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Review

Endothelial heat shock response in cerebral ischemia

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Summary. Blood vessels and nerve fibers often course alongside one another in an orderly fashion throughout the brain. This clustering gives rise to a reciprocal signaling network between endothelial and nerve cells that follows highly stereotyped anatomical patterns. One such molecular signal that is produced by endothelial cells and acts on surrounding neurons is heat shock protein 70. Here we briefly review recent studies that have revealed a critical role of this signaling pathway during harmful insults to the brain, particularly during episodes of cerebral ischemia.

Key words: Blood vessels, Heat shock proteins, Hypoxia, Penumbra, Infarct core

Endothelial cell networks

Endothelial cells lining the blood-vessel wall occupy a strategic location in the vasculature of the brain as they separate flowing blood from underlying tissue, regulate the trafficking of cells and nutrients, maintain vasomotor tone and provide molecular signaling cues to nerve fibers (Fig. 1). The various functions of endothelial cells suggest that these cell types are differentially regulated in time and space, giving rise to endothelial cell heterogeneity and vascular diversity (Carmeliet, 2003). Indeed, endothelial cells often take advantage of their heterogeneity to produce a wide range of chemical signals (such as artemin, integrin, neurotrophin 3 and Gax) that affect axon guidance, axon terminal arborization and ultimately synaptic function (Pasqualini and Ruoslahti, 1996; Carmeliet and Tessier-Lavigne, 2005; Wu et al., 2005). However, it should be noted that endothelial cells are not the only inner constituents of the blood-vessel wall; mural cells (or pericytes) also line the vessel wall and produce different proteins depending on where they are in the brain and what events are occurring in a given site (Fig. 2). In general, a major role for endothelial cells is aligning with nerve fibers to form vascular and neural networks where highly stereotyped chemical patterns often flow along one another (Cleaver and Melton, 2003; Carmeliet and Tessier-Lavigne, 2005). For instance, neurons surrounding the vasculature may produce signals such as vascular endothelial growth factor (VEGF) and neuropeptide Y (NPY) to directly stimulate endothelial cell proliferation, migration and overall blood vessel development (Zukowska et al., 1998; Ekstrand et al., 2003; Weis and Cheresh, 2005).

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Endothelial cells and hypoxia

Endothelial cells are highly glycolytic and consume relatively low amounts of oxygen compared with neurons. In this regard, endothelial cells are among the most hypoxia-tolerant of all mammalian cells, surviving in acutely and chronically hypoxic conditions without evidence of cellular damage (Tucci et al., 1997). How endothelial cells remain viable and retain functional integrity under the above adverse conditions is not yet clear. There are, however, tantalizing cues regarding a family of related proteins called hypoxia- inducible transcription factors (HIFs) which are expressed when oxygen becomes scarce (Pugh and Ratcliffe, 2003). Under this scenario, endothelial cells may have a highly efficient system for turning on HIFs that can counteract the effects of low oxygen levels. It is also conceivable that in response to diminished ambient oxygen, endothelial cells can slow or arrest their growth-division cycle thereby altering the distribution and proliferation state of daughter cells. Indeed, there is evidence that under adverse oxygen conditions endothelial cells increase time spent in S phase (period of DNA synthesis and replication) compared with time spent in G0/G1 phases (periods of growth and metabolic activity; Tucci et al., 1997). In addition, because hypoxia or cerebral ischemia causes up-regulation in brain glycolysis (MacMillan and Siesjo, 1972) and parallel up-regulation

of the brain endothelial glutamate 1 (Glut1) glucose transporter (Bondy and Lee, 1993; McCall et al., 1996), over-expression of heat shock proteins (HSPs or molecular chaperones) often occurs throughout the neurovasculature. In this regard, glucose-regulated proteins are constitutively expressed in endothelial cells (Boado et al., 2003); the synthesis of these proteins (as a result of low levels of glucose and oxygen) activate HSPs (Sharp et al., 1999). Indeed in response to hypoglycemia or hypoxia ischemia, the neurovascular network mounts a stress response with induction of HSPs, most notably the 70-kDa species (Rajdev and Sharp, 2000; Torres et al., 2004; Kafel et al., 2006).

