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MINI REVIEW

Heat shock proteins as biomarkers for the rapid detection of brain and spinal cord ischemia: a review and comparison to other methods of detection in thoracic aneurysm repair

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Abstract The heat shock proteins (HSPs) are members of highly conserved families of molecular chaperones that have multiple roles in vivo. We discuss the HSPs in general, and Hsp70 and Hsp27 in particular, and their rapid induction by severe stress in the context of tissue and organ expression in physiology and disease. We describe the current state of knowledge of the relationship and interactions between extra- and intracellular HSPs and describe mechanisms and significance of extracellular expression of HSPs. We focus on the role of the heat shock proteins as biomarkers of central nervous system (CNS) ischemia and other severe stressors and discuss recent and novel technologies for rapid measurement of proteins in vivo and ex vivo. The HSPs are compared to other proposed small molecule biomarkers for detection of CNS injury and to other methods of detecting brain and spinal cord ischemia in real time. While other biomarkers may be of use in prognosis and in design of appropriate therapies, none appears to be as rapid as the HSPs; therefore, no other measurement appears to be of use in the immediate detection of ongoing severe ischemia with the intention to immediately intervene to reduce the severity or risk of permanent damage.

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Introduction

We consider here the role of the heat shock proteins (HSPs) as biomarkers of central nervous system (CNS, i.e., brain and spinal cord) ischemia, hypoxia, and other severe stressors. The HSPs are members of highly conserved families of molecular chaperones, which have a variety of roles in cellular physiology, including rapidly induction by severe stress. Hsp70 refers to several genes that encode highly homologous Hsp70i (aka Hsp70-1, Hsp72) kDa proteins from these genes. Up to 13 sequences have now been described (Kampinga et al. 2010), but only three are highly stress inducible. Nomenclature is well reviewed in several sources (Milner and Campbell 1990; Tavaria et al. 1996), including two very recent reviews (Daugaard et al. 2007; Kampinga et al. 2010). The HSPs have at least two main roles in physiology, in the intracellular and extracellular compartments. An emerging body of evidence supports the view that Hsp70 is primarily an intracellular protective protein that also associates with the inner plasma membrane. The inducible members of the Hsp70 and Hsp27 families are associated with cellular protection and recovery after a near lethal stress, most likely as a function of their intracellular molecular chaperone roles. Whole animals, isolated organs, and cells subjected to heat shock or to other severe stressors are protected against a subsequent near lethal ischemic or hypoxic event. Transcription and translation of the HSP proteins increases dramatically in response to hypoxia or ischemia (Nowak and Jacewicz 1994; Li et al. 2004), and this increased transcription and

translation serves as an endogenous mediator of intracellular protection in all tissues. In Drosophila, in which the heat shock response was first described, Hsp70 has a 2-h half life in vivo, with rapid decay, but the half life can be extended by continuous heat shock (Li and Duncan 1995). Hsp70 is tightly regulated and thermally tolerant cells limit subsequent Hsp70 response to severe stress with transcriptional and pre-translational feedback mechanisms (Li and Duncan 1995; Theodorakis et al. 1999). The HSPs in turn induce anti-inflammatory responses, probably through T cells (van Eden et al. 2010). Hsp70 usually co-localizes with the Immediate Early Genes c-fos, c-jun, early indicators of cellular stress (Munell et al. 1994; Gilby et al. 1997; Li et al. 1999; Mariucci et al. 2007) but with different and specific responses depending on the particular stress and local cellular factors (Munell et al. 1994).

Hsp70 can be actively excreted by a non-classical secretory pathway that involves associated stress response proteins in lipid rafts. Hsp70 is found, for example, in the plasma membrane of tumor cells, despite an absence of peptide leader sequences (Multhoff 2007; Mambula et al. 2007) and appears to be associated within receptor complexes in lipid rafts (Bausero et al. 2005; Multhoff 2007). Perhaps the first report of an extracellular release via a pathway independent of the common secretory pathway was reported by Hightower and Guidon in 1989 (Hightower and Guidon 1989). The immunoregulatory capabilities of Hsp70 released into the extracellular compartment with other immunomodulatory proteins largely occurs through Toll-like receptors (Asea 2008). When secreted by this exosomal pathway, it is a potent inflammatory stimulator and active regulator of immune function.

