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Regulation of Apoptotic and Inflammatory Cell Signaling in Cerebral Ischemia

The Complex Roles of Heat Shock Protein 70

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Although heat shock proteins have been studied for decades, new intracellular and extracellular functions in a variety of diseases continue to be discovered. Heat shock proteins function within networks of interacting proteins; they can alter cellular physiology rapidly in response to stress without requiring new protein synthesis. This review focuses on the heat shock protein 70 family and considers especially the functions of the inducible member, heat shock protein 72, in the setting of cerebral ischemia. In general, inhibiting apoptotic signaling at multiple points and up-regulating survival signaling, heat shock protein 70 has a net prosurvival effect. Heat shock protein 70 has both antiinflammatory and proinflammatory effects depending on the cell type, context, and intracellular or extracellular location. Intracellular effects are often antiinflammatory with inhibition of nuclear factor-κB signaling. Extracellular effects can lead to inflammatory cytokine production or induction of regulatory immune cells and reduced inflammation.

HEAT shock proteins (HSP), also called *stress proteins*, are induced by specific types of stress, including heat, and they are highly conserved from bacteria to humans.[1–4] The HSP70 family facilitates the folding of newly synthesized polypeptides in an adenosine triphosphate (ATP)–dependent manner, plays an important role in maintaining the dynamic stability of protein folding and protein–protein interactions within the cell, and inhibits protein aggregation.[5,6] These are referred to collectively as *chaperone functions*. By interacting with a range of cochaperones and client proteins, both constitutive and inducible HSPs regulate the functioning of other proteins and indeed whole signaling cascades. These interactions allow a cell to rapidly respond to stresses and changes in its environment without requiring protein synthesis, though induction of stress protein synthesis provides the next line of response. HSPs are divided into families on the basis of molecular weight. HSPs that are present as a single copy in bacteria (*e.g.*, dna K), are generally represented by multiple related genes in eukaryotes (*e.g.*, HSP70 family).

HSP70 family members have long been recognized to have cytoprotective effects. The human HSP70 family consists of at least 12 members.[7] The best known members are the heat inducible form, Hsp70/Hsp72; the constitutively expressed Hsc70/Hsp73/Hsc73; the endoplasmic reticulum form, Grp78/BiP; and Hsp75/mtHsp70/mortalin, which is localized largely to mitochondria. Of these, the cytosolic inducible Hsp72 plays a major role in mediating cytoprotective, antiapoptotic, and immune regulatory effects and is by far the best studied. Enhanced expression of Hsp72 in experimental models of stroke, sepsis, acute respiratory distress syndrome, renal failure, and myocardial ischemia has been shown to reduce organ injury and in some cases improve survival.[8–11] Deletion of the *hsp70.1/3* gene is associated with poorer outcome in mice.[12] In addition to their intracellular protective and antiapoptotic role, HSPs also function as extracellular signals.[13] We will use HSP70 to refer to the entire family, and Hsp70 in instances where either Hsp72 or 73 is referred to, because some reports and some antibodies do not distinguish between these two cytosolic family members, though the majority of studies focus on the stress inducible Hsp72.

Clinical studies have begun to identify correlations between Hsp70 and outcome in a variety of diseases. A reduced ability to induce Hsp72 in peripheral lymphocytes was noted in patients with sepsis.[14] Higher serum Hsp72 levels correlated with improved survival after trauma[15] and severe sepsis.[16,17] Several studies have evaluated Hsp70 expression after myocardial infarction and cardiac surgery with bypass and found significant increases in Hsp70 expression in all cases.[18–20] Therefore, increased levels of Hsp70 can indicate tissue damage, but they may also indicate the successful mounting of a stress response that correlates with tissue protection and better outcome.[21] Hsp70 seems to participate in protection against organ dysfunction both in critically ill patients and in patients during the perioperative period.

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Overexpression by gene therapy or chemical induction of a stress response is under investigation as a potential treatment for ischemia in several organ systems, including the use of glutamine to increase Hsp70 in critically ill patients.[17,22] We will focus primarily on data from cerebral ischemia in this review.