Heat shock proteins

High expression of Hsp70 might provide endothelial cells with another compensatory mechanism for dealing with hypoxia-ischemia, cerebrovascular flow dysregulation and/or aberrant vascular remodeling (Yenari et al., 1999). All of these injuries could initiate manifold pathogenic cascades of chemical events resulting in the demise of the brain microenvironment and, ultimately, synaptic and neuronal loss (Kiang and Tsokos, 1998; Yenari et al., 1999; Sherman and Goldberg, 2001). Induction of Hsp70 is regulated by *trans*-acting heat shock factors (HSFs) and a cis-acting heat shock element (HSE) present within promoters of heat shock genes (Kiang and Tsokos, 1998). Under pathological states of cellular stress, Hsp70 dissociates from HSFs and binds selectively to denatured or damaged proteins in an ATP-dependent manner (Ohtsuka and Suzuki, 2000). Several heat shock-activated signaling pathways are known to interact with cytosolic Hsp70 (Fig. 3). For example, the c-jun N-terminal kinase (JNK) pathway as well as certain "death" signaling proteins [e.g., Bcl-2-associated athanogene-1 (BAG 1) and Bcl-2-associated X-protein (BAX)] interact with either the ATPase domain or the peptide binding domain of Hsp70 to eliminate unfolded polypeptides (Giffard and Yenari, 2004). In addition, Hsp70 appears to be essential for the ubiquitination and rapid degradation of many abnormal proteins back to amino acids, although at widely different rates (Sherman

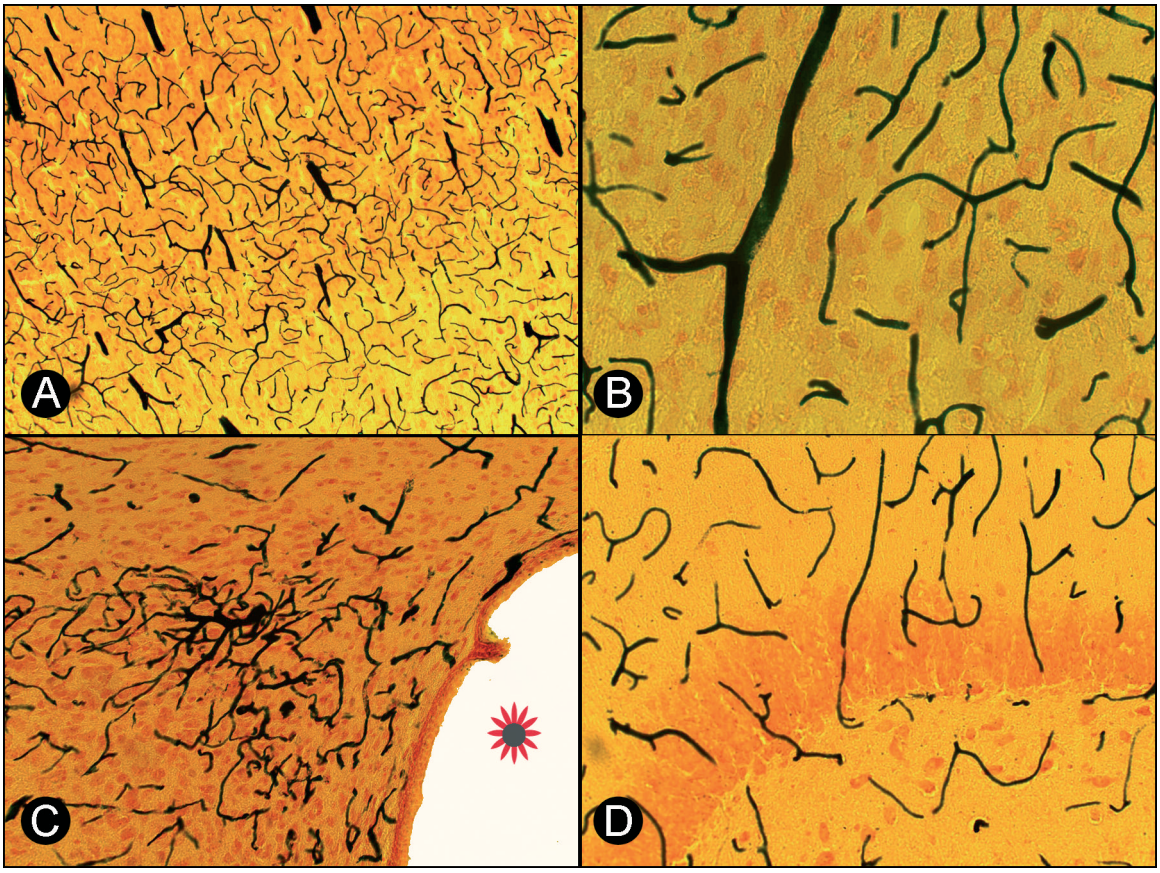

Fig. 1. Bright-field photomicrographs of the rat neurovasculature network. Visualization of the neurovasculature was accomplished with blue pigmented gelatin (Pelikan) ink directly injected into the ascending aorta. Brain sections (50 μm thick) were then counterstained with Neutral Red. Note the regular arrangement of blood vessels in the motor agranular cortical area **(A)** and clusters of perivascular plexuses intrinsic to the anterior parvocellular nucleus of the hypothalamus **(C)**. Denotes third ventricle (asterisk). Further examples of the rat neurovasculature network depicting higher magnification of large and smaller blood vessels embedding cortical neurons **(B)**. The vascular architecture shows smooth-running blood vessels with a uniform diameter and two-dimensionally even distribution coursing through the hippocampal area CA1 (cornu ammonis; **D**). The CA1 region of the hippocampus and the cerebral cortex are considered to be particularly vulnerable to ischemia. a, x 20; B-D, x 40

and Goldberg, 2001). These dual roles of Hsp70 point to the importance of molecular chaperones in suppressing chemical toxicity, and raise the possibility that endothelial Hsp70 may be a useful target for pharmacological intervention.