Exosomal binding and release from tumor cells (Bausero et al. 2005; Graner et al. 2009) probably occurs by binding to phosphatidylserine in inner membranes (Yoo and Hayman 2010), although Mambula et al. (2007) attribute extracellular expression to ABC-family transporter proteins and an endolysomal compartment. Lancaster, Febbraio et al. reported an active secretory pathway for Hsp70 release with stress (exercise) (Lancaster and Febbraio 2005a, b), including active release from brain during exercise that occurs without concurrent evidence for ischemia (Lancaster et al. 2004). Release of the HSPs, particularly in trained individuals, appears to improve heat tolerance and has a net effect that is both anti-apoptotic and anti-inflammatory (Selkirk et al. 2009). Release of Hsp72 during exercise by a variety of stressors, and possible role in immune function, was also reviewed by Whitham and Fortes (2008) and methods for the cell surface localization of membrane Hsp70 are described in Multhoff (2007). Asea summarized two pathways for Hsp70 (which he termed a "chaperokine") release from cells, one a passive release from necrotic but not apoptotic cells, the other an active release that is pro-inflammatory and requiring intact surface membrane lipid rafts (Asea 2007). Pockley et al. (2008) also described the extracellular role of Hsp72 that includes both immunostimulatory and immunosuppressive effects that depend in turn on cellular niche environment. Multhoff (2007), Chen et al. (2007), and Joly et al. (2010) have reviewed the dual roles of cytosolic, protective chaperone HSPs versus extracellular or membrane-bound HSPs.

The extracellular HSPs serve in "danger" signal roles, in antigen presentation, and in systemic infections. Williams compared Hsp70 (Williams and Ireland 2008) to the "danger" signal protein HMGB1 (Forsdyke 1999; Yanai et al. 2009) as both Hsp70 and HMGB1 trigger specific host inflammatory responses (Zedler and Faist 2006; Wieten et al. 2010). Both are involved in signaling severe cellular stress to the rest of the tissue or organism, although the HMGB1 protein may be more specific for immunogenic nucleic acids (Yanai et al. 2009). As an example of this, incorporation of Hsp70 into a fusion protein increases cellular immunogenicity and immune response to an HPV vaccine (Zhou et al. 2010). Westerheide and Morimoto reviewed the potential for therapeutics based on small molecule targeting of HSF1, the heat shock factor (HSF) transcription factor (Westerheide and Morimoto 2005). Finally, Awad et al. reported on Hsp70 in CSF in a dog model of spinal cord ischemia (Awad et al. 2008). We will discuss Awad's results in detail later, but of interest was their statement that the release of intracellular Hsp70 during ischemia contributed to the modulation of inflammation by extracellular Hsp70.

The role of the HSPs that we will focus on for the remainder of this discussion is as biomarkers of severe intracellular stress through the coordinated release via inner membrane binding and exosomal release. Elevation of Hsp70 mRNA and protein is both a response to severe stress to quickly provide intracellular protective proteins as well as an extracellular biomarker for exposure to severe (near-lethal) stress. The protective and biomarker roles are most probably two sides of the same coin (i.e., protection may be due to "sub acute activation of the Heat Shock Response" (Westerheide and Morimoto 2005)). Extracellular Hsp70, in serum for example, is secreted or is released from stressed cells (Williams and Ireland 2008) long before a terminal physiological insult has occurred.

Hsp70 and other HSPs as biomarkers for severe stress

Hsp70 and Hsp27 change chronically with a variety of conditions, including but not limited to age, vascular

disease, diabetes, smoking, hypertension, sepsis, and renal insufficiency. For example, although it is not an acute maker, Hsp27 in serum was found to be a marker for diabetic neuropathy in a subanalysis of the EURODIAB study, independent of other risk factors (Gruden et al. 2008). Hsp70 protein and mRNA are elevated in tumors and have been proposed as targets in solid cancers because Hsp70 overexpression confers protection from chemotherapy or radiation. Elevated HSPs in serum or tissue appear to be nonspecific indicators of organ ischemia or dysfunction and in chronic diseases may suggest a lack of vascular delivery reserve or of microvascular disease. Blake (Blake et al. 1991) showed stress-induced Hsp70 expression in adrenal cortex in a rapid, adrenocorticotropic hormone-sensitive, age-dependent response in unanesthetized animals. In humans, Hsp70 synthesis in cardiac tissue is significant within 2 h after cardioplegia exposure (Schmitt et al. 2002). Levels of Hsp70 are elevated in as little as 30 min after severe trauma. Although elevated Hsp70 in serum correlated with survival, it was not correlated with severity of injury or subsequent organ dysfunction, suggesting that a mechanism independent of release from necrotic cells was involved (Pittet et al. 2002).

Surgical stress induces endocrine responses including the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. Strong Hsp70 induction occurs as early as 30 min after surgical incision and additionally after hemorrhage (Udelsman et al. 1991). Induction is most prominent in adrenal gland, aorta, and vena cava (Udelsman et al. 1991) Hsp70 is induced by restraint alone in rats, with simultaneous induction of the HPA axis and adrenal cortex (Blake et al. 1991).