Hsp72 in Cell Death Signaling Pathways in Cerebral Ischemia

Hsp72 has been shown to provide neuroprotection from cerebral ischemia in animal and cell-culture models of stroke.[10,23–25] Although the mechanism of this protection was initially attributed to chaperone functions (*i.e.*, maintaining correct protein folding and blocking aggregation), recent work has shown that Hsp72 may also directly interfere with cell death pathways such as apoptosis and necrosis (fig. 1) and may modulate inflammation.[10,26–34]

Programmed cell death occurs by multiple pathways. Apoptosis occurs primarily by one of two pathways.[35] The intrinsic pathway responds to stress and intracellular changes; it relies on the release of mitochondrial proapoptotic molecules, opening of the mitochondrial permeability transition pore, and activation of caspases.[36] The second well-described pathway is the extrinsic pathway, which is triggered by the activation of plasma membrane receptors, which then signal through their death domains. This signaling activates caspase 8 and can proceed independently of the intrinsic pathway, but it can also lead to activation of the intrinsic pathway.[37] In addition, caspase-independent forms of cell death have been described,[38,39] and depletion of Hsp70 can trigger caspase-independent cell death in cancer cells.[40,41]

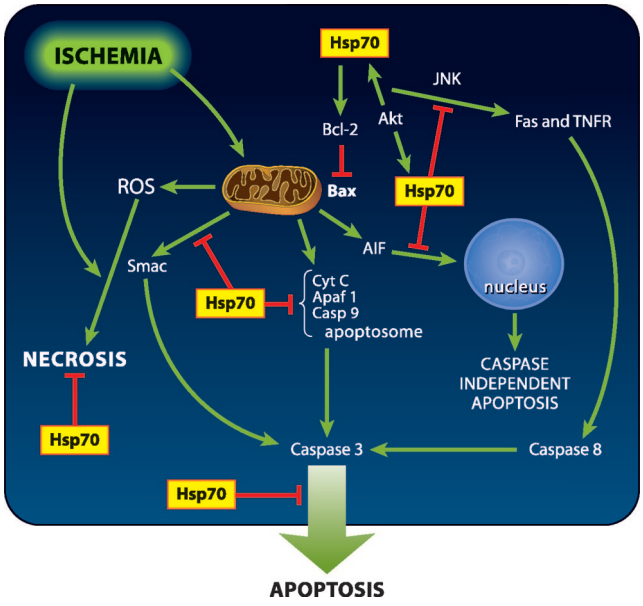

Fig. 1. Ischemia induces cell death by several distinct pathways, and heat shock protein 70 (Hsp70) reduces all of these. *Arrows* indicate increased activity or amount, and the *barred ends* indicate steps that are blocked or reduced when Hsp70 is overexpressed. These do not indicate direct protein–protein interactions in many cases (see text for details). Fas and the tumor necrosis factor receptor (TNFR) are transmembrane receptors with intracellular death domains that can induce apoptosis by activating caspase 8 *via* a pathway including tumor necrosis factor–associated factor. AIF = apoptosis inducing factor; Akt = protein kinase B; Apaf 1 = apoptosis protease activating factor 1; casp 9 = caspase 9; Cyt C = cytochrome *c*; JNK = c-Jun N-terminal kinase; ROS = reactive oxygen species. Bcl-2 is an antiapoptotic protein, and Bax is a proapoptotic member of the same family. Smac/DIABLO is a mitochondrial protein that upon release neutralizes the caspase inhibitory effects of inhibitor of apoptosis family proteins.

Hsp72 Reduces Mitochondria-dependent Apoptotic Signaling

Mitochondria are central to both necrotic and apoptotic cell death; the pathway followed often depends on the severity of the injury.[42] The resulting death reflects the signaling cascade activated by the stress or apoptotic stimulus.[43–46] In most instances, severe cerebral ischemia rapidly renders mitochondria unable to produce ATP, which ensures necrotic cell death. Mitochondrial alterations that occur during both global and focal cerebral ischemia and contribute to cell death include changes in mitochondrial respiratory function,[47,48] production of reactive oxygen species,[49,50] changes in mitochondrial membrane potential and permeability,[51,52] and release of regulatory and signaling molecules from the mitochondrial intermembrane space.[53]