Endothelial cells and heat shock proteins

Several experimental approaches (anatomical and biochemical) have made it possible to describe ischemic events and measure rate levels of Hsp70 in endothelial cells. In animal models, reversible middle cerebral artery occlusions (a valid and predictive model of cerebral ischemia) trigger the induction of Hsp70 mRNA and Hsp70 protein in endothelial cells surrounding the ischemic core (Kinouchi et al., 1993a,b; Rajdev and Sharp, 2000; Kokubo et al., 2003). In our own studies, extreme cases of global cerebral ischemia (e.g., postmortem delay) cause endothelial cells to synthesize massive amounts of Hsp70 presumably to reduce oxidative damage to cell proteins and components of the ubiquitin-proteasome pathway (Torres et al., 2004; Kafel et al., 2006). In contrast, when rat brains are collected immediately after death (i.e., no postmortem delay), levels of Hsp70 in endothelial cells are almost non-existent (Fig. 4). Such a differential rate of induction is consistent with the view that under normal conditions Hsp70 is hardly detectable, but following brain injury it can become the most abundant single protein in the endothelium unit (Sharp et al., 1999; Yenari et al., 1999; Torres et al., 2004). Several recent observations have further indicated that blood-borne signals could feature in the induction process of Hsp70. For instance, rat brains devoid of blood content fail to show a heat shock response to global cerebral ischemia. However, if such brains are later reperfused with blood, Hsp70 production is reinstated in the neurovasculature network (Kafel et al., 2006). These findings suggest that although some of the molecular events that lead to brain damage occur within the endothelium-neuron circuit, circulating

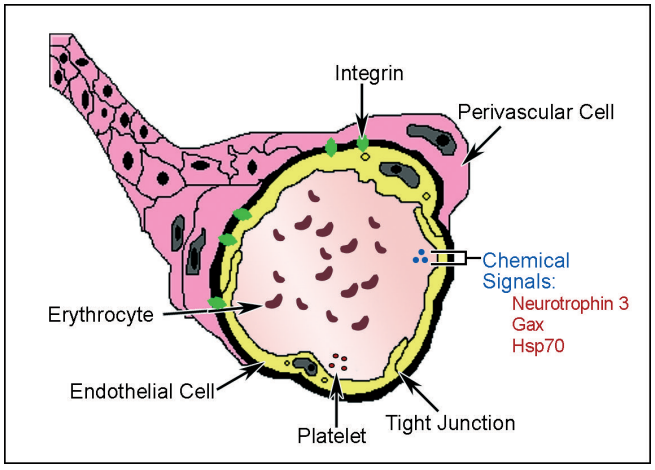

Fig. 2. This schematic diagram depicts the morphology of the endothelium unit as well as local chemical signals produced by the unit which are eventually released in neighboring nerve fibers. Tight junctions are characteristic of brain blood vessels. Endothelial cells in the mature brain have long half-lives of several years. Intrinsic matrix molecules, soluble growth factors, homeodomain-transcription factors and molecular chaperones synthesized by endothelial and mural cells interact with signals inside (e.g., platelets) and outside the vessel lumen, all in an integrated manner.