The significance of elevated serum Hsp70 is unknown but is the subject of much investigation. Hsp70 and Hsp27 are detectable in the serum of healthy young individuals at low levels. Lancaster et al. reported that volunteers who exercised to exhaustion had measurable release from brain into blood (Lancaster et al. 2004; Lancaster and Febbraio 2005b), presumably by active secretion (Lancaster and Febbraio 2005a). In contrast, two other studies found no increase in human CSF HSP levels. Steensburg et al. measured Hsp70 and Hsp27 protein levels of less than 0.5 ng/ml in human CSF and serum, even after exercise to exhaustion (Steensberg et al. 2006). Although resting Hsp70 in CSF was higher than in resting serum, levels of Hsp70 in CSF did not increase with exercise, at a time when serum Hsp70 increased fivefold (Steensberg et al. 2006). Dalsgaard et al. found that the inducible form of Hsp70 was near the lower limit of detection by ELISA in the CSF of human volunteers and remained so despite exercise to exhaustion (Dalsgaard et al. 2004).

In cardiac tissues

Numerous studies have used Hsp70 immunohistochemistry as a marker for injury, for example after cardiac surgery (Dybdahl et al. 2004; Becker et al. 2007). The dual roles of intracellular protection and extracellular immune modulating response are illustrated in the effects of Hsp70 in heart disease. TLR4 mediates the inflammatory response to ischemia reperfusion and myocardial injury through release of Hsc70 (Zou et al. 2010). Intracellular Hsp70 is not only cytoprotective but is also part of an inhibitory feedback via NF-kappaB. When released, extracellular Hsp70 acts on cytokines and TLRs to stimulate both pro- and anti-inflammatory effects, including cytokines, chemokines, and cytolytic immune cells (de Jong et al. 2009). Release of stress proteins during cardiac ischemia and reperfusion probably also accounts for changes in Hsp70 and c-fos in hippocampus and cerebral cortex in the rat (Wang et al. 2006). In patients with acute chest pain and myocardial ischemia, increased serum antibodies in cardiac tissues to Hsp60 (Birnie et al. 2005) and Hsp27 (Ghayour-Mobarhan et al. 2008) were associated with adverse outcomes over the following year. However, Zhang et al. (2010) in contrast found that increased circulating Hsp70 or decreased anti-Hsp70 antibody in the days following acute myocardial infarction were associated with increased risk of acute coronary syndrome. Hsp70, 27, and 90a in serum increased intraoperatively more in open coronary artery bypass grafting (CABG) procedures than in cardiac patients who underwent “off-pump” (i.e., non-cardiac bypass) cardiac surgery (Szerafin et al. 2008). We presume that this difference reflects some aspect of the relative stresses of the two procedures, although the degree of tissue damage in the two procedures differs as well. Becker et al. correlated increased Hsp70 protein in thoracic wound fluid after CABG with increased myocardial damage by cardiac enzymes (Becker et al. 2007). Schmitt et al. found that only Hsp70, but not Hsp27 or 90a, was increased in right atrial biopsies during CABG, cardioplegia, and reperfusion (Schmitt et al. 2002). Hsp70 mRNA was also upregulated in pediatric cardiac right atrial biopsies at time of surgery, induced by cardioplegic arrest (Vitorini et al. 2007).

In two studies (Dybdahl et al. 2004; Pizon et al. 2006), higher Hsp70 protein levels in serum after cardiac surgery correlated with an increased incidence of postoperative atrial fibrillation (AF), a common complication. In contrast, St. Rammos et al. and Mandal et al. both found that a higher pre-operative right atrial tissue level of Hsp70 correlated with a lower incidence of postoperative AF (St Rammos et al. 2002; Mandal et al. 2005). Elevated mRNA and/or protein Hsp70, and possibly Hsp27 in tissue prior to a procedure, probably reflects ongoing tissue or organ stress. This is one but not the only pathway to ischemic

pre-conditioning and perhaps anesthetic pre-conditioning as well (Frassdorf et al. 2009). Induction and expression of some degree of the HSPs prior to cardiac surgery is probably, therefore, protective. How this relates to the findings for AF remains unclear, as does the question of whether there is a limit to the beneficial aspects of induction and expression of mRNA and/or protein.

In obstetrics

The significance of Hsp70 and Hsp27 in blood or CSF during pregnancy is unknown. Increases may be related to fetal development, or to ischemia or inflammation of the placenta. As previously reported (Bausero et al. 2005; Multhoff 2007), heat shock proteins are released as exosomes in what appears to be an immune regulatory role. Exosomes containing Hsp72 have been noted in amniotic fluids (Asea et al. 2008). Amniotic fluid levels of Hsp70 are elevated in amniotic infection, chorioamnionitis, term parturition (Chalworapongsa et al. 2008), and serum levels are elevated in preeclampsia and HELLP syndrome (Molvarec et al. 2009, 2010; Madach et al. 2010). We measured Hsp70 and Hsp27 in obstetric patients (Approved IRB protocol, unpublished results). To our surprise, we found elevated CSF levels of Hsp70 and Hsp27 in a significant minority of apparently healthy young pregnant patients in active labor. We do not at present know if the source of Hsp70 and Hsp27 in the CSF is maternal, fetal, or placental in origin or whether it originates in the CNS (analysis currently underway).

