CHAPTER 13

RELEASE OF HEAT SHOCK PROTEINS AND THEIR EFFECTS WHEN IN THE EXTRACELLULAR SPACE IN THE NERVOUS SYSTEM

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Abstract: The ability of heat shock proteins (Hsps) to make cells more resistant to most types of metabolic stress has great implications for all post-mitotic cells, especially those of the nervous system. Preventing the loss of neurons is a more parsimonious approach to treatment of injury and disease than is replacement because of the difficulty in reconstructing the complex architecture of the nervous system, the basis for its function storage of information. The discoveries that the 70 kD Hsps are released and that neurons can take them up from the extracellular fluid provides a rationale to investigate how to use them to rescue injured neurons teetering between life and death. We present some of the history behind those discoveries and review the current understanding of the release and uptake of the 70 kD Hsps, discussing the distinct significance these observations have for neurons and some hypotheses about how extracellular Hsps protect neurons from potentially lethal injuries

Keywords: Exogenous; extracellular; Hsp70; Hsc70; neuronal injury; apoptosis

Abbreviations: bov, bovine; exo-Hsps, exogenously administered and/or extracellular Hsps; Hsc70, constitutive form of 70 kD Hsp; Hsp, all heat shock proteins; Hsp70, inducible form of 70 kD Hsp; Hsp/c70, both inducible and constitutive forms of 70 kD Hsps; hum, human; PC12 cells, rat phaeochromocytoma neuron-like cell line; recom, recombinant protein

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BACKGROUND AND HISTORY OF EXTRACELLULAR HSPS

For more than two decades after the initial discovery of the heat shock protein response by Ritossa (1962, 1963, 1996), the 70 kD heat shock protein and other members of this group discovered subsequently, all were considered to be intracellular proteins. This assumption arose from other discoveries in cell biology during that period showing that proteins destined to be packaged into secretory vesicles all were synthesized via the rough endoplasm reticulum and had a similar peptide sequence at their amino termini, the signal sequence, that targeted them to secretory vesicles (Stroud and Walter, 1999). However, beginning in the mid-1980s, reports began to be published suggesting that Hsp70 could be released, that it was present in extracellular fluid, and that it could be taken up from that compartment (reviewed in Tytell, 2005). Then, with the discovery that Hsp70 was present in normal human blood (Pockley et al., 1998), a large number of publications have appeared documenting the presence of Hsp70 in blood and other extracellular fluids like the cerebrospinal fluid (Steensberg et al., 2006). However, because this protein is found in all cells and tissues, which ones are the primary sources of extracellular Hsp70 remains to be determined. Nonetheless, there is no longer any doubt that extracellular Hsp70 has functional significance for both the immune system and tolerance to metabolic stress.

HOW ARE HSPS RELEASED FROM CELLS?

This issue is addressed in detail in Chapter 1 of the first of the volumes in this series (Asea, 2007), so only a few pertinent points will be discussed here. Asea (2007) makes the point that all of the Hsps are known to be intracellular proteins and lack the signal peptide that would target them to secretory vesicles for conventional release via secretory vesicles. His Figure 1 summarizes the two other potential release processes: (1) unregulated leakage from cells whose membranes are damaged because of physical disruption subsequent to trauma or necrosis or (2) regulated release of membrane-bounded vesicles called exosomes. The blebbing of small membrane-bounded vesicles from the surfaces of cells has been recognized for a long time in a few types of cells known to release material by the process called apocrine secretion. These are the secretory epithelial cells of the mammary gland, apocrine glands in the skin, ciliary glands of the eyelid, and the wax-producing cells of the external ear canal. More recently, exosome production has been observed in a wide variety of cells (de Gassart et al., 2003; Fevrier and Raposo, 2004). The significance of this process is that it provides a means for a cell to release small volumes of its cytoplasmic constituents. Since Hsps are relatively abundant cytoplasmic constituents, with Hsp70 comprising 0.29% of total brain protein (Gutierrez and Guerriero, 1995) and Hsc70 being 2%–3% of total spinal cord protein (Aquino et al., 1993), exosomes released by neurons and glial will contain Hsps. In fact, they also can contain functional mRNAs (Valadi et al., 2007), implying that the cell receiving the exosomes potentially could manufacture more of the protein than