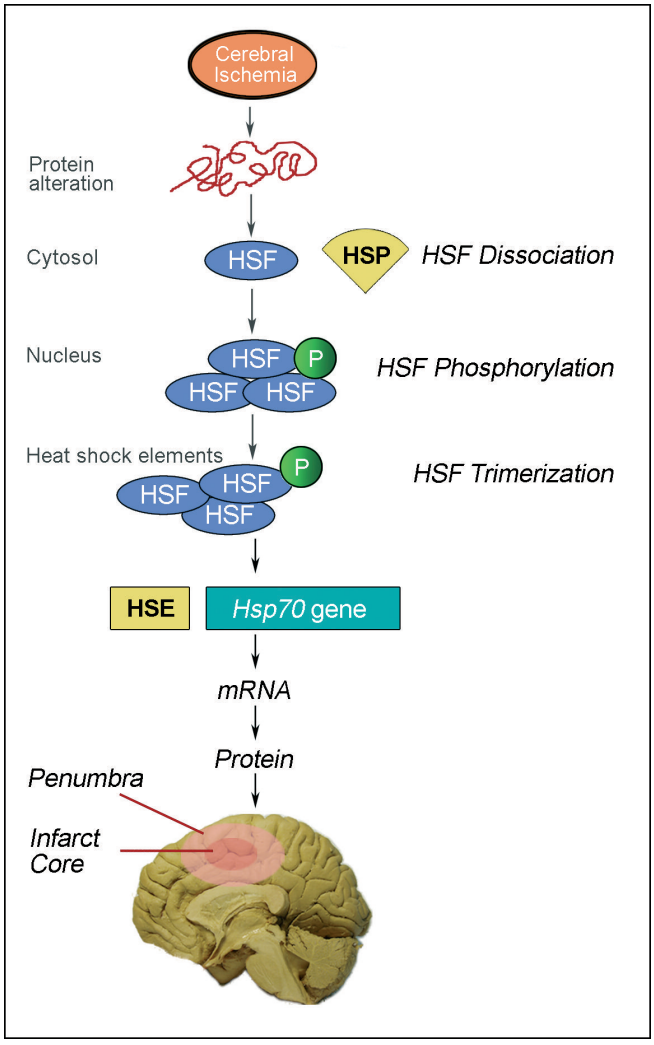

Fig. 3. This schematic diagram depicts a stepwise but partial representation of the biochemical events leading to the induction of Hsp70 following cerebral ischemia. Ischemia and other brain insults result in protein denaturation within neurons. In the presence of denatured proteins, HSF dissociates from HSP. HSF then begins a process of phosphorylation (P) and trimerization. Trimers subsequently bind to HSE located within promoters of the gene encoding Hsp70. This nuclear binding activates transcription and translation of Hsp70 protein species. Newly generated Hsp70 binds to denatured proteins to restore their errant structure/function and promote survival of afflicted neurons. Hsp70 induction is markedly seen in endothelial cells of the penumbra.

factors inside the endothelium cell also participate in this event. In this regard, migration of circulating neutrophils across cerebral blood vessels and into the ischemic brain appears to activate adenosine A2A receptors present in endothelial cells (Yu et al., 2004). These receptors contribute to ischemic injury by enhancing the synthesis of inflammatory molecules (Perera et al., 2006), including cytokines (IL-1, IL-6 and IL-12), and possibly cyclooxygenase-2 (COX-2). It is conceivable, therefore, that circulating blood-borne signals within the endothelium unit not only respond to cell injury but also worsen the outcome of cerebral ischemia. The phenotypic identities of most of the circulating blood-borne factors affecting Hsp70 remain unknown, however.

Endothelial cells and neuropathology

As described earlier, the endothelium of blood vessels exerts multiple regulatory influences in the highly vascularized brain parenchyma. Histological observations also place secretory events of endothelial cells in close association with pathology. For instance, the brain epithelium of Alzheimer's disease secretes the precursor substrate for the ß-amyloid plaque and a toxic peptide that selectively injures cortical neurons (Vagnucci and Li, 2003). Further, downregulation of the homeobox gene *Gax* in the Alzheimer's brain leads to loss of cerebral blood vessels, reduction of blood flow and impaired clearance of ß-amyloid plaques (Wu et al., 2005). Endothelium cell dysfunction is also associated with unipolar and bipolar depression. More specifically, hemodynamic and arterial endothelial function studies using pulse wave analysis indicate that certain patients with either type of mood disorder have impairment of endothelial function during depressive episodes, an effect that is not reversed even when the patient is in remission after pharmacological treatment (Rajagopalan et al., 2002; Sherwood et al., 2005; Rybakowski et al., 2006). Thus, there is a real need to characterize endothelial signaling at the cellular level, by identifying paracrine molecules that act on adjacent neurons. Characterizing the nature of this signaling and the added complexity of endothelial cell networks will undoubtedly have many applications in brain pathology. This is important, particularly in cerebral ischemia where reciprocal signaling cues between the endothelium and the neuron might improve damage