HSPs as biomarkers for detection of CNS ischemia in real time

Inducible HSPs, in particular Hsp70 and Hsp27, have also been used as markers for cells and tissues in the CNS that have been exposed to a near-lethal stress. An understanding of the time course and correlation with injury of HSPs released during brain and/or spinal cord cellular stress (ischemia) is critical in understanding the role of the HSPs in survival of cells in the CNS. We will not further review the intracellular protective role of the HSPs, in particular Hsp70 and Hsp27, here as that role in numerous organs and species has been comprehensively reviewed elsewhere; for example in (Feige and Polla 1994; Morimoto et al. 1994; Kim et al. 1995; Minowada and Welch 1995; Feige 1996; Sedlak 1996; Perdrizet 1997; Sharp et al. 1999; Beere 2005).

Induction and expression, or release of pre-formed Hsp70, is robust and extremely rapid and has been shown in numerous tissues and organs including brain and spinal cord. As noted earlier, Hsp70 is induced by ischemia, surgical stress, hyper- and hypothermia, hypertension, and other near-lethal stressors. Boehm et al. demonstrated minute by minute increases in HSF and Pol II in Drosophila (Boehm et al. 2003). Marsala et al. demonstrated through microarray that brief spinal cord ischemia upregulates Hsp70 mRNA within 30 min and is confined to the ischemic area of rat spinal cord (Carmel et al. 2004). Marsala et al. characterized the Hsp70 response in spinal cord after brief ischemia (6 to 12 min) or hyperthermia (Cizkova et al. 2004). This brief exposure produced a robust expression of Hsp70 by 4 h, but no neuronal injury, confirming induction and expression in CNS cells that survive the ischemia. In neonatal rats, Hsp70 was elevated within 1 h in brain in a hypoxic-ischemic model (Jiang et al. 1991). In short, release, transcription, and translation of the inducible HSPs are among the fastest of intracellular severe stress responses and are conserved from Drosophila through humans.

Detection of intraoperative ischemia during thoracic aneurysm repair

Because certain surgical procedures carry significant risks of brain or spinal cord ischemia and injury, we discuss the use of the HSPs as biomarkers in detecting CNS ischemia in real time and compare their use to other methods of detection. Surgical procedures that might benefit from rapid detection of CNS ischemia include thoracic aortic aneurysm repair (discussed below); carotid artery repair (carotid endarterectomy); brain tumor resection; intracerebral aneurysm or arterio-venous malformation repair; interventional neuroradiology embolization or coiling; CABG; prolonged operative hypotension; surgical procedures in the sitting position; procedures involving massive blood loss; and any procedure requiring deep hypothermic circulatory arrest (DHCA). Other clinical situations in which biomarkers of CNS ischemia would probably also be very beneficial include: after stroke; to assess neurodegeneration, ALS or Alzheimer's Disease; or prior to any of these above procedures to diagnose pre-existing ischemia that might predict increased surgical risk. The degree and nature of CNS (brain or spinal cord) ischemia varies from patient to patient, and our ability to predict in which patients and in what way ischemia will present is limited (von Oppell et al. 1994). Poor neurologic outcome results from one or more ischemic insults that can occur during the intraoperative or postoperative periods.

One example of a surgical procedure with high risk of spinal cord ischemia and potential paralysis is the repair of thoracic aortic aneurysms (TAA) and thoraco-abdominal aortic aneurysms (TAAA). The natural history of expanding

aortic aneurysms suggests an eventual rate of rupture varying from 50% to 95% and greater than 90% mortality rate following rupture (Bickerstaff et al. 1982; Crawford et al. 1986; Safi and Miller 1999; Cina et al. 2004). Elective surgical repair of unruptured aneurysms has significant morbidity and 5–40% mortality (Rectenwald et al. 2002; Khan and Stansby 2003) depending on location, acuity of the repair, and patient risk factors. Outcomes after open TAAA repairs are historically worse than after TAA repair, with mortality rates between 18% and 22% at 30 days and up to 32% at 1 year (Cambria et al. 2002; Cowan et al. 2003; Rigberg et al. 2006).

Acute ischemia—and reperfusion injury if the spinal cord is reperfused—are the primary causes of spinal cord injury in TAA repairs (von Oppell et al. 1994; Jacobs et al. 2002). Ischemic insults during the intraoperative or postoperative periods can result from intraoperative hypotension, blood loss, the loss of critical intercostal arteries that are occluded or intentionally sacrificed during repair, aortic cross-clamping, or increased intracerebral pressure. Failure to prevent or rapidly detect spinal cord ischemia frequently results in permanent paraplegia, which can occur rapidly and depends on the severity of the loss of perfusion (Katz et al. 1981; Livesay et al. 1985; Connolly 1998; McGarvey et al. 2007; Sloan and Jameson 2007). Surgical and anesthetic attempts to prevent paralysis include Deep Hypothermic Circulatory Arrest (DHCA), epidural spinal cooling, elevation in MAP (mean arterial pressure), removal of CSF with a lumbar drain, augmentation of distal aortic perfusion using partial bypass, intercostal artery re-implantation, and adjunct pharmacologic measures (Gloviczki 2002). Despite clinical efforts to prevent spinal cord ischemia the risk of paraplegia (paraparesis) remains significant (Hamilton and Hollier 1998; Robertazzi and Cunningham 1998; Safi and Miller 1999; Coselli et al. 2002).

