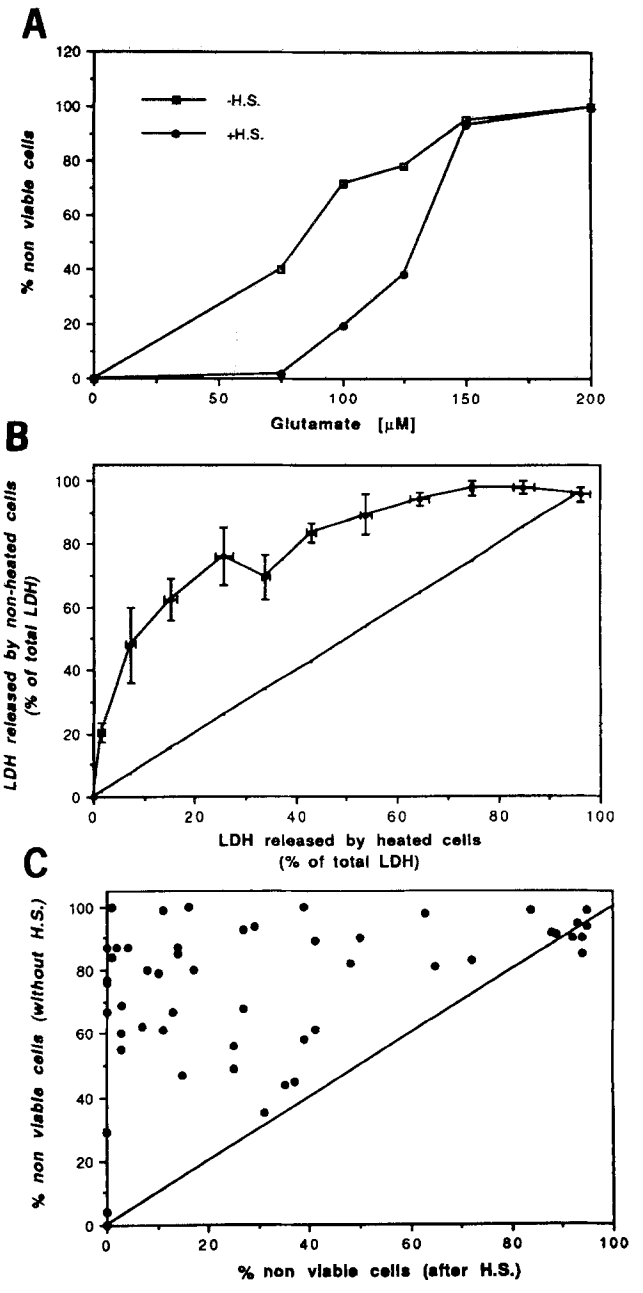

Figure 1. Neuroprotection in neuronal cultures induced by heat shock response. Half of the cultures in a plating were heat shocked for 20 minutes at 42.2 °C. Pairs of sister cultures were exposed to identical doses of glutamate, one heated and one not heated. Neuronal death was measured 24 hours after the 10 minute glutamate exposure which was performed in a low Cl-buffer solution. Cell counts and LDH-release quantified the amount of toxicity. *A* shows the leftward shift in the glutamate dose-toxicity relationship caused by heat shock in a single plating. *B* and *C* show compositite data from experiments on 51 pairs of control and heat shocked duplicate culture dishes in 9 seperate platings. In *B* and *C*, if the heat shock did not affect toxicity, then all the points would fall on the drawn line of identity; points above the diagonal indicate protection. The figure is taken from reference 50 with permission of the publishers; copyright Cell Press.