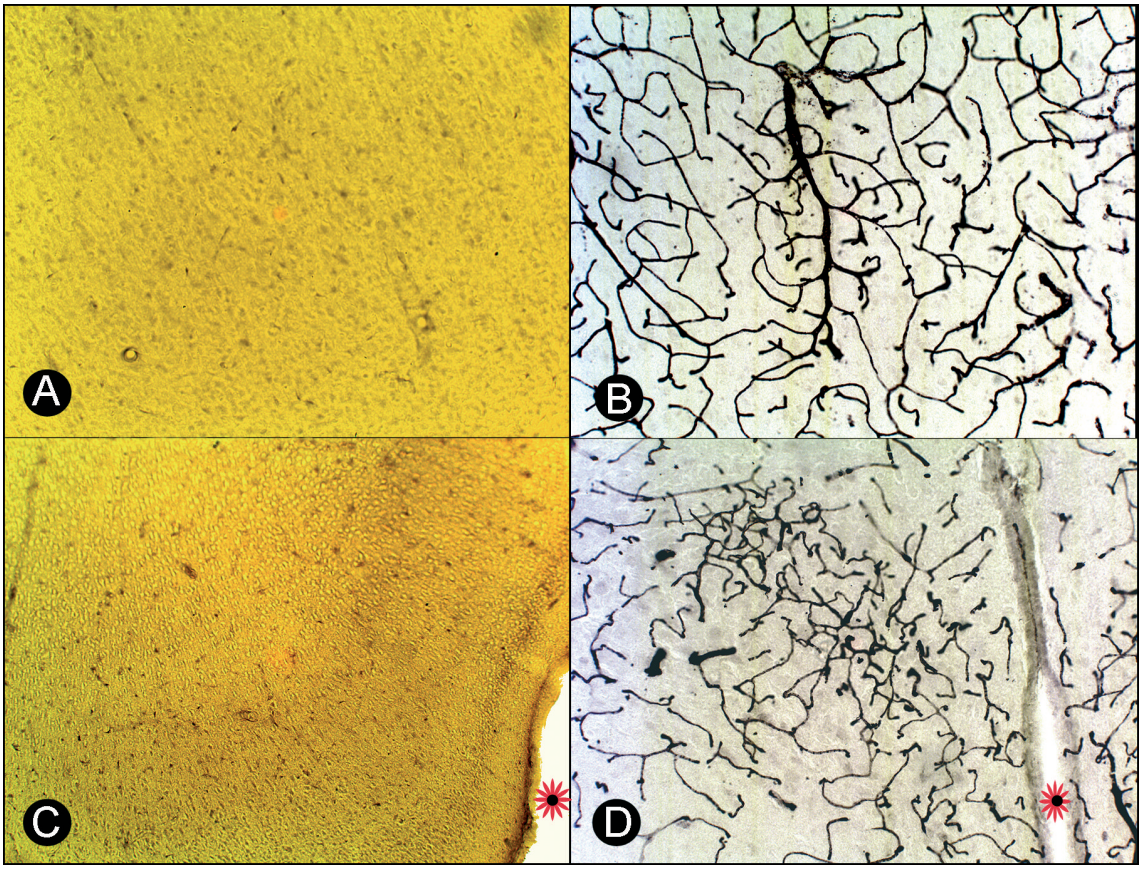

Fig. 4. Bright-field photomicrographs depicting avidin-biotin-immunoperoxidase staining of Hsp70 in the rat neurovasculature following global cerebral ischemia (for further details see Torres et al., 2004; Kafel et al., 2006). Hsp70 expression is almost non-existent in rat brains (i.e., control animals) collected immediately after death **(A and C)**. In contrast, rats undergoing global cerebral ischemia show a striking and prominent induction of Hsp70 immunoreactivity throughout the endothelial cell network of the motor agranular cortical area **(B)** and anterior parvocellular nucleus of the hypothalamus **(D)**. Denotes third ventricle (asterisk). A-D, x 20

outcome and survival.

Cerebral ischemia

Cerebral ischemia is caused by a sudden interruption of blood flow to the brain resulting in the permanent loss of neurons (Hsu et al., 1994; Sweeney et al., 1995). There are numerous causes of cerebral ischemia and these can be broadly classified as either ischemic (e.g., thrombotic occlusions) or hemorrhagic (e.g., rupture of a blood vessel). Ultra-structural studies show significant endothelial cell damage and intravascular accumulation of thrombin following occlusions of the middle cerebral artery (Fig. 5). The middle cerebral artery is the largest branch of the internal carotid artery, giving off extensive perforating branches to anterior limbs of the internal capsule and basal nuclei (Lindsay and Bone, 2004). The middle cerebral artery also provides perforating branches to frontal, temporal and parietal cortices (Lindsay and Bone, 2004). These brain regions play critical roles in the processing and expression of discrete sensory, motor and cognitive behaviors. Not surprisingly, therefore, middle cerebral artery injury can lead to neurological deficits, including paralysis (hemiparesis), impaired vision (contra-lateral gaze paresis), inability to speak (aphasia), loss of balance (ataxia) and memory deficits.