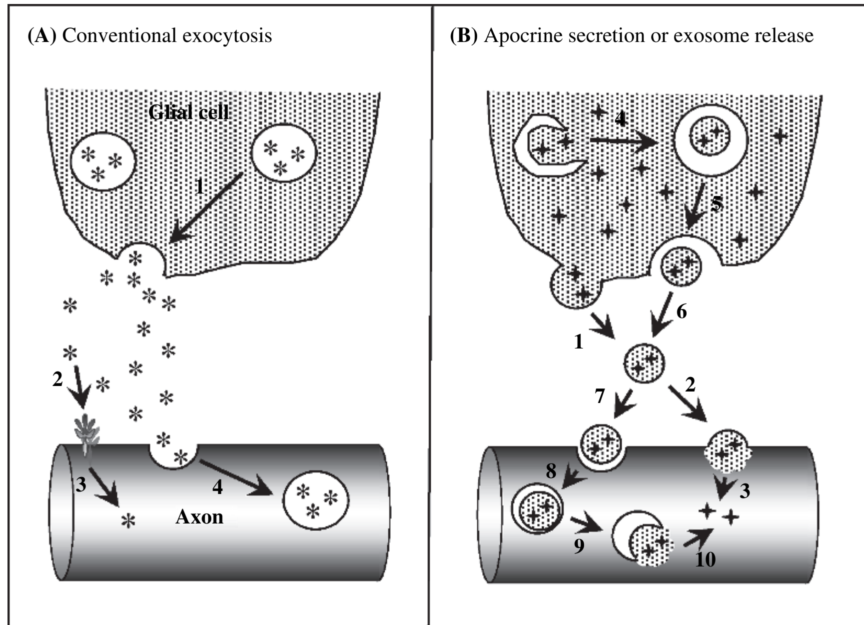

Figure 1. Potential mechanisms for the release and uptake of Hsps. For the purposes of these examples, release occurs from a glial cell and uptake by an axon, but these hypothetical transfers could occur between any groups of cells. **Panel A** presumes that an unknown mechanism allows Hsp70 or Hsc70, represented by asterisks, to become concentrated in secretory vesicles. (Hereafter, the proteins will be jointly designated as Hsp/c70.). Arrow 1 indicates conventional exocytosis of the Hsp/c70, so that it is present in the extra-cellular fluid. Arrow 2 indicates interaction of the exo-Hsp/c70 with the axonal membrane, reflected by a change in its shape. Arrow 3 indicates diffusion of Hsp/c70 through the plasma membrane by an unknown mechanism so that it is in the cytoplasm of the axon and is free to interact with other cytoplasmic components as endogenously synthesized Hsp/c70 would. Arrow 4 indicates uptake of Hsp/c70 by conventional pinocy-tosis, after which it is present in endocytotic vesicles. From there it may diffuse through the endosomal membrane to enter the cytoplasm or it may remain inside as the endosome cycles through the endolysosomal pathway of the neuron. How that process might affect stress tolerance is not known. **Panel B** depicts two ways that exosomes containing glial cytoplasmic Hsp/c70 (4-pointed stars) could wind up in the cytoplasm of the axon. On the left side, arrow 1 indicates conventional apocrine secretion, in which small vesicles containing a mixture of cytoplasmic constituents bud off the cell surface . This process is the way that milk is secreted by the mammary gland and mucus by goblet cells lining the intestinal tract. Alternatively, some of the cytoplasm of the donor may be enclosed within a vesicle by the well-known process of autophagy (arrow 4). Autophagy's role in the production of exosomes is discussed in the text. The multivesicular body (small vesicles within a larger vesicle) resulting from autophagy can then be released by the glial cell via exocytosis (arrows 5 and 6). Thus, both apocrine secretion and exosome release yield the same result, a membrane enclosed vesicle containing cytoplasmic constituents in the extracellular space. The released vesicle then may interact with the axon in either of two hypothetical ways. It may fuse with the plasmalemma of the recipient cell, releasing its contents, including Hsp/c70, into the neuron's cytoplasm (indicated by arrows 2 and 3). Alternatively, the released vesicle may be phagocytosed by the neuron, forming another multivesicular body. Then the inner vesicle membrane may fuse with the outer vesicle membrane in a form of intracellular exocytosis, releasing the Hsp/c70 and other cytoplasmic constituents from the glial cell into the cytoplasm of the neuron (indicated by arrows 8–10). (This figure modified from Tytell (2005)