Activation of the intrinsic mitochondrial pathway in ischemic brain has been demonstrated in both neonatal and adult models by the release of mitochondrial cytochrome *c*.[46,52,54] Cytochrome *c* translocates from the mitochondria to the cytosol, where it interacts with the CED-4 homolog, apoptosis protease activating factor 1, and dATP to form the apoptosome and activate caspase 9.[35,36] Caspase 9 activates caspase 3, one of the executioner caspases, as well as caspases 2, 6, 8, and 10.[55] Caspase 3 also activates caspase-activated DNase, which fragments DNA. In cerebral ischemia, caspases 3 and 9 have been shown to play a key role in neuronal death after both global ischemia[56,57] and focal ischemia,[58–61] with caspase 3–dependent apoptosis more prominent in neonatal than adult ischemia, and more prominent in global than focal ischemia. In cerebral ischemia, the downstream caspases cleave many substrate proteins, including poly(ADP-ribose) polymerase (PARP).[56,57,62] With cleavage of multiple targets within the cell and DNA fragmentation, apoptotic cell death results.[63–67]

Hsp70 affects several different steps in the apoptosis cascade (fig. 1). Hsp72 interacts with components of the programmed cell death machinery upstream[68,69] and potentially downstream[70] of mitochondrial events. Hsp72 can inhibit cytochrome *c* release in both neonatal and adult ischemia,[54,71,72] and inhibit apoptosis inducing factor translocation to the nucleus[34,73] while reducing ischemic brain injury in both adult and neonatal models.

Several of the studies on effects of Hsp72 in cerebral ischemia have been performed in transgenic mice overexpressing this gene. These findings in cerebral ischemia are consistent with observations in other systems where Hsp72 has been shown to interfere with recruitment of procaspase 9 into the apoptosome, and to sequester apoptosis inducing factor.[74] Hsp72 also inhibited release of the proapoptotic protein Smac/DIABLO from myocyte mitochondria.[75]

Mitochondrial Hsp70/Hsp75/mortalin helps to maintain mitochondrial membrane potential, which may contribute to the preservation of mitochondrial function[76] and mitochondrial protein import.[77,78] Several authors have postulated an involvement of Hsp75 in preventing electron leak between complexes III and IV, by binding and consequently reducing cytochrome *c* loss from mitochondrial membranes, thereby averting an increase in state IV respiration rates and induction of cytochrome *c*–linked apoptosis.[79] Overexpression of Hsp75 in astrocytes reduced their vulnerability to oxygen glucose deprivation, an *in vitro* model of ischemia, and maintained higher ATP levels in stressed cells.[80] Overexpression of Hsp72 in astrocytes was associated with reduced reactive oxygen species formation and better maintained mitochondrial membrane potential after ischemia *in vitro* [81] and with better preservation of glutathione levels.[27] In myocardial cells, overexpression of Hsp72 was shown to increase the activity of the mitochondrial antioxidant enzyme manganese superoxide dismutase.[82]

Hsp72 and the Bcl-2 Family Regulators of Apoptosis

Viral vector–mediated Hsp72 overexpression was associated with increased levels of Bcl-2 protein in brain cells.[83] Bcl-2 is a key antiapoptotic protein; its increased expression blocks release of cytochrome *c* and apoptosis inducing factor and reduces caspase activation. The balance between proapoptotic and antiapoptotic members of the large Bcl-2 family determines whether cells undergo apoptosis by regulating the mitochondrial membrane permeability transition.[84,85] Transgenic overexpression of Bcl-2 decreased infarction after focal cerebral ischemia,[86] whereas Bcl-2 knockout mice had increased infarct area.[87] Therefore, increased Hsp72 expression can reduce induction of apoptosis upstream of mitochondria in cerebral ischemia both directly and *via* increased Bcl-2 levels. Hsp72 blocks heat-induced apoptosis primarily by inhibiting translocation of the proapoptotic Bcl-2 family member Bax, thereby preventing the release of proapoptotic factors from mitochondria.[69] Hsp72 also interferes with the activity of apoptosis protease activating factor 1, which is required for formation of the apoptosome and activation of caspase 9,[54,74,88] but also see Steel *et al.*,[68] who demonstrated lack of direct interaction with apoptosis protease activating factor-1.