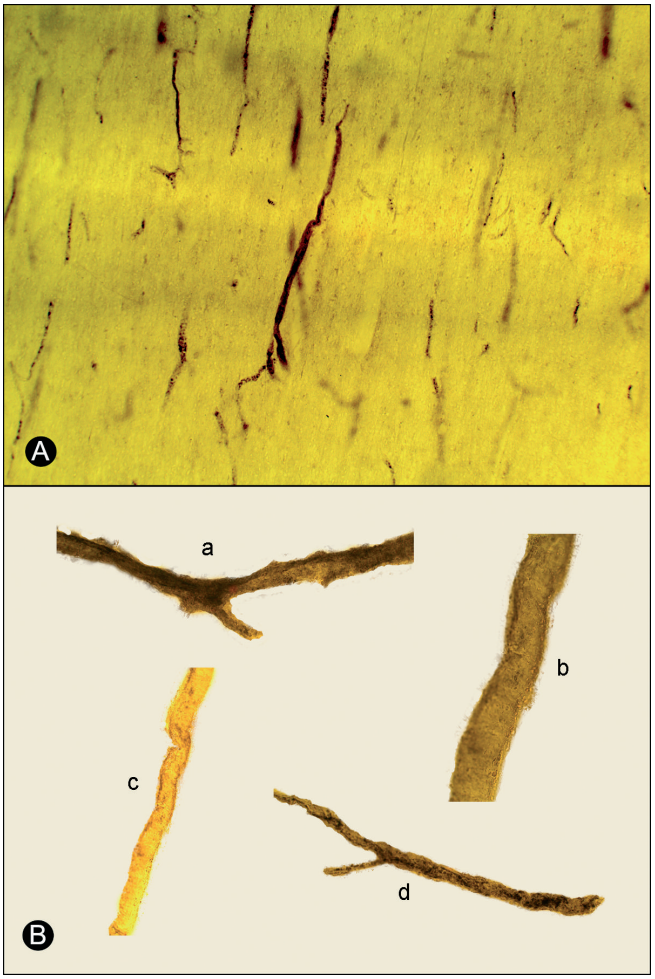

Fig. 5. Bright-field photomicrographs depicting avidin-biotin-immunoperoxidase staining of Hsp70 in the human neurovasculature network. Representative brain sections (100 μm thick) are from pre-frontal cortex **(A)** that had been frozen upon collection (~18 hours postmortem) and then fixed and prepared for standard immunocytochemical protocols. This suggests that both agonal (i.e., premortem) and postmortem states induce measurable levels of Hsp70 protein in endothelial cells (For further details see Torres et al., 2004). Examples (a-d) expressing the signal for Hsp70 immunoreactivity show smooth-running vessels with a uniform diameter and two-dimensionally even distribution **(B)**. A, x 20; B, x 40

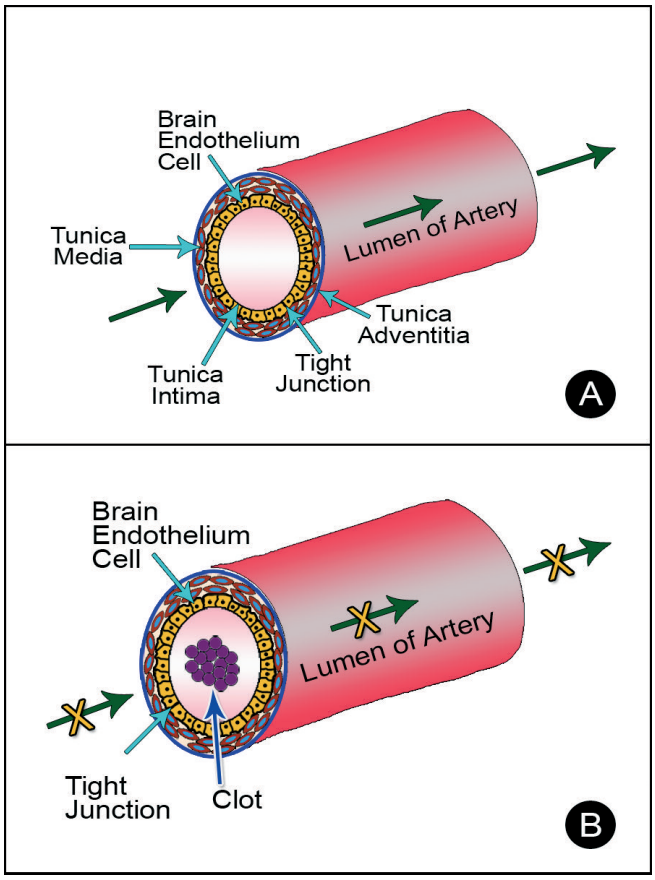

Fig. 6. This schematic diagram depicts a partial sequence of events when an artery is occluded, such as the occlusion of the middle cerebral artery. Under normal physiological conditions, cerebral blood flow (black arrow) is unimpeded and irrigation of focal brain areas is not restricted **(A)**. However, when the above blood flow is restricted (X) by clots **(B)**, it results in energy failure that ultimately leads to the activation of heat shock proteins and other intracellular pathways involved in cell survival. In general, the severity of cerebral ischemia has two components: degree of cerebral blood flow reduction and duration of the ischemic insult. Blood vessels, including arteries, are composed of three concentric layers (tunica intima = simple squamous endothelial cells, tunica media = smooth muscle cells, and tunica adventitia = fibroelastic connective tissue).