Although the damage from ischemia during TAA repair usually occurs intraoperatively, confirmation of neurologic injury does not occur until the postoperative period when anesthetic effects have resolved and the patients can be fully evaluated. Often, patients remain sedated for prolonged periods following their operation; therefore, methods to detect the onset of acute spinal cord ischemia during the intraoperative and immediate postoperative period would be extremely valuable, particularly as this complication can sometimes be reversed. We reported an 11% incidence of delayed onset (postoperative) paraplegia and a 72% success rate in completely or partially reversing cases of delayed onset paraplegia (Cheung et al. 2002), suggesting the importance of rapid detection of ischemia. Although surgical mortality rates have decreased because of improved perioperative care (Svensson et al. 1993; Velazquez et al. 1999), TAA and TAAA procedures are being surgically repaired at increasing rates in the USA (Olsson et al. 2006), and an aging population may make it difficult to continue this improvement in mortality rates. Initial experience with thoracic endovascular aortic repair (TEVAR), a less invasive alternate surgical procedure, is promising when compared to traditional open surgical repair. But spinal cord ischemia remains an important complication of TEVAR as well, occurring in up to 12% of patients (Lewis et al. 2002; Sullivan and Sundt 2006; Nienaber et al. 2009). We found that patients who are paraplegic upon awakening from surgery remain paralyzed and have greater than 65% mortality (Messe et al. 2008). Biomarkers for the real-time detection of ongoing spinal cord ischemia or prediction of an increased risk for paralysis would potentially provide time to intervene and would be of great benefit in preventing this devastating complication. Although we use the surgical repair of aortic aneurysms as a relevant clinical example, the same concept applies to detection of ongoing ischemia in brain as well. Diagnosis and prognosis of brain injury would be aided immensely by specific patterns of specific brain injury, although to be useful for intervention they would have to change and be assayed rapidly, as opposed to guiding prognosis and therapy.

Other methods of ischemia detection

Commonly used measures to attempt to detect intraoperative ischemic events and predict adverse neurologic outcome include intraoperative neurophysiologic monitoring of the spinal cord and brain with somatosensory-evoked potentials (SSEPs) (Galla et al. 1999; Guerit et al. 1999; de Haan and Kalkman 2001; van Dongen et al. 2001; Shine et al. 2008), motor-evoked potentials (MEPs) (de Haan and Kalkman 2001; Jacobs et al. 2006; Kakinohana et al. 2007; Kawanishi et al. 2007; Shine et al. 2008), electrical cortical activity (EEG) (Wada et al. 2001; Stecker et al. 2001; McGarvey et al. 2007; Rijsdijk et al. 2009), and the measurement of presumptive biomarkers of ischemia (described below).

SSEP measurement, routinely used during TAAA surgery, is widely considered to be safe and potentially beneficial and can be continued until patients have recovered from anesthesia (de Haan and Kalkman 2001; Winnerkvist et al. 2007). SSEPs record neurologic waveforms by electrodes placed strategically over the sensory portions of the peripheral nerves, spinal cord, and brain. The waveforms are generated by repetitive electrical stimulation of peripheral nerves and propagate along sensory pathways to the recording electrodes, where they are averaged, amplified, and displayed thereby allowing the neurologist, anesthesiologist, or neurophysiologist the

ability to assess the integrity of neurologic pathways when patients are under anesthesia. However, SSEPs may fail to reliably predict all presentations of paralysis and cannot provide evidence as to whether ischemic events are coincident or isolated peripheral vascular events (clots and emboli causing distal damage) or secondary to a reduction in spinal cord perfusion. Lower extremity perfusion disturbances may occur for a variety of reasons during these procedures (Dong et al. 2002) and the raw SSEP signals provide ambiguous quantitative correlation to intraoperative events (Winnerkvist et al. 2007; Shine et al. 2008). Winkerkvist et al. attempted to increase sensitivity by combining SSEPS with GFAP and neurofilament biomarkers but found that these biomarkers increased too slowly to be of use intraoperatively (Winnerkvist et al. 2007).

