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EXTRACELLULAR HEAT SHOCK PROTEINS: A NEW LOCATION, A NEW FUNCTION

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Abstract

The expression of heat shock proteins (hsp) is a basic and well conserved cellular response to an array of stresses. These proteins are involved in the repair of cellular damage induced by the stress, which is necessary for the salutary resolution from the insult. Moreover, they confer protection from subsequent insults, which has been coined stress tolerance. Since these proteins are expressed in subcellular compartments, it was thought that their function during stress conditions was circumscribed to the intracellular environment. However, it is now well established that hsp can also be present outside cells where they appear to display a function different than the well understood chaperone role. Extracellular hsp act as alert stress signals priming other cells, particularly of the immune system, to avoid the propagation of the insult and favor resolution. Since the majority of hsp do not possess a secretory peptide signal, they are likely be exported by a non-classical secretory pathway. Different mechanisms have been proposed to explain the export of hsp, including translocation across the plasma membrane and release associated with lipid vesicles, as well as the passive release after cell death by necrosis. Extracellular hsp appear in various flavors, including membrane-bound and membrane-free forms. All of these variants of extracellular hsp suggest that their interactions with cells may be quite diverse, both in target cell types and the activation signaling pathways. This review addresses some of our current knowledge about the release and relevance of extracellular hsp.

Keywords

ectosomes; exosomes; immune cell activation; non-classical secretory pathway; stress; vesicles; cellular communication

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HISTORIC BACKGROUND

When cells are challenged by adverse environmental conditions, they respond by the expression of a family of polypeptides called heat shock or stress proteins (hsp). These proteins are involved in the repair of cellular damage induced by the stress, which is necessary for insult resolution. Moreover, hsp confer protection from subsequent insults, which has been coined stress tolerance (1). Thus, the expression of hsp is a universal response to stress, which is fundamental to assure survival. The cellular response to stress was discovered over 50 years ago by the Italian scientist Ferruccio Ritossa. By serendipity, he found that *Drosophila* cells exposed to temperatures higher than their normal growing conditions responded with an increase in chromosomal activity. Ritossa was puzzled by the observation, and, as a good scientist, he repeated the “accident” several times, included the appropriate controls, and was able to validate his initial unintended observation. Thus, the “heat shock” response was born. In spite of the excitement and tremendous importance of his finding, Ritossa had difficulties publishing his study, which was considered “a finding that lacks biological relevance” by the editor of a prestigious scientific journal (2). His findings were ultimately published in the journal *Experientia* (3), which does not exist anymore. Currently, we all recognize the tremendous importance and impact of the heat shock response discovery, which plays a major role in current biology (4). Today, we know that these proteins are expressed in response to a large variety of environmental, physiological, and clinical stresses, in addition to temperature increases (1). The transcriptional activity that Ritossa observed was correlated with the expression of hsp by Tissieres et al., twelve years after the initial observation (5). Several years after, a *Drosophila* gene encoding for an inducible heat shock protein was cloned (6–8).

Subsequent studies revealed that polypeptides similar to the inducible hsp were present in cells during normal physiological conditions, participating in several basic cellular processes, including protein folding, assembly of macromolecule complexes, and signal transduction (1, 9). Overall hsp, constitutive and stress-inducible, are classified according to their molecular mass into discrete families. As in many other fields, a variety of names have been given to the hsp family members, creating a vast confusion in the field. Therefore, a consensus nomenclature has been proposed (10). Each sub-group is composed of very similar proteins that differ in their sub-cellular localization, expression pattern, and minor amino acid sequence (Table 1). Hsp are better known as molecular chaperones due to their well recognized ability to fold polypeptides.