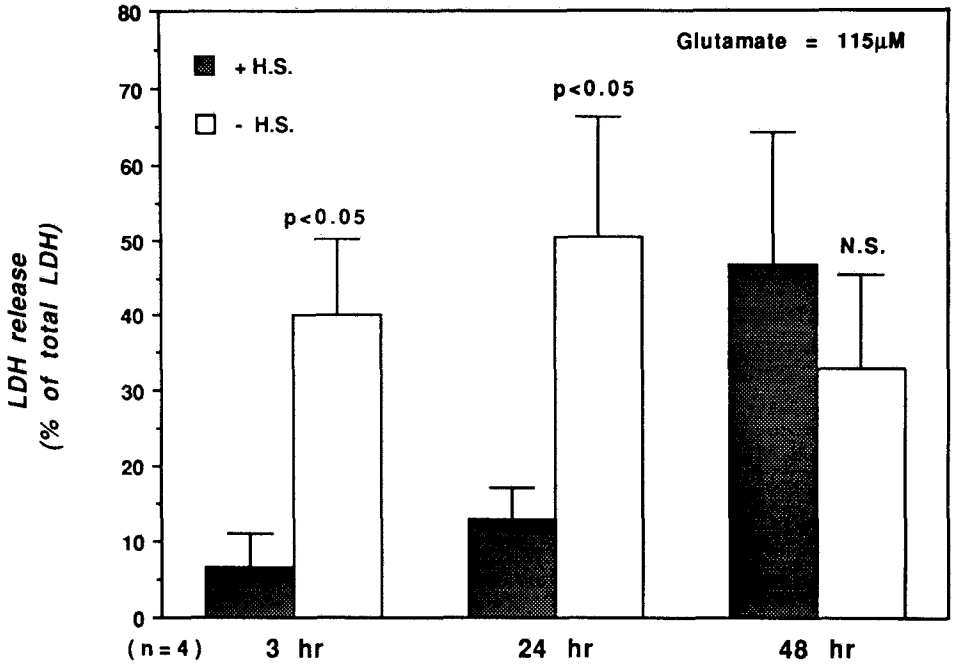

Figure 2. Duration of the heat shock-induced neuroprotection against excitotoxicity. Half of the dishes from a single plating were heat shocked then returned to 37 °C. At 3, 24 and 48 hours later, pairs of heated and non heated dishes were exposed for 5 minutes to various doses of glutamate (results of 115 μM exposure are shown here). Comparison of the excitoxicity data shows that heat shock induced protection at 3 and 24 hours but not at 48 hours. No protection was detected at 30 minutes. The figure is taken from reference 50 with permission of the publishers; copyright Cell Press.

cultures exposed to thermal stress (Reback, Patenaude and Koroshetz, unpubl. data). Interestingly, HSP have been described to transfer from glial to neuronal cells[65]. Considering that heat stress may have a variety of effects on neuronal function, we examined whether the heat shock response itself, as defined by a general decrease in general protein synthesis and increased synthesis of the HSP, was responsible for the observed neuroprotection. To approach this issue we showed that neuroprotection after heat shock was completely dependent upon new protein synthesis. Protection occurred when cells were exposed to glutamate 180 minutes after the thermal stress but not at 30 minutes. Neuroprotection was blocked by RNA and protein synthesis inhibitors (fig. 3). In addition, we found that inhibition of protein and RNA synthesis actually exacerbates the degree of neuronal death in the heated (but not the unheated) cultures. Thus previous thermal stress potentiates excitotoxic stress when the heat shock response does not occur[20]. This deleterious effect is unmasked only when the protective effect of HSP synthesis is prevented. If a non-stressful means of eliciting important components of the stress response can be engineered, a greater degree of protection may be evident. We also observed that, though the synthesis 72 kDa and 85 kDa proteins was enhanced by thermal stress, considerable amounts of these proteins were present in non-heated control cultures[50]. Since we had determined that growing cortical and hippocampal cultures in the presence of glutamate blockers markedly enhances neuronal survival[15,23], we suspected that endogenous glutamate receptor activation may be a stress leading to HSP expression in the control cultures. When concentrations of glutamate receptor blockers, which are effective in blocking such cell death and epileptiform activity, were added to the cultures we found a marked decrease in the baseline synthesis of 72 kDa and 85 kDa proteins[50]. The heat shock response could still be triggered by thermal stress to cells in cultures exposed to blockers.

These data from experiments in cortical cultures[50] and cerebellar cultures[33] showed that sub-lethal thermal stress in neuronal cultures induces HSP synthesis and protects neurons from glutamate excitotoxicity. In addition, we demonstrated that the neuroprotection following thermal stress lasted for 24 hours and, most importantly, was dependent upon the pattern of protein synthesis induced by stress.