what is contained in the exosomes. It seems that this alternative release process may be a common means by which cells release Hsps, as exosomes released from maturing red blood cells and from normal and hyperthermically stressed blood-borne mononuclear cells contain Hsc- or Hsp70 (Mathew et al., 1995; Geminard et al., 2001; Lancaster and Febbraio, 2005).This possibility is especially important for axons, as explained in the next section.

Though exosomes may account for Hsp release by cells in many situations, recent work shows that secretion via conventional secretory vesicles is also an option. Evdonin et al. (2006) found that two types of transformed human keratinocyte cells released Hsp70 from granules that included the secretory vesicle marker, chromogranin A and that the secretion was blocked by an inhibitor of conventional exocytosis, brefeldin A. However, this paper does not address the mechanism by which Hsp70 becomes concentrated in secretory vesicles, despite its lack of a signal sequence. Since this phenomenon has not been reported in other cells, it remains to be determined if it is specific to these keratinocytes.

If exosome-based Hsp70 release is its primary route for leaving a cell, one still needs to explain how it becomes a soluble protein in the blood or other extracellular fluids. The possibility that it can diffuse through lipid bilayers, though unexpected for a 70 kD protein, is supported by a number of observations suggesting that Hsp70 is an amphipathic protein. The first hint that this might be the case was in the observation by Guidon and Hightower that both Hsc70 and Hsp70 purified from rat brain included nonesterified fatty acids (Guidon and Hightower, 1986a, b). A few years later, Alder et al. reported the surprising observation that Hsp70 added to a solution on one side of an artificial lipid bilayer formed an ion conducting channel in that bilayer (Alder et al., 1990). Despite these intriguing early observations, six years passed before this property of Hsp70 was again examined. Negulyaev et al. (1996) found that potassium channel activity was increased in a cultured monocyte cell line treated with a mixture of soluble, extracellular Hsc- and Hsp70. Then Arispe and co-workers, using artificial lipid bilayers and liposomes, found that soluble Hsc- and Hsp70 formed potassium-conducting channels and promoted the spontaneous fusion of liposomes (Arispe and De Maio, 2000; Arispe et al., 2002). Both these activities of the two proteins were modulated by ATP and ADP, though in different ways. ATP increased the frequency of channel opening, whereas ADP inhibited it. Conversely, ADP in low concentration promoted liposome fusion induced by Hsc70, but ATP inhibited it. For Hsp70, both nucleotides inhibited its liposome-fusing activity. These results are the only ones that have shown clear distinctions in functional activities of the two forms of the protein. What they imply for the in vivo functions of Hsc- and Hsp70 remains to be examined.

The above observations leave us with several intriguing possibilities concerning the release, uptake and effects of extracellular70 kD Hsps. First, there is no doubt that the 70 kD Hsps can be released from cells, but three routes are suggested, exosomes, conventional secretory vesicles, and diffusion through the plasmalemma (see Figure 1). Once in the extracellular space, the proteins can interact with the membranes of cells, possibly altering ion conductance, cell-to-cell interactions,

and/or diffusing through the membrane into the cytoplasm. Each of these events will have distinct functional outcomes for the cells with which the 70 kD Hsps are interacting and it is likely that the specific events triggered by the Hsps will be both context and cell-type dependent.