HSP CAN ALSO BE FOUND OUTSIDE CELLS

The main function of hsp, such as the folding of newly synthesized polypeptides, is carried out in the cytosol. However, these proteins have also been found outside cells, which has become a very puzzling observation. Two major questions have emerged from this unusual observation: How do these proteins get there, and what is their function? The first publication regarding the presence of hsp outside cells was by Tytell et al. (11), who reported a “heat-shock-like protein” as a glia-axon transfer protein of the squid giant axon. Almost simultaneously, Hightower and Guidon (12) described the presence of Hsp70 (HSPA1) in the extracellular medium, released by a process that could not be blocked by

inhibitors of classical secretory pathways and was independent of the possible release after cell death. They also found that extracellular Hsp70 was associated with fatty acids. These publications were against the traditional thinking since the common knowledge at the time indicated that hsp were exclusively intracellular components. Therefore, Hightower and Guidon's findings were considered as potential artifacts, similarly to Ritossa's discovery, and disregarded for many years. It was not until the year 2000 that the interest in extracellular hsp was regained. Srivastava's group showed that extracellular Hsp70, assumed to be released after tissue necrosis, modulated the immune system (13). Similarly, Asea and Calderwood showed that recombinant Hsp70 could activate macrophages as measured by an increase in the level of intracellular calcium and the release of cytokines (14). The potential immune regulatory role of Hsp70 was challenged due to the possibility that the effect was due to bacterial lipopolysaccharide contamination (15) or by other bacterial products (16). Several subsequent studies have demonstrated that, indeed, Hsp70 is capable of activating cells of the immune system (17–19).

After finding extracellular Hsp70 to be an immunomodulator, other hsp were also detected outside cells. Grp78 (HSPA5), which is an endoplasmic reticulum (ER) resident homologous to Hsp70, has been found outside cells (20, 21). Grp75 (HSPA9), also known as mortalin, is a mitochondrial chaperone protein that belongs to the Hsp70 family. This protein is released in the extracellular medium upon complement treatment of cells (22). Intracellular Hsp90, which is presented in two isoforms: α (HSPC2) and β (HSPC3), differs in its expression pattern, with Hsp90β being abundant during normal physiological conditions and Hsp90α expressed during stress (23). Initially, Hsp90α was detected outside human hybridoma SH-76 cells as a growth stimulating factor (24). Hsp90α was also identified as a secreted oxidative stress-induced factor (25). Hsp90β also has been reported secreted by osteosarcoma cells (26). A homologous form of cytosolic Hsp90 is the ER resident Grp94 (HSPC4), which has also been found in the extracellular space (27, 28). The release of Grp94 can be explained since this protein is an ER resident that can be transported through the Golgi complex. As for Hsp70, extracellular Hsp90 was considered to be a product of cell death. However, further studies have shown that the protein could be secreted by an active mechanism (29, 30). Another hsp, Hsp60 (HSPD1), which is located into the mitochondria and cytosol, has been found in plasma after some disease conditions (31, 32). In addition, circulating anti-Hsp60 antibodies has been detected in a variety of pathological conditions (33). Small hsp are also secreted by cells and modulate the immune system (34). In particular, Hsp27 (HSPB1) has been noticed in serum of patients during pathological conditions (35, 36). One of the members of the large hsp family, Grp170 (HSPH4), has also been detected outside cells (37).

THE EXPORT OF HSP

Since the detection of hsp outside cells or in plasma, a discussion has been generated regarding whether or not the protein is released secondary to cell death or exported by an active mechanism that is non-dependent on cell lysis. The initial studies of Srivastava's group (13) proposed that the release of Hsp70 was the product of cellular necrosis. On the other hand, Hightower and Guidon (12) demonstrated that the release of Hsp70 was not due to cell death, which was later confirmed by Hunter-Lavin et al. (38). Therefore, the question

is whether both pathways are possible. The major argument against the secretion of hsp, particularly Hsp70, by an active mechanism is the fact that this protein does not contain a consensus peptide signal for secretion via the classic ER-Golgi pathway. Moreover, typical inhibitors of the ER-Golgi pathway, such as brefaldin A, did not block the release of Hsp70 from healthy cells (12). These observations suggest that Hsp70 may be exported via an alternative mechanism, which has been named the non-classical or unconventional secretory pathway (39). Several other proteins, such as interleukin-1α and β, high-mobility group box 1, galectin-1 and 3, and fibroblast growth factor-1 have also been suggested as released by this alternative pathway. However, a unifying mechanism for the release of proteins by the non-classical secretory pathway has not yet emerged (39). Different scenarios have been proposed for the active export of Hsp70 from the cytosol into the extracellular milieu. One suggested mechanism is export via the