Neural stress response in disease

The potential biologic importance of the stress response in mammalian brain is supported by the presence of the response in neurons and/or glia after a wide variety of insults, including ischemia[13,43,44,54,58,59], status epilepticus[34,56,60,66], trauma, thermal stress[36,44], drug treatment[38]. Neuroprotective effects were first suggested by the report that thermal stress increased retinal levels of three HSP and protected the photoreceptors against

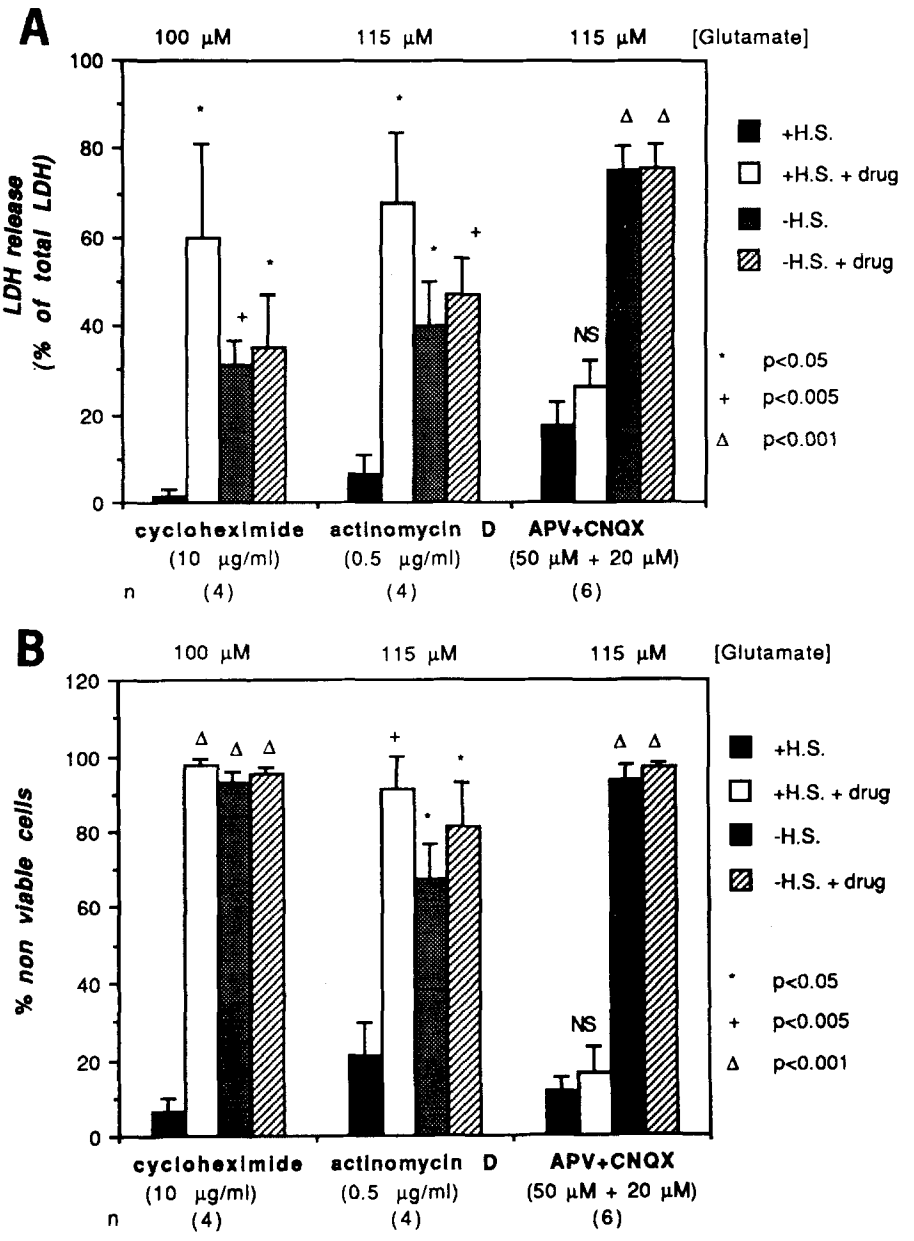

Figure 3. The protective effects of heat shock were blocked by protein and RNA synthesis inhibitors, but not by preincubation with glutamate antagonists. Cycloheximide (10 µg/ml) or actinomycin D (0.5 µg/ml) were added to the cultures 2 hours before the heat shock and washed out just prior to glutamate exposure, 3 hours after the heat shock. A significant protective effect of heat shock is seen as the difference between the black and stippled bars. Both cycloheximide and actinomycin D blocked the protective effect of heat shock. Treatment of the cultures with glutamate blockers did not affect glutamate toxicity or the protection conferred by heat shock. The figure is taken from reference 50 with permission of the publishers; copyright Cell Press.

light damage[2]. In animal models of stroke and epilepsy HSP induction preceded cell death and accompanied potentially destructive levels of excitation[58,60]. Hsp70 immunoreactivity has been used to chart the region of brain injury in models of stroke[43,44,58] and status epilepticus[66]. In addition, in brain ischemia neuronal populations that never accumulate hsp70 immunoreactivity can show prolonged expression of hsp70 RNA up until the time of cell death[43]. Induction of hsp70 immunoreactivity has also been demonstrated in neuronal populations outside the ischemic zone but synaptically linked to infarcted regions[21]. In studies in vivo, hsp70 has been seen in glia, neurons, and endothelial cells after various stress conditions[54]. A functional role for brain HSP response is suggested by experiments in which pretreatment of animals with sub-lethal thermal or ischemic brain stress induces hsp70 immunoreactivity and attenuates infarct size following a second, more severe, ischemic insult[9,22,32,59]. These data suggest a neuro-protective benefit of the CNS stress response and challenge the investigator to understand the regulation and mechanisms of cytoprotection of the HSP. A safe method of

pharmacologically inducing the stress response would likely be of therapeutic importance.