It should be noted that there is a cascade of biochemical events that follow interruption of blood flow to the brain. While it is often thought that accumulation of glutamate at the N-methyl-D-aspartate (NMDA) receptor results in intracellular Ca^{2+} overload that sets the stage for cell death, other molecular mechanisms (e.g., acidosis, changes in cerebral protein synthesis and formation of free radicals) also participate in the above cascade (Ohtsuka and Suzuki, 2000; Kokubo et al., 2003). Thus, since we are dealing with a cascade of events with several mechanisms involved, several pharmacological interventions will be needed to achieve a therapeutic value in cerebral ischemia (Curry, 2004).

Cerebral ischemia treatment

Currently in the USA, the primary pharmacological treatment for cerebral ischemia is intravenous tissue plasminogen activator (tPA; a native molecule found in certain cells). This recombinant drug increases the activity of plasmin on fibrin. Plasmin is a proteolytic enzyme that dissolves fibrin, a protein that when polymerized in conjunction with platelets forms hemostatic clots. Thus, tPA helps increase the ability of certain proteins to dissolve blood clots occluded by extraneous material. With an acute condition like cerebral ischemia there is a window of opportunity for maximizing cell survival outcome. Generally speaking, the earlier the initiation of tPA treatment after onset of ischemic symptoms the better. However, how late is too late? The answer to this question is not known since patients seek medical treatment at different times after detection of ischemic signs (Curry, 2004). The current approach is to administer tPA within three hours of the insult, a time frame that in most cases is not logistically feasible. Based on the observation that brain areas destined for infarction rarely recover after cerebral ischemia, the theoretical objective of tPA is to reperfuse the penumbra as soon as possible so neurons surrounding the ischemic core can be salvaged from the final pathway of glutamate degeneration. In this regard, following experimental middle artery occlusion, induction of Hsp70 is seen in neurons of the penumbra and endothelial cells of the ischemic core, although at different times (Kinouchi et al., 1993a,b). For example, following middle cerebral artery occlusion in the rat, Hsp70 protein is detected in endothelial cells of the penumbra at 24 hours after the insult (Kinouchi et al., 1993b). The temporal induction pattern of human Hsp70 is similar to that reported in rodents; Hsp70 mRNA and protein are observed in trauma tissue at 4-6 hours and 12-20 hours after injury, respectively (Dutcher et al., 1998; Fig. 6). These observations emphasize the relevance of animal models for understanding ischemic events, and raise the possibility of utilizing mechanisms that occur later in the ischemic biochemical cascade to address the window of opportunity issue. In principle, induction of a heat shock response in the penumbra would have a larger window of opportunity than that of tPA, and would also offer a mechanism that preferentially targets both infarcted (i.e., ischemic core) and non-infarcted (i.e., penumbra) areas. These possibilities are particularly important to consider since they also suggest that a valuable new therapeutic approach to treat cerebral ischemia would be pharmacological agents that induce Hsp70.

Cerebral ischemia and heat shock proteins

Recent pre-clinical studies support the possibility of using pharmacological induction of HSPs to treat or delay cell injury (Table 1). *In vitro* studies suggest that the ansamycin antibiotic geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a

Table 1. Therapeutic approaches to disease.

DRUG CHAPERONE:	MECHANISM(S) OF ACTION:	CURRENT APPLICATION:
4-phenyl Butyric acid Trimethylamine sulfoxide	Stabilizes protein conformation, improves ER folding capacity, and facilitates the trafficking of mutant proteins	Restoring glucose homeostasis in mouse model Type 2 Diabetes
Taurine conjugated-ursodeoxycholic acid	Modulation of ER function	Restoring glucose homeostasis in mouse model Type 2 Diabetes
Arimoclomol	Amplification of heat shock response	Delays disease progresion in mice with Amyotrophic Lateral Sclerosis

Hsp70 expression is linked to a growing collection of brain diseases and treatment. Listed are some potential drugs tested in animals that may offer a therapeutic approach to diseases: enhancement of the heat shock response. Of interest is the possibility that these substances that coordinately up-regulate HSPs will be therapeutic in cerebral ischemia, an acute brain insult which causes dire downstream consequences, including protein instability. Note that some of the drugs above target the convoluted network of membranous tubes known as the ER. This is for good reason, roughly one-third of proteins that end up in cellular membranes or are released to the outside are synthesized in the ER. Also, it is in the ER where the so-called unfolded protein response (UPR) occurs. In this regard, studies of both human brain samples taken at autopsy and brains of mice that have been genetically engineered to mimic certain neurodegenerative diseases have revealed increased expression of various components of the UPR pathway. Thus, the pharmacological actions of PBA and TUDCA may involve the suppression of ER stress.