MEPs are elicited through transcortical electrical stimulation and may provide increased sensitivity and specificity for identification of spinal cord ischemia when compared to SSEPS (Jacobs et al. 2006; Denda et al. 2006; Kakinohana et al. 2007; Kawanishi et al. 2007; Shine et al. 2008). Myogenic potentials are recorded from muscles in the extremities by delivering multipulse, electrical stimulation to the scalp overlying the motor cortex. The evoked potentials elicited from this stimulation travel from the motor cortex through cortical spinal tracts, anterior horn cell, peripheral nerve, and finally to muscle. An interruption in this pathway will result in loss of the motor evoked potential. MEPS cannot be monitored postoperatively due to the pain involved in delivering the stimulus. Myogenic MEP recording does impose some requirements for specific anesthetic agents, including limitations in the use of muscle relaxants and lower concentrations of inhaled anesthetic agents. The availability of IV anesthetics that include propofol, ketamine, remifentanyl and etomidate make MEP monitoring during TAAA repairs easily accomplished when indicated.

EEG measurement recorded from electrodes placed on the scalp is often performed during procedures mentioned earlier and is used to identify injury to the cerebral cortex. Intraoperative EEG is useful for identifying larger cortical strokes, but the ability of EEG to detect subcortical posterior circulation or small cortical strokes is limited. While EEG is rapid, it can be difficult to interpret. If significant changes in SSEPs, MEPs or EEGs occur, surgical and anesthetic interventions are initiated to attempt to improve spinal cord or brain perfusion. Any improvement in early detection of injury to the sensory or cortical pathway can alert the interpreting neurophysiologist and anesthesiologist of potential reversible injury, who in turn can provide guidance to the surgeon (Galla et al. 1999; Guerit et al. 1999; de Haan and Kalkman 2001; Wada et al. 2001; van Dongen et al. 2001; Cheung et al. 2002).

Biomarkers of spinal cord or brain ischemia

Tissue ischemia caused by decreased spinal cord or brain perfusion is a potent and powerful stressor that triggers many metabolic and inflammatory pathways. CSF is produced continuously and the total CSF compartment volume is replaced three times a day under normal conditions. CSF bathes the neural tissues of the brain and spinal cord and should allow detection of the biochemical products of acute CNS ischemia more rapidly than in serum, particularly if the blood brain barrier is intact. Specific biochemical markers that have been examined to date include lactate, pCO_2, neuron-specific enolase (NSE) (Nagy et al. 2002), excitatory neurotransmitters such as glutamate, aspartate, glycine (Brock et al. 1997), tau, glucose, pH, and S100β. These markers also markedly increase in serum at other times, including during surgical procedures unrelated to acute brain injury (Anderson et al. 2001; Routsi et al. 2006). S100β, for example, is elevated in marathon runners (Hasselblatt et al. 2004) and may be released from a variety of tissues (Kleine et al. 2003), including brain injury during sepsis (Lipcsey et al. 2010). Plasma and urinary levels of isoprostanes, breakdown products of free-radical induced oxidation of arachidonic acid, have been examined in experimental animal models of spinal cord ischemia (Basu et al. 2001) and have been correlated in human neurodegenerative diseases (Pratico et al. 2000a, b). Matrix metalloproteinases, which have a role in tissue remodeling and inflammation (Rosenberg 2002), have also been investigated. Skouen et al. measured increases in S-100β, NSE, GFAP, and Neuro Filament in a pig model (Skouen et al. 1991) but found significant increases only after 1–4 weeks. Von Reyn examined sodium channel proteolysis from rat brain with calpain activation (von Reyn et al. 2009) but again at longer time points. These studies are inconclusive; lactate appeared to correlate best with ischemic injuries (Brock et al. 1997; Lindsay et al. 1999; Nagy et al. 2002). Recently, Sharp and colleagues described microRNA and gene expression profiles from rat brain and serum after ischemic stroke, induced seizures, and intracranial hemorrhage (Liu et al. 2010; Zhang et al. 2010), but again, these markers were not measured until 24 h after injury and are, therefore, not useful for rapid detection. Modesti et al. (2009) used mass spec 2D electrophoresis in TAAA patients and isolated spots specific for differences in thrombin precursors and inflammatory mediators in the perioperative period. The available literature for biomarkers for spinal cord injury was reviewed in Pouw et al. (2009). They found reports of increases in S100β, NSE, NL, and GFAP after spinal cord injury but concluded that results were confounded by "polytrauma, hemolysis, extracerebral sources, and poor resuscitation.....and do not yet provide a sensitive prognos-

tic tool." Hu et al. also measured serum S100β and NSE in conjunction with SSEP and MEPs in human spine surgery patients undergoing elective decompression for spondylitic myelopathy (Hu et al. 2010). They used the recently described technique of remote ischemic preconditioning (three 5-min cycles of upper limb ischemia) of an upper extremity prior to surgery. They found that S100β and NSE levels, as well as sensory and motor recovery rates all improved even though SSEPs showed no significant signal changes intraoperatively, but again, these biomarker changes were only after 6 h to 1–3 days. As yet, none of the putative markers of injury in blood or CSF reliably detect early brain or spinal cord ischemia or validated surrogate endpoint measures.