THE SPECIAL SIGNIFICANCE OF HSP RELEASE AND UPTAKE IN THE NERVOUS SYSTEM

In the nervous system, neurons, especially large ones with long axons (i.e., tens or hundreds of times greater in length than the cell body diameter), have a refractory stress response, failing to turn on the stress protein synthesis system after stresses such as hyperthermia or ischemia (Mathur et al., 1994; Voisin et al., 1996; Batulan et al., 2003; Tidwell et al., 2004; Robinson et al., 2005). Thus, the greater vulnerability of neurons to metabolic stress may be partly a consequence of their poor Hsp response. Another reason for the stress vulnerability of neurons with long axons arises from the fact that axons lack the full complement of protein synthetic machinery found in the cell body. For that reason, they are largely dependent on the latter, as well as the system of axonal transport, for protein renewal (Gallant, 2000; Brown, 2003). This fact leads to one more handicap for the neuronal stress protein response that is specific to the axon and is typically overlooked. Hsp90, and -70 are transported from the neuronal cell body towards the axon terminal at a slow rate, about 2 mm/day (Clark and Brown, 1985; Waegh and Brady, 1989; Black et al., 1991; Bernstein et al., 2001). Hsp25 axonal transport rate has been estimated to be faster, about 20 mm/day (Murashov et al., 1998), but is still about 10-fold slower than the 200 mm/day rate of fast axonal transport (Cyr and Brady, 1992). These slow transport rates mean that, even if a neuron could respond robustly to an injury to part of its axon several centimeters away, it would take at least 24 hours for the newly synthesized stress-induced Hsps to reach the site of injury, clearly too long to be of use. However, these neurons and their axons and dendrites are surrounded by glial cells that do respond in the typical way to metabolic stress, showing prominent increases in Hsp content (Manzerra et al., 1993; Koroshetz and Bonventre, 1994; Voisin et al., 1996; Krueger et al., 1999). Thus, if glial cells can release exosomes containing either or both Hsps and their mRNAs, then they can serve as a local source of additional protein or make it possible for axons (and dendrites as well) to synthesize the protein via translation of the message at any points along their lengths, compensating for the problem of slow transport over long distances.

In fact, glia to axon transfer of Hsp70 in the giant axon of the squid was the first observation suggesting that intact, normally functioning cells could release Hsps and that they could be taken up by neighboring cells (Tytell et al., 1986). That observation was replicated in another invertebrate with large axons, the crayfish (Sheller et al., 1998), but for vertebrate models, the evidence is more limited. Hsp70 was observed to be synthesized in the glial sheath of the severed frog sciatic nerve, taken up by the sciatic nerve axons and retrogradely transported (Edbladh

et al., 1994). In the rat cerebellum, hyperthermia strongly stimulated the production of Hsp27 and -32 in Bergman glial cells, after which those Hsps were also localized in Purkinje cell synaptic terminals (Bechtold and Brown, 2000); the relatively short interval between these events suggested that the glia were the source of the Hsps, not the neuron cell bodies. This possibility is supported by the observations that mammalian glial cells in culture release Hsp70 (Guzhova et al., 2001; Taylor et al., 2007). Furthermore, the Hsp70 released from astrocytes is in the form of exosomes (Taylor et al., 2007). As yet, no one has shown directly in the mammalian nervous system that glia-derived Hsps are taken up by neurons and render them more resistant to metabolic stress. However, it is likely that this uptake occurs, since our observations and those of others show clearly that soluble extracellular Hsc- and Hsp70 can be taken up by neurons (Houenou et al., 1996; Guzhova et al., 2001; Yu et al., 2001; Tidwell et al., 2004; Tytell, 2005; Novoselova et al., 2005; Robinson et al., 2005; Robinson et al., 2007).