Hsp72 and Regulation of Transcription Factors in Cell Death Signaling

Hsp72 interacts with pathways leading to activation of transcription factors important in regulating cell death. Hsp72 has been shown to inhibit c-Jun N-terminal kinase (JNK) dephosphorylation, thereby blocking its activation.[89–91] Activated JNK phosphorylates the transcription factor c-JUN to up-regulate a specific group of proteins.[91] JNK activation plays both direct and indirect roles in neuronal apoptosis,[92] and it is a proposed target for stroke therapy.[93,94] JNK is implicated in apoptosis triggered by Fas, a member of the tumor necrosis factor superfamily of membrane receptors,[95] as well as figuring prominently in the apoptosis of neurons induced by growth factor withdrawal.[92] JNK is one of the mitogen-activated protein kinases. These kinases constitute one of the central signaling pathways in intracellular response,[96] often determining whether a cell responds with apoptosis or differentiation and survival. JNK signaling in the nervous system is not solely for promoting apoptosis. There is a high level of basal JNK signaling activity in the nervous system compared with other tissues, suggesting normal physiologic functions.[94] Increasing evidence suggests that the downstream events of JNK activation leading to apoptosis involve both transcription[97,98] and mitochondrial mechanisms.[92,93]

In ischemic stroke, increased c-JUN phosphorylation colocalized with terminal deoxynucleotidyl transferase–mediated dUTP-biotin end labeling in the penumbral area in an experimental model of focal ischemia.[99] Subsequent studies showed that *Jnk3*-deficient mice have increased resistance to global ischemia–hypoxia.[94] JNK3 deficiency causes reduced Bim and Fas expression after stroke, and *Jnk3*-null hippocampal neurons released less cytochrome *c* after oxygen-glucose deprivation.[94] Furthermore, mice lacking the JNK signaling scaffold protein JIP1 have increased resistance to glutamate excitotoxicity[100] and reduced infarct volume in a focal ischemia model of stroke.[101] These studies suggest that JNK signaling may play an important role in determining cell death or survival for neurons at risk in the ischemic penumbra.

Hsp72 also interacts with topoisomerase 1, which is also implicated as a regulator of apoptosis.[102,103] These interactions were shown to be independent of the ATP binding domain.[102] Hsp72 is also an effector for the important antiapoptotic prosurvival kinase Akt/protein kinase B[104,105] and acts upstream of the transcription factor nuclear factor κB (NFκB), reducing its activation, as discussed below.

Hsp72 and Inflammation

Hsp72 also plays a role in modulating inflammation caused by cerebral ischemia. Inflammation can contrib-

ute to the damage resulting from stroke.[106–109] Inflammatory responses include the activation of resident microglia and astrocytes, as well as recruitment of peripheral inflammatory cells. Inflammation and the concomitant release of reactive oxygen species and reactive nitrogen species by inflammatory cells exacerbate damage caused by direct ischemic production of reactive oxygen species. Blocking the neutrophil integrin CD11/CD18 with an antibody reduced injury in focal ischemia in association with a marked reduction of neutrophil infiltration.[110] Recruitment of peripheral leukocytes weakens the blood–brain barrier, leading to further damage. HSP70 family members play a crucial role in modulating these responses.[33,111]

Hsp72 and Inflammatory Cytokines

Intracellular Hsp72 has a range of antiinflammatory actions. It can prevent responses to inflammatory cytokines such as tumor necrosis factor α (TNF) and interleukin 1 (IL-1). Mice subjected to heat shock are protected from normally lethal inflammatory shock after systemic administration of high doses of TNF, whereas mice missing the hsp70.1 gene are no longer protected.[112] Liposomally delivered Hsp72 protein protected rats from IL-1–induced impaired pancreatic β-cell function in a diabetes model.[113] However, such protection from inflammatory responses may come at a price, because Hsp72 can actually make cells more liable to undergo apoptosis in response to TNF.[114,115] In addition to modulating the response to inflammatory cytokines, Hsp72 also down-regulates their production (fig. 2). Overexpression of Hsp72 in human macrophages blocked lipopolysaccharide-induced increases in the production of TNF, IL-1, IL-10, and IL-12.[116] In the setting of focal cerebral ischemia, overexpression of Hsp72 was associated with reduced production of TNF and IL-1b,[111] likely a reflection of reduced NFκB activation.