In brain

Hsp70 increases in ischemic brain as well. Mariucci showed increased Hsp72 and increased synaptic protein synthesis in brain tissue in a permanent MCAO model in rats (Mariucci et al. 2007). In humans, intracellular Hsp60 appears to be a specific marker for microglial activation and TLR4 binding and may indicate CNS neurodegeneration (Lehnardt et al. 2008). Hsp70 was also associated with mild cognitive impairment but appeared to correlate better with vascular inflammation rather than with Alzheimer's disease (Lee et al. 2008). In epilepsy, Yang et al. suggested that Hsp70 was an "indicator of stressed neurons" (Yang et al. 2007). Elevated serum Hsp70 levels in patients with severe traumatic brain injury (TBI) were found to predict poor outcomes (Da Rocha et al. 2005). Siman et al. identified novel protein biomarkers of CNS injury in experimental animals (Siman et al. 2004, 2005). They then applied a similar analysis in the TAAA population (Siman et al. 2008) and in TBI patients (Siman et al. 2009). Although these biomarkers increase too slowly (over 12 to 48 h) to be useful intraoperatively, they may prove to have additional value in prognosis and management of ischemic or traumatic CNS injury (Siman et al. 2008).

In spinal cord and CSF

In spinal cord, Awad et al. (2008) showed increases in intra- and extracellular Hsp70 protein in a dog model of spinal cord ischemia, with correlation to severity of hind limb paraplegia. However, statistically significant increases were not reached in CSF until 9 h after aortic crossclamp. Also in the dog, compression of lumbar and sacral nerve roots upregulates Hsp70 in spinal cord and dorsal root ganglion cells (Cizkova et al. 2005). As we noted earlier, Cizkova et al. (Carmel et al. 2004; Cizkova et al. 2004) found that as little as 3–6 min of spinal cord ischemia was required to increase Hsp70 mRNA and protein expression in spinal cord in as little as 30 min, with a peak at 24 h. The multiple roles for Hsp70 and Hsp27 led us to investigate whether quantitative measurements of Hsp70 and Hsp27 could be used to predict ischemic injury. Previous work done by us, as well as by others (discussed above), led us to hypothesize that Hsp70 (Nowak and Jacewicz 1994; Westerheide and Morimoto 2005; Mariucci et al. 2007) and 27 levels (Wagstaff et al. 1999; Latchman 2005; Patel et al. 2005) would be found at significant levels in the CSF during intraoperative CNS ischemia and that the quantitative Hsp70 and Hsp27 level would be predictive of the severity of ongoing ischemia and the potential for paralysis. We measured Hsp70 and Hsp27 at multiple times in the CSF of patients undergoing TAA repairs and found that Hsp70 and Hsp27 levels in CSF obtained intraoperatively predicted the risk of perioperative paralysis (Hecker et al. 2008). In ongoing studies, we are collecting CSF and serum samples at short (20–30 min) intervals to determine exactly how quickly Hsp levels are reflected in CSF effluent.

Devices for the rapid detection of proteins and prospects for miniaturization for in vivo use

Several new and emerging technologies look promising for adaptation to rapid protein detection or "fast ELISA" analyses. Most depend on a miniaturized platform with some type of antibody-coupled detection, often with a fluorescent readout. For example, febit has an automated biochip that can analyze multiple micro-RNAs from total RNA extracted from blood (Keller et al. 2009). The assay is highly reproducible and validated but, unfortunately, time consuming. One-step immunotests are already commercially available for a variety of rapid detection assays, including biomarkers of acute myocardial infarction (Xie et al. 2010). Xie et al. reported 82% sensitivity for acute MI detection with a 15-min sandwich immunoassay from whole blood, lending itself to serial testing for improved detection (Xie et al. 2010). Similarly, Burbelo et al. used a luciferin immuno precipitation system and a liquid phase light output assay for Lyme disease (Burbelo et al. 2010). They reported a wide dynamic range, with 98% sensitivity and specificity using serum samples, but the incubation steps required several hours. Surface plasmon resonance imaging is a surface spectroscopic measurement of protein bioaffinity interactions in serum samples on thin gold films (Oh et al. 2010). Detection works by measurement of light intensity variations due to the refractive index changes within small distances reflected from the back of the thin gold films. Oh et al. reported the rapid (1 h), label-free, high-throughput measurement of Mycoplasma anti-

gens (Oh et al. 2010). This technology is approaching the speed necessary for indwelling or Point of Care ex vivo real-time diagnostics and can handle 300–400 samples simultaneously. Munge et al. described an electrochemical immunosensor that is carbon nanotube array-based (Munge et al. 2010) that is made up of multiwell carbon nanotubes with multiple enzyme labels (approximately 4,200) for signal amplification. This sensor is a sandwich immunoassay that has been miniaturized and has a 0.4 ng/ml (7.7 pM) detection limit of matrix metalloproteinase MMP-3 in 10 μl of calf serum. This technology is highly sensitive due to the amplification but requires 2 to 3 h for measurement, although the authors claim a "potential for a real-time detection system" (Munge et al. 2010) with further development.