NEUROPROTECTIVE AND NEURODEGENERATIVE EFFECTS OF EXTRACELLULAR, EXOGENOUS HSPS

In the 1980s, conventional wisdom held that a protein the size of Hsp70 could not pass through the cell membrane without the involvement of a pore or membrane transporter. Despite this bias, we and a few others were intrigued by the observed release and cell-to-cell transfer of the protein (Tytell et al., 1986; Hightower and Guidon, 1989). Additionally, the strong association between Hsp70 content and neuronal survival shown by the many stress-preconditioning papers published from the late 1970s on, prompted a few researchers to try administering the protein itself to the injured neural tissue or cells. This unconventional approach was motivated by the goal of developing therapeutic uses of the potent survival-promoting activity of Hsps, since the preconditioning model was not applicable for the treatment of unpredictable, acute injury of the nervous system. The results obtained to date confirm that extracellular Hsc- and Hsp70 can promote survival and function of neurons subjected to a wide variety of stressful conditions, including hyperthermia, lack of neurotrophic factors, ischemia, free radical damage, and physical trauma; these are summarized in Table 1. Additionally, one report made the novel observation that Hsp70, -90, and -32 all stimulated the activity of microglia, promoting phagocytosis of β-amyloid, whereas Hsp27 did not (Kakimura et al., 2002). Regarding Hsp27, the only other report in which this member of the group was tested also showed it did not have a protective effect in concussive brain injury, though it did alter the K^+ channel function, but in a negative way (Armstead and Hecker, 2005). One other observation suggests that exo-Hsps also may be useful in treatment of chronic neurodegenerative conditions. Human neuroblastoma cells transfected with the Huntington's disease polyglutamine-repeat gene were protected by addition of a mixture of Hsc- and Hsp70 to the culture medium, the treated cells showing fewer and smaller polyglutamine protein aggregates (Novoselova et al., 2005). This effect is consistent with the well-known protein folding functions of the Hsps and indicates that the exo-Hsps must have entered the cytoplasm

Table 1. Summary of effects of exo-Hsps on neural and glial cells and tissues in vitro and in vivo

Model used	Hsps used	Effects	Ref.
In vitro			
Neuroblastoma cells	bov skeletal muscle Hsc/Hsp70	Increased survival after heat stress and resistance to drug-induced apoptosis	Guzhova et al. (2001)
Rat microglia	hum-Hsp90, recom hum-Hsp70, recom rat-Hsp32, recom hum-Hsp27	All except Hsp27 stimulated cytokine production and phagocytosis of β-amyloid	Kakimura et al. (2002)
Rat brain slice	recom hum-Hsp70	Synaptic transmission preserved during hyperthermia	Kelty et al. (2002)
Rat cortical neurons	recom bov-Hsc70	Inhibition of ischemia-induced Hsp70 increase	McLaughlin et al. (2003)
PC12 cells	recom bov-Hsc70, recom hum-Hsp70	Increased apoptosis	Arispe et al. (2004)
Rat olfactory cortex anoxia	bov skeletal muscle Hsc/Hsp70	Preservation of glutaminergic neurotransmission	Mokrushin et al. (2004)
Rat cortical neurons	bov skeletal muscle Hsc/Hsp70	Preservation of glutaminergic neurotransmission	Mokrushin et al. (2005)
Human neuroblastoma cells transfected with Huntington gene	bov skeletal muscle Hsc/Hsp70	Promoted survival by reducing poly-Q protein inclusions	Novoselova et al. (2005)
Chick embryonic motor neurons	recom bov-Hsc70 or hum-Hsp70	Promoted survival in absence of trophic factors	Robinson et al. (2005)
Chick embryonic motor neurons	Full length & substrate-binding portion of hum-Hsc70	Promoted survival after free radical damage	Robinson et al. (2007)
In vivo			
Rat retina	bov skeletal muscle Hsc/Hsp70	Reduction of light-induced photoreceptor degeneration	Yu et al. (2001)
Mouse sciatic nerve	bov brain Hsc70, bov skeletal muscle Hsc/Hsp70, recom bov- Hsc70, recom hum-Hsp70	Reduction of axotomy-induced apoptosis of dorsal root ganglion neurons and spinal motor neurons	Houenou et al. (1996) and Tidwell et al. (2004)
Pig brain, neonatal	recom hum Hsp70 and Hsp27	Preservation of post-injury K^+ channel-related cerebrovasodilation by Hsp70, but not Hsp27, after concussive injury	Armstead and Hecker (2005)
Chicken embryo	hum recom Hsc70	Prevention of developmental programmed motor neuron death	Robinson et al. (2005)