Possible mediators of neural HSP expression

Excessive excitatory stress is thought to be mediated by increased intracellular calcium, lowered intracellular glucose, kinase activation, lowered intracellular pH, proteolytic cleavage of structural proteins, and free radical mediated protein denaturation. Each may be involved in induction of the heat shock response by activating a constitutive protein, the heat shock factor, and enabling it to bind to a DNA sequence, the heat shock element, in the promoter region of heat shock genes[61]. Alterations in the structure of intracellular proteins due to changes in pH, protease activation or free radical reactions could give rise to abnormalities in the folding pattern of proteins within the neuron. Misfolded intracellular proteins can induce the expression of certain stress-related genes[21,24]. Elevated intracellular calcium, as occurs within neurons with excitotoxic stress, has been shown to be necessary for hsp26 (ref. 14) and hsp70 synthesis[48,62] in some but not all[19,27,67] cell systems. In some cell types raising intracellular calcium with calcium ionophores causes induction of HSP synthesis[42,48]. Phosphorylation, and hence kinases, may also play a central role in HSP synthesis. In some cases thermally activated human heat shock factor is phosphorylated and activation is blocked by kinase inhibitors[61]. A variety of kinases are activated by binding of glutamate to its receptors[16].

Potential protective actions of neural HSP

A complex combination of molecular events probably mediate excitotoxic cell death. Lowered glucose potentiates excitotoxicity, consistent with the stress on energy processes that excitatory neurotransmission generates[3]. Neuroprotection can be demonstrated when glutamate antagonists are used even after the excitotoxin is withdrawn[53]. This indicates the importance of continued glutamate release in potentiating toxicity after an initial insult. Free radicals scavengers[41], phospholipase/lipoxygenase[25,51] inhibitors, intracellular calcium chelators[64], protease inhibitors[28,57], endonuclease inhibitors[10], growth factors[37], the presence of glial cells[52], as well as the heat shock response protect against excitotoxic neuronal death. The effects of the heat shock response on the potential mediators of excitotoxic neuronal death are completely unknown. However, the mechanisms underlying HSP-mediated neuroprotection might be evaluated by determining how the HSP and closely homologous constitutive proteins (HSC) modulate the various toxic consequences of excitatory amino acid exposure.