Hsp72, iNOS, NADPH Oxidase, and Matrix Metalloproteinases

Hsp72 may limit production of reactive oxygen species *via* several routes. Inflammation leads to the production of reactive oxygen species by activation of both the inducible form of nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Induction of iNOS occurs in response to cytokine release.[117] Mice lacking the iNOS gene are protected from cerebral ischemia relative to wild-type mice. At high levels of production, nitric oxide reacts with superoxide to produce the highly toxic strong oxidant, peroxynitrite.[117] However, iNOS can be beneficial in facilitating neurogenesis in ischemia.[118,119] Hsp72 suppresses iNOS activation in glial cells exposed to bacterial lipopolysaccharide.[120]

NADPH oxidase is one source of superoxide induced by inflammation. NADPH oxidase produces the oxidative burst of phagocytic leukocytes.[121] Recent work suggests that it may be activated in neurons as well as in microglia. That neuronal NADPH oxidase plays a role in aging and hypoglycemic injury was also suggested.[122,123] Heat shock induction of Hsp72 reduces NADPH oxidase activity in neutrophils and increases superoxide dismutase, which scavenges superoxide, in phagocytes.[124,125] Hsp72 has also been linked to regulation of matrix metalloproteinases. Matrix metalloproteinases are involved in remodeling of the extracellular matrix; they are associated with breakdown of the blood–brain barrier and hemorrhage after cerebral ischemia.[126] Hsp72 overexpressing astrocyte cultures down-regulated matrix metalloproteinase 9 after oxygen glucose deprivation, compared with wild-type cell cultures,[127] consistent with involvement of Hsp72 in regulation of this aspect of inflammation.

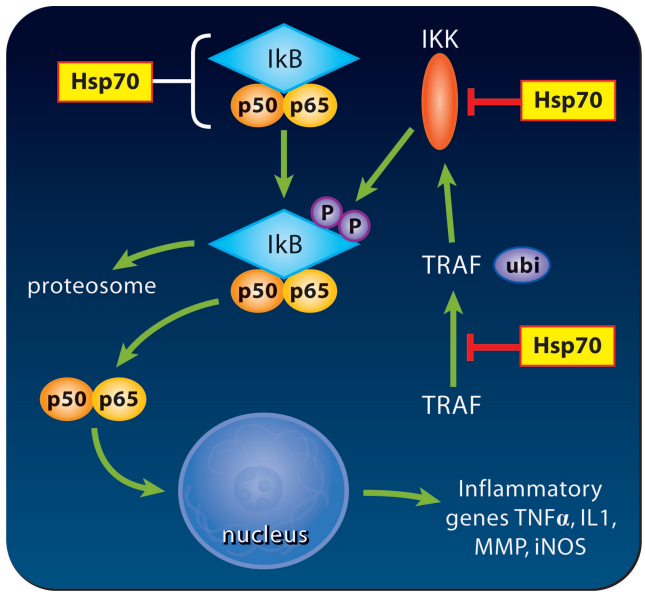

Fig. 2. Intracellular heat shock protein 70 (Hsp70) blocks activation of the transcription factor nuclear factor κB (NFκB), reducing production of downstream inflammatory mediators. Three mechanisms have been described, inhibition of activation of IκB kinase (IKK), inhibition of ubiquitination (ubi) of tumor necrosis factor–associated factor 6 (TRAF), and stabilization of the inhibitory complex with inhibitor of NFκB (IκB). IL1 = interleukin 1; iNOS = inducible nitric oxide synthase; MMP = matrix metalloproteinase; TNFα = tumor necrosis factor α. p50 and p65 are two of the NFκB subunits that, after release, move to the nucleus to act as a transcription factor resulting in activation of inflammatory genes. The Y-shaped bracket from Hsp70 to the IκB:NFκB complex is meant to indicate binding and stabilization of the complex.