(In chronological order, then alphabetically by author)

of the neurons. All these observations suggest that Hsc- and Hsp70 present in the extracellular fluid surrounding neurons are as broadly neuroprotective as the endogenously synthesized proteins have been show to be in preconditioning and transfection-induced overexpression experiments. It is also apparent that Hsp90 and -32, like Hsp/c70, may have neuroprotective activities when present in the extracellular space, but, except for one observation of the release of Hsp90α by cultured vascular smooth muscle cells (Liao et al., 2000), little is known about whether these two Hsps occur naturally outside the cell and how they interact with cells from the extracellular space.

In only one case has extracellular Hsp/c70 been found to cause neuronal death. Arispe et al. (2004) showed that as little as 0.3 μg/ml of Hsc70 or Hsp70 added to the culture medium of PC12 cells caused a significant increase in cell death. This unexpected toxicity was a result of the presence of phosphatidylserine on the extracellular side of the plasma membrane of PC12 cells, a normal feature for them, but, for other neurons, it occurs only at the beginning of apoptosis. They found also that the toxicity was increased when either ATP or ADP was added with Hsc70, whereas only ATP increased the toxicity of Hsp70, a functional distinction between the two proteins that they had noted earlier in their interactions with liposomes (Arispe et al., 2002). These results, they suggested, may be caused by the formation of ion channels in the plasma membrane and may explain other observations that overexpression of Hsp/c70 can be toxic (Feder et al., 1992), a possibility that must be taken into account when considering neuroprotective strategies using the Hsps.

ENDOGENOUS SOURCES OF EXTRACELLULAR HSPS

As mentioned at the beginning of this chapter, the case for the physiological relevance of extracellular Hsps received a major boost when Pockley and coworkers showed that soluble Hsp70 was present in normal human blood (Pockley et al., 1998). That observation prompted many more studies that have found changes in blood levels of Hsp70 with various types of injuries, diseases, and even after vigorous exercise (Giraldo et al., 1999; Walsh et al., 2001; Pockley et al., 2002; Febbraio et al., 2002; Pittet et al., 2002; Zhu et al., 2003; Campisi and Fleshner, 2003; Njemini et al., 2003; Dybdahl et al., 2004; Kimura et al., 2004; Lancaster et al., 2004; Fleshner and Johnson, 2005; da Rocha et al., 2005; Johnson et al., 2005; Marshall et al., 2006). These numerous observations have not, unfortunately, provided a coherent explanation of the functional significance of circulating Hsp70 and what the alterations mean. There is some evidence that the protein may enter the bloodstream simply as a result of cell damage or death after injury, analogous to the appearance in the blood of cardiac cell cytoplasmic components, like creatine kinase and troponin, after a heart attack. For example, Hsp70 increased in the blood as a function of time on a heart bypass pump during open-heart surgery (Dybdahl et al., 2004) or the extent of surgical trauma during liver resection (Kimura et al., 2004). From this perspective, the finding of a negative correlation between blood Hsp70 concentration and recovery after traumatic brain injury is not surprising (da Rocha et al., 2005). However, the opposite also has been reported (Pittet

et al., 2002). Conflicting results exist as well for the functional impact of circulating Hsp70. Asea and colleagues found extracellular Hsp70 to have pro-inflammatory effects on monocytes (Asea et al., 2000) and a recent review by Fleshner proposed that an increase in circulating Hsp70 serves as a "danger signal" and stimulates the innate immune response (Fleshner and Johnson, 2005). Conversely, others found evidence for anti-inflammatory effects of extracellular Hsp70 (Yoo et al., 2000; van Eden et al., 2005), even in relation to microglial cell activation in the brain (Yenari et al., 2005). The best explanation for these opposing reports is that the biological impact of extracellular Hsp70 must be highly context-and concentration-dependent, meaning that it is contingent on when, where, and how much of it is present. Details, recent findings, and current hypotheses on the immunomodulatory functions of extracellular Hsp70 can be found in reviews by Asea and Calderwood in volume 1 of this series (Asea, 2007; Calderwood et al., 2007).