Wu et al. (2010) developed an electrospun membrane (micro/*n* polymer fibers from viscous or melted polymers sprayed onto membranes or scaffolds) immunoassay with a linear detection range to 1 ng/ml for human serum albumin (HSA) and HSA-FITC and a fast reaction speed. Signal amplification is currently by a "press and fold" method, and the assay requires 50 min. Projected advances in coaxial and miniaturized electrospun membrane assemblies offer the potential for faster signal times. Polystyrene bead suspension arrays offer rapid multiple analyte sample detection but are not easily miniaturized for microfluidics. Burton et al. described (Burton et al. 2010) a miniaturized detection particle for multiplexed detection based on antibodies and fluorescent secondary antibodies linked to individual DNA molecules. They built a suspension array with recombinant DNA for high sensitivity ("single molecule detection"), biocompatibility, and small enough size to be used in 1 μm microfluidic chips. Multiple proteins can be analyzed in this device, although this adds to the analysis time. Magnetic nanoparticle biosensors (Haun et al. 2010) are used as proximity modulators of spin–spin relaxation times of neighboring water molecules for measurement with MRI. Although this technology could be used in clinical MRI scanners, resolution is only likely to be obtained in ex vivo chip-based NMR ("microNMR") detectors using microliter samples, rather than in vivo. Microfluidics can also be used in an automated platform for enzyme MW, amount and activity. And it is possible that the ATPase activity of Hsp70 might be measured by this means, although it is unclear at first glance what advantage this technology might have over those already discussed (Hughes and Herr 2010). In another ex vivo approach, Zhang and colleagues (2009) developed a solid-phase extraction enzyme-linked immunosorbent assay with a detection limit of 0.0055 ng/ml, well below the detection limits needed for Hsp70. They compared this rapid assay for illegal steroids and found excellent correlation with established, but slower, ELISA and HPLC methods.

In short, a variety of newly developed technologies are now available that offer detection of proteins in microliter volumes of fluids down to detection limits well below levels of interest for measurements of the HSPs in biological fluids. Although many of these approaches already offer adequate dynamic ranges and detection limits, few are rapid enough for intraoperative point of care application. Several of these technologies are close to being employed and validated in point of care use, i.e., at bedside or adjacent to the operating rooms, for example. Several are also small enough that they could be deployed into body cavities, into the spinal canal, or blood stream. The 10-min assay time for the Digital DNA assay (Burton et al. 2010) for one or two proteins looks closest to miniaturization and could conceivably be used in in-dwelling catheters, in the CSF for example. Finally, Lieber et al. reported (Tian et al. 2010) a method for manufacturing nanowires that are small enough for use in individual cells. Two advances make these nanowire field effect transistors particularly interesting, besides their size. They are coated with a phospholipid bilayer, so that the "devices are easily pulled into a cell via membrane fusion." And they can be fitted with receptors or ligands for individual intracellular biochemicals. This technology, more than any of the serum-based technologies described above, might be appropriate for intracellular measurement of the HSPs.

Summary

We have reviewed the available literature on the use of the heat shock proteins, in particular Hsp70 and Hsp27, in the detection of severe cellular stress, primarily ischemia. We believe this role of these HSPs as a biomarker for ischemia is intimately related to but separate from the role of the HSPs in intracellular protection. It appears that HSPs can be secreted and do not reflect solely release from necrotic or damaged cells and organs. Because secretion and circulation of extracellular Hsp70 (and Hsp27) is involved in antigen presentation, immune response, and patterns of "danger" signaling, it is an area of great interest. The secretion or release of intracellular and extracellular Hsp70 during or immediately after organ ischemia has local and systemic physiological responses that are only now beginning to be understood. The protective properties of the HSPs have been described in a wide variety of species, organs, and tissues. However, most reports of the protective roles of the intracellular Hsps are from the cardiac and CNS tissues, as are our descriptions of their role as biomarkers. In those reports in which biomarkers are correlated with functional measures such as SSEP or MEPs, HSPs appear to have value and perhaps even advantages in terms of sensitivity and specificity. The numerous roles that the

HSPs occupy in normal development, immune response, antigen presentation, intracellular trafficking, protein folding, apoptosis, and neuropathology and disease lead us to conclude that much work remains to be investigated. But we maintain that the inducible intracellular HSPs Hsp70 and Hsp27 are valuable as immediate, secreted biomarkers for critical, ongoing cellular ischemia, and offer one of the few ways to identify and eliminate or minimize the resulting damage.

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