Hsp72 and NFκB

Much, if not most, of intracellular Hsp72's modulatory effects on inflammation can be attributed to its regulation of the NFκB pathway (fig. 2). Transcription factors of the NFκB family are key players in the initiation of the inflammatory response.[128] NFκB is comprised of four related proteins that function as dimers. The most well studied of these is the p50/p65 heterodimer, which is normally se-

questered in the cytoplasm by its interaction with inhibitor of κB (IκB). Phosphorylation of IκB by the IκB kinase complex leads to ubiquitination and degradation of IκB, freeing the NFκB dimer to translocate to the nucleus, where it induces the expression of a multitude of genes involved in inflammatory and immune responses, including TNF, IL-1, iNOS, and matrix metalloproteinase 9.[128,129]

Induction of Hsp72 inhibits the nuclear translocation of NFκB in response to inflammatory cytokines or other stimuli.[130] Mice overexpressing Hsp72 showed reduced NFκB activation after stroke.[111] This reduced activation may be accomplished through direct interaction of Hsp72 with NFκB proteins or by interactions with other proteins in the NFκB regulatory pathway. Guzhova *et al.*[130] were able to coimmunoprecipitate Hsp72 with three members of the NFκB family (p65, p50, and c-Rel) after heat shock. IκB, however, did not coprecipitate. Feinstein *et al.*[120] demonstrated that heat shock or Hsp72 expression decreased the accumulation of NFκB p65 in the nucleus. Wong *et al.*[131] found that heat shock prevented degradation of IκB, thereby preventing activation of NFκB. Later studies identified interactions between Hsp72 and the γ subunit of the IκB kinase complex.[114] Hsp72 may also interact directly with upstream inducers of the NFκB pathway. Another recent study found that Hsp72 directly associated with the IκB–NFκB complex and suggested stabilization of the complex as another mechanism.[111] Chen *et al.*[132] found a direct interaction between Hsp72 and tumor necrosis factor receptor–associated protein 6. Ubiquitination of tumor necrosis factor receptor–associated protein 6 is a crucial step in the activation of the NFκB pathway by bacterial lipopolysaccharide and IL-1.[133–135] Hsp72 prevents this ubiquitination, which in turn prevents activation of the IκB kinase complex. It is likely that Hsp72 can operate at many levels of the NFκB pathway to inhibit or dampen its activation. Likely independent of its effects on inflammation, NFκB has frequently been associated with cell survival, acting downstream of the kinases Akt and RIP-1. Although there is also a report that NFκB may be involved in induction of apoptosis by ceramide, the majority of reports find it to have antiapoptotic actions.[136]

Extracellular HSP70s

Although most experiments to date address the intracellular functions of HSP70s, studies have now clearly demonstrated that Hsp72/Hsc73 can be released from cells. The mechanisms of release and the extracellular effects of HSP70 are growing areas of study. One of the first observations suggesting extracellular release of Hsp70 was made in the nervous system; exposure to heat caused an increase in production of heat shock–like proteins in the glial sheath surrounding the squid giant axon (reviewed by Tytell).[137] These proteins were transferred from the glial sheath to the interior of the axon. Work from several laboratories now suggests that Hsp72/Hsc73 is released from astrocytes or Schwann cells and can be transferred to and affect neighboring neurons/axons.[138–142] Hsp70 release has been documented from a variety of nonneuronal cell types, including epithelial cells,[143] rat embryo cells,[144] B lymphocytes and dendritic cells,[145,146] maturing erythrocytes,[147] and tumor cells.[148] Hsp70 and anti-Hsp70 antibodies have been identified in human serum.[149] Since then, numerous studies have examined levels of extracellular Hsp70 in relation to diseases and pathologic states, as mentioned in the introduction, though in some instances Hsp72 and Hsc73 were not distinguished. Current thinking suggests that HSPs are released physiologically, as well as by dying cells, and can act on a variety of receptors.[13,150]

Mechanism of Release of Extracellular HSP70

Because Hsp72 and Hsc73 do not contain a leader sequence for membrane targeting or localization to membrane vesicles of the secretory pathway, several alternative mechanisms for extracellular release have been proposed. One hypothesis is release from lysosomes. Lysosomal inhibitors were shown to block Hsp72 release and release correlated with increased expression of the intralysosomal protein LAMP1 on cell surfaces,[151] though others found little effect with lysosomal inhibitors. Release of Hsp72 by exosomes is the mechanism supported by the most evidence at this point.[145–148,152] Exosomes are membrane-bound vesicles containing various cytosolic proteins, including Hsp72/Hsc73 as well as peripheral and integral membrane proteins.[146] Some investigators found that lipid rafts, which are sphingolipid cholesterol–rich microdomains in cell membranes, play a role in HSP70 release.[143,153,154] In contrast, others saw no effect on Hsp72 release when either lipid rafts or the classic secretory pathway were disrupted.[152]