Another event that causes temporary increases in blood Hsp70 concentrations is exercise, a context that seems very different than the acute injury and infection-related events described above, but may have some physiological effects in common. For example, exercise can cause microscopic damage to skeletal muscle (Miyake and McNeil, 2003), which may account, in part, for the exercise-induced increase in skeletal muscle Hsps (Thompson et al., 2003), and it is well known to alter the responsiveness of the immune system (Nieman, 2007). Thus, it is not surprising that Febbraio and colleagues have shown that vigorous exercise in healthy individuals acutely elevates blood Hsp70 concentration (Walsh et al., 2001). Some of this Hsp70 may be derived from specific organs, such as the liver (Febbraio et al., 2002) and the brain (Lancaster et al., 2004). From subsequent in vitro studies, they provide evidence that the Hsp70 is released via exosomes (Lancaster and Febbraio, 2005), but this creates a puzzle, especially with respect to release from the brain. How would Hsp70-containing exosomes released from the brain parenchyma enter the bloodstream, given the blood-brain barrier? Other work shows that a primary trigger for Hsp70 release into the blood is the activation of α1-adrenergic receptors (Johnson et al., 2005; Johnson and Fleshner, 2006) and the authors suggest that the exosome is the vehicle for that release. However, as described previously for the brain, it is not known how exosomes released from cells would get into the bloodstream unless they were produced by cells in direct contact with the blood. Thus, the simplest explanation for the phenomenon is that the blood vessels themselves are the source of the blood-borne Hsp70. If that is the case, then the apparent release of Hsp70 into the blood by the brain and liver may be accounted for by the high vascularity of those organs. Much work needs to be done to sort out these questions.

HOW DO EXTRACELLULAR 70 KD HSPS PROMOTE NEURONAL SURVIVAL?

In all of the injury models in which exo-Hsc70 or Hsp70 have been shown to promote neuron survival, apoptosis is the primary mode of neuronal death. Thus, our studies of the details of the neuroprotective effects of the protein have used

a well-established model of neuron apoptosis, the primary embryonic chick spinal motoneuron (Milligan et al., 1994). When these neurons are prepared for culturing, they require trophic factors in order to survive and extend neurites. The optimal mixture of trophic factors is provided by an extract of chick skeletal muscle (MEx) added to the culture medium. Without MEx, the intracellular signals leading to apoptosis are initiated by 16 hours, committing the neurons to die (Li et al., 2001). Hsc70, Hsp70, or a mixture of both, can be substituted for MEx to promote neuron survival, but must be added to the culture medium within the first 12 hours; the Hsps are ineffective after that time (Robinson et al., 2005). This interval precedes the first detectable step in motoneuron apoptosis, the translocation of Bax from the cytoplasm to organelles (Li et al., 2001). Therefore, the proteins, jointly termed exo-Hsp/c70, must be inhibiting signals at or before the decrease in mitochondrial membrane potential and release of cytochrome C.