cell culture model (COS-1) of Huntington's disease (Sittler et al., 2001). In Huntington's disease the abnormal form of huntingtin contains long stretches of glutamine residues (poly-glutamine extensions), whose conformations accumulate as aggregates either within the nucleus or the perinuclear zone of neurons (Davies et al., 1997; Cooper et al., 1998). In a related study, treatment with arimoclomol, a hydroxylamine derivative that acts as a co-inducer of HSPs, significantly delays amyotrophic lateral sclerosis (ALS) disease progression in mice expressing the human mutant gene Cu/Zn superoxide dismutase-1 (SOD1; Kieran et al., 2004). ALS, like Huntington's disease, is a neurodegenerative condition in which motor neurons of the spinal cord and motor cortex unexpectedly die. Mutations in the gene encoding SOD1 account for about 20% of familial causes of ALS. Indirectly, these results are consistent with Drosophila models of human poly-glutamine diseases in which over-production of Hsp70 strongly protects nerve cells that express the mutant form of human huntingtin (Warrick et al., 1999; Fernandez-Funez et al., 2000; Kazemi-Estfarjani and Benzer, 2000). Augmenting the levels of Hsp70 by either transgenic over-expression or over-expression of Hsp70 in the brain via Herpes or Adenoviral vectors also reduces cell injury in rat stroke models of global and focal ischemia (Yenari et al., 1998; Kelly et al., 2001, 2002; Lee et al., 2001). What is not yet clear is exactly how HSPs enhance cell survival and maintain protein stability. It is conceivable that the induction of HSPs facilitates the ubiquitination and degradation process of poly-glutamine extensions and mutant forms of SOD1. Along the same lines, Hsp70 may preferentially bind to poly-glutamine extensions and mutant forms of SOD1 and reduce their toxicity by preventing them from interacting with molecules that promote cell degeneration. Lastly but not mutually exclusive, is the possibility that Hsp70 could be protecting vulnerable neurons by antagonizing the actions of cell death programs (e.g., BAG 1, BAX) that often follow injury (Giffard and Yenari, 2004). It is noteworthy that bimoclomol, an analog of arimoclomol, also induces Hsp70 expression and prevents neuronal degeneration in experimental models of ischemia and reperfusion injury (Lubbers et al., 2002; Kalmar et al., 2003). Thus, there is credence that manipulating heat shock pathways with pharmacological agents achieve protection of cells whose proteins are in a state of disarray. Other chemical chaperones that activate a heat shock response and may also prove beneficial in cerebral ischemia are 4-phenyl butyric acid (PBA), trimethylamine N-oxide dihydrate (TMAO) and its taurine-conjugated derivative TUDCA. These pharmaceutical drugs stabilize protein conformation, improve capacity of endoplasmic reticulum (ER) and facilitate the trafficking of mutant proteins to the ubiquitin-proteasome pathway (Ozcan et al., 2006). Other examples of drugs that activate HSPs include the anti-ulcer compound, carbenoxolone (Nagayama et al., 2001); the herbal ingredient celastrol (Allison et al., 2001); and the anti-inflammatory drug indomethacin (Lee et al., 1995), which, among other mechanisms, activates the Hsp70 promoter and triggers the DNA binding of HSF-1. Many pharmacological aspects of the aforementioned drugs are clearly congruent with our prediction that agents that activate a heat shock response may protect penumbra neurons from ischemia injury. Unfortunately, most of the drugs described above have not been systematically tested in well-design studies. Nevertheless, these chemical chaperones may improve the efficacy of thrombolytic therapy by (1) increasing the window of opportunity post-ischemia and (2) salvaging more viable brain tissue; problems that currently limit the use of tPA.

Concluding remarks

Recent studies are now beginning to support the hypothesis of a direct role for paracrine signaling between endothelial cells and nerve fibers. There is also emerging evidence that the endothelium unit provides a heat shock response, which enhances the ability of both blood vessel cells and surrounding target neurons to withstand polypeptide aggregation and reduce oxidative damage to components of the ubiquitin-proteasome pathway. Understanding the nature of this signaling and its heterogeneity as well as the inducibility and functioning of specific molecular chaperones in potentially toxic conditions may not only provide insights into basic cellular mechanisms, but could also lead to novel targeted therapeutic approaches to cerebral ischemia treatment.

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