The family of HSP proteins most extensively studied in the nervous system is the hsp70 family. We found expression of hsp70 mRNA and increased synthesis of protein of ~ 72 kDa in our cultures after thermal stress[50]. Hsp70 are the most highly conserved stress proteins[31]. They are the family of HSP induced to the greatest amounts by stress. Highly homologous proteins (hsc70) are present in nervous tissue in non-stressful conditions[5]. These constitutive proteins are involved in the disassembly of clathrin from coated vesicles in endocytosis[4,12]. A constitutive, and possibly an inducible, form of hsc70 has been found to be a microtubule associated protein involved in slow axonal transport[70]. Hsc70 proteins bind ATP, have weak ATP-ase activity and bind calmodulin in a calcium-dependent manner[62]. Interestingly, hsp70-calmodulin binding inhibits activation of calmodulin-dependent enzymes, and nitric oxide synthase is a calmodulin-dependent enzyme that has been implicated in promoting excitotoxicity[11].

Multiple kinases are activated by glutamatergic actions[16]. Hsp70 can be phosphorylated in the presence of elevated calcium[29]. The functional role of phosphorylation is not clear. A variety of cell proteins, including receptors and proteases, have been shown to form complexes with HSP and their function regulated by this association[6,39,47,55]. HSP binding to an important protease, phospholipase, kinase or transmitter receptor, with a resultant attenuation of destructive cellular effects, could underly a protective mechanism.

Irreversible protein denaturation due to thermal stress, protease activation, or free radical reactions may be an important common event in cell death and also triggers the heat shock response[24,42]. To account for the cytoprotective properties of the stress-induced hsp70, Pelham proposed that preferential binding of hsp70 to hydrophobic regions of partially denatured proteins inhibits an otherwise irreversible aggregaton of denatured proteins[46]. As the bond between HSP and partially denatured protein binding is released by ATP hydrolysis, cellular repair by proper refolding of partially denatured proteins occurs and function is restored. The chaperonins (hsp60), may work in concert with the hsp70 family to catalyze appropriate refolding[35]. In the context of excitotoxic and ischemic neuronal death we postulate that HSP might protect against the protein denaturation and aggregation that occurs as the result of free radical reactions, protease or phospholipase activation, change in intracellular pH, or the action of calcium-mediated proteases.

Inhibition of cellular metabolism, as occurs in stroke or hypoglycemia, is a potent stimulus for both HSP synthesis and excitotoxic neuronal death. HSP may have additional effects on cellular metabolism which may be protective against excitotoxicity. A family of stress proteins, the glucose-regulated proteins (grps) are synthesized in response to glucose deprivation, insulin, calcium ionophore, 2-mercaptoethanol, tunicamycin and anoxia, but not thermal stress[31]. Grp-94 is related

to hsp90; they are both calcium binding proteins and present in the endoplasmic reticulum and plasma membrane. The association between metabolic function and HSP is quite consistent. Interestingly, insulin has been reported to induce the expression of hsp70 (ref. 63) and homologous hsc70 and hsp60 proteins are found in mitochondria[18,35,40]. Mitochondrial hsp70 is involved in the translocation of nuclear-encoded precursor proteins across the mitochondrial membrane and in the folding of imported proteins in the mitochondrial matrix. Thus hsp70 may also function in the mitochondria to maintain proper complement of proteins critical for cellular energy production. The rat brain glutamate transporter, Glut-1, has also been reported to be a stress-inducible protein whose function is to increase glucose transport into a stressed cell[69]. These reports associating HSP with mitochondria and glucose regulation suggest that one way in which stress proteins might protect against excitotoxic neuronal death is by improving energy metabolism in threatened cells or enhancing the ability of the cell's energy-producing organelles to recover once the stress is withdrawn.

Conclusions

Future studies of the effects of HSP on particular molecular mechanisms mediating excitotoxicity may improve our understanding of evolution's attempt to develop successful cytoprotective strategies. Clinical benefit will come if these cytoprotective mechanisms can be induced safely as prophylactic treatments for patients in situations of known high risk or if the neuroprotective effects of the heat shock response can be mimicked or amplified by pharmacological administration of HSP-like molecules.

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