Effects of Extracellular Hsp72

If there are physiologic mechanisms for the release of HSP70, there must also be physiologic functions for these extracellular proteins. Although HSP release from dying cells can serve as a danger signal, release from live cells can signal a successful stress response[21] and suggests a modulatory or signaling role. Several reports demonstrated that extracellular Hsp72 could induce release of cytokines, including TNF, interleukin-6 (IL-6), and IL-1β, from monocytes.[155–158] Other reports cast doubt on those conclusions, suggesting that at least in some cases, the response is due to contamination with lipopolysaccharide, a potent inducer of cytokine release.[159–161] Extracellular Hsp72- induced cytokine release was found to be mediated through Toll-like receptor 2 (TLR2), TLR4, and downstream activation of NFκB

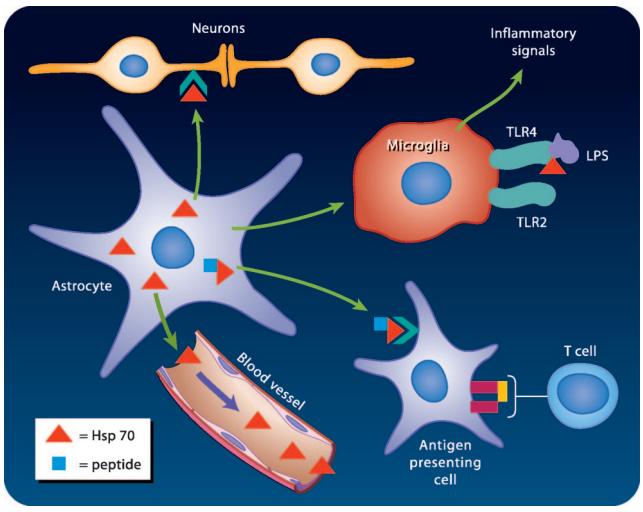

Fig. 3. Extracellular heat shock protein 70 (Hsp70) can be released by astrocytes within the central nervous system and bind to a variety of cells, especially neurons and microglia. Several cell surface receptors are implicated in Hsp70 binding to monocytes such as microglia. Hsp70 plays an important role in antigen presentation and can be present in serum. LPS = lipopolysaccharide; TLR = Toll-like receptor.

(fig. 3).[155] This contrasts with the aforementioned inhibition of NFκB activation observed in mice overexpressing Hsp72 after cerebral ischemia,[111] which is likely due to intracellular effects.

TLR4 initiates the signaling cascade triggered by lipopolysaccharide from gram-negative bacteria, whereas TLR2 mediates the signaling cascade triggered by bacterial lipoproteins, gram-positive bacteria, mycoplasma, yeast, and spirochetes. A role for HSP70 in the response to lipopolysaccharide has been identified. The details of the activation complex induced by lipopolysaccharide are still being worked out, but elegant studies of the mobility of lipopolysaccharide and some of the relevant receptors in the plasma membrane suggest that Hsp70 and 90 can be immobilized in the plasma membrane and colocalize with lipopolysaccharide and TLR4, after an initial transient interaction of lipopolysaccharide with CD14.[159] Lipopolysaccharide signaling is thus mediated by a large complex that can include Hsp70. The composition of the complex determines whether signaling results in induction or inhibition of immune response.[162,163] There is still discussion in the literature on the extent to which Hsp70 binding is directly mediated by TLR2 or 4, and whether the interaction of these receptors with Hsp70 is of high affinity, because overexpression of either receptor alone does not increase binding of Hsp70 to cells that previously did not bind Hsp70.[150]

Arispe *et al.*[164] showed a direct interaction of Hsp72/Hsc73 with lipid components. Hsc73 was shown to incorporate into the lipid bilayer and create an ATP-dependent cation channel.[164] These investigators also showed that Hsp72 and Hsc73 are able to aggregate liposomes by interacting with phosphatidylserine.[165] Although phosphatidylserine is generally found on the cytosolic side of the plasma membrane, it is present on the surface of apoptotic cells. Hsp72 and Hsc73 seem to accelerate cell death by interacting with phosphatidylserine on the surfaces of apoptotic cells.[165]