The possibility that exo-Hsp/c70 inhibits early kinase-mediated events in the commitment of a cell to undergo apoptosis implies that this function may be separate from its well-known protein folding role. This speculation was raised in a review by Gabai and Sherman (2002) and, in support of it, they referred to earlier, intriguing reports that mutant Hsp72 lacking ATPase activity still prevented heat-induced death of fibroblasts transfected to express such mutant proteins; it also inhibited c-Jun NH_2-terminal kinase (JNK) (Volloch et al., 1999; Yaglom et al., 1999; Park et al., 2001). We examined the same question in oxidatively stressed cultured motor neurons by pretreating them with the substrate-binding domain of Hsc70 (Robinson et al., 2007). Not only was it neuroprotective, it was effective at 0.1 μM, which was one-tenth the concentration of intact Hsc70 needed to achieve a similar effect. The ATPase domain, in contrast, had no protective effects at the same concentrations. Changes in kinase activity were not monitored in these experiments, but we did assess mitochondrial membrane potential and found that it was preserved by pretreatment with both the intact Hsc70 and the substrate-binding domain, but not by the ATPase domain. These results support the possibility that the extracellular proteins enter the neurons and can interact with mitochondria in protecting them from oxidative stress. We also found that the substrate-binding domain could rescue neurons when added immediately after, rather then before, exposure to peroxide-induced oxidative stress (unpublished observations), but this required a higher concentration, 1 μM, then when it was added 4 hours before the peroxide. Why the post-injury treatment required a 10-fold higher concentration of the substrate-binding domain to produce significant protection is not known. Although it is clear that extracellular Hsp/c70 can enter cells from the extracellular space (Houenou et al., 1996; Guzhova et al., 1998; Fujihara and Nadler, 1999; Guzhova et al., 2001; Yu et al., 2001; Tidwell et al., 2004; Novoselova et al., 2005; Robinson et al., 2005) and affect elements of several signal transduction pathways that improve neuronal survival, there is no evidence as yet for the existence of any membrane receptor type of interaction, as there is for cells of the immune system (Asea et al., 2000, 2002; Asea, 2005) (see also volume I of this series (Asea and De Maio, 2007)). However, other types of interactions

with neuronal membranes must be considered in light of the observations presented earlier in this review on the membrane interactions of Hsp/c70. Vigh and coworkers have for some time proposed that plasma membrane fluidity changes may be a trigger for induction of the Hsp response (Vigh et al., 1998; Balogh et al., 2005). Furthermore, Hsp70 has been found to be associated with lipid rafts in neuronal tissue (Chen et al., 2005) and to stabilize lysosomal membranes in stressed tumor cells and transformed fibroblasts (Nylandsted et al., 2004). Thus, further study is needed on the possibility that stabilization of the plasma membrane and the membranes of intracellular organelles is one of the actions of exo-Hsp/c70.

In addition to direct effects on injured neurons, one needs to consider that in vivo, Hsp/c70 and other Hsps may promote neuronal survival by eliciting beneficial responses of the surrounding glial and other non-neuronal cells. This possibility is suggested by the previously mentioned stimulation of microglial phagocytosis of amyloid by Hsp90, -70, and -32 (Kakimura et al., 2002). It serves as a reminder that the potential positive and negative effects of exogenous Hsps will require an understanding of how they may alter the functions and interactions of all cells in the nervous system, not just neurons.

CONCLUSIONS

There is extensive evidence that the 70 kD Hsps are normal constituents of the extracellular fluid and that they vary with physiological stress. That fact, together with the growing number of reports that administration of the proteins enhances neuronal survival under a wide variety of stressful conditions, makes the potential for the development of therapeutic uses of the proteins very likely. The focus of much of the research on traumatic injury and degenerative diseases of the nervous system has been and remains on regeneration and replacement. However, we suggest that the potential for Hsps to rescue injured neurons offers an approach that is more expedient and achievable sooner. One of the key issues that needs to be addressed to begin translating this research into practical clinical treatments is to determine the interplay between the immunomodulatory and cytoprotective effects of the Hsps because inflammatory responses occurring after acute nervous system injury and in many chronic neurodegenerative diseases cause much greater loss of neurons than those directly affected by the injury or disease process.

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The therapeutic potential of Hsp70 received further support recently in the report by Gifondorwa et al. that regular, repeated intraperitoneal injections of human recombinant Hsp70 in a transgenic mouse model of amyotropic lateral sclerosis inhibited the onset of paralysis and extended lifespan (Gifondorwa, D. J., M. B. Robinson, C. D. Hayes, A. R. Taylor, D. M. Prevette, R. W. Oppenheim, J. Caress, and C. E. Milligan. Exogenous delivery of heat shock protein 70 increases lifespan in a mouse model of amyotrophic lateral sclerosis (2007) J Neurosci 27 13173–13180).

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