Internalization of extracellular Hsp72 is thought to be *via* cell surface receptors. Hsp72 was found to interact with two main families of cell surface proteins: the scavenger receptor family members LOX-1 and SR-A,[166] and the C-type lectins of the natural killer family. These proteins could mediate internalization of Hsp72 protein from the extracellular space.[167] Extracellular Hsp72 has been extensively studied for its role in antigen presentation *via* the major histocompatibility complex pathway, a function important for recognition of tumor cells.[168] Extracellular Hsp70 is important in triggering the activity of natural killer cells. Multhoff *et al.* identified an N-terminal 14–amino acid peptide of Hsp70 that was as active in stimulating natural killer cell cytolytic activity as full-length Hsp70 protein.[169] The activation of the cytolytic activity of natural killer cells by Hsp70 is mediated through C-type lectin receptor CD94 and the adhesion molecule CD56.[170]

Interestingly, administration of Hsp70 *in vivo* promoted wound healing by stimulating macrophage phagocytic activity,[171] and in some chronic inflammatory diseases it is now appreciated that HSPs can prevent or arrest inflammatory damage and promote production of antiinflammatory cytokines.[172] Pretreatment with Hsp70 has also been shown to reduce the inflammatory response of monocytes to a subsequent challenge with lipopolysaccharide.[173] Therefore, several different functions have already been described for extracellular HSP70, including protection of neurons and modulation of immune cell function.

Extracellular Hsp70 and Cardiovascular Disease

As the importance of inflammation in cardiovascular disease is increasingly recognized, the likelihood that immunomodulatory effects of HSP70 may be relevant increases. A significant correlation between elevated levels of serum Hsp70 and reduced progression of atherosclerosis assessed as carotid intima–media thickness was found.[174] A study of coronary artery disease patients observed significantly higher serum Hsp70 levels in patients found not to have coronary artery disease on angiogram, and disease severity was inversely correlated with serum Hsp70 levels.[175]

Although higher serum Hsp72 levels were associated with reduced risk of atherosclerosis, Hsp72 is released with myocardial infarction; serum levels after acute myocardial infarction were higher than in patients with angina.[18] Levels of extracellular Hsp72 also correlated with levels of IL-6 and IL-8. In atherosclerosis, endothelial cells are activated and macrophages release inflammatory cytokines. Oxidized low-density lipoproteins accumulate in macrophages. Svensson *et al.*[156] found that oxidized low-density lipopro-

tein–treated macrophages released increased amounts of Hsp72, and this released Hsp72-induced IL-1β and IL-12 production by naive macrophages. While elevated serum Hsp72 was associated with slower progression of carotid intimal thickening, it may also have some proinflammatory effects. The role of Hsp72 in cerebral atherosclerosis and stroke is thus complex.

Conclusions

Many studies support the protective effect of Hsp72 in cerebral ischemia. These studies employed transgenic overexpression of Hsp72 in neonatal and adult models of ischemia,[54,72,73,176] the use of mice in which the Hsp70.1 gene was knocked out,[71] and transfection or viral vector mediated overexpression.[10,34] Although each method has its own caveats, the consistent result strongly suggests that Hsp72 is efficacious at reducing cerebral ischemic injury. However, in evaluating the different mechanisms discussed in this article, much work remains to define the relative contributions of each to protection in the setting of cerebral ischemia, and differences between different models should be expected. Although there is already strong evidence for both antiinflammatory and anti–cell death effects of Hsp72 in cerebral ischemia, the relative importance of these mechanisms remains to be determined. In marked contrast, the role of extracellular Hsp72 in stroke has not yet been studied in animal models, and at this moment we are in the curious position of having more data on the association of serum Hsp70 with ischemic disease in patients than in animal models. Future studies should address this issue.

Hsp70 has many physiologic roles, both intracellular and extracellular, and participates in the regulation of many intracellular processes. Hsp70 holds great promise as a potential therapeutic approach to many diseases involving abnormalities of protein folding or increased aggregation as found in both acute and chronic neurodegenerative diseases. Hsp70 is also an important immune modulator and is now appreciated to play a role as an extracellular signaling molecule. Current understanding suggests active release of HSP from live cells to modulate the function of other cells as well as release from dying cells as a danger signal. The use of serum Hsp70 as a marker in diverse disease states and its possible use in prognosis are just being investigated. Therefore, Hsp70 holds promise as both a therapeutic strategy and a biomarker for severity of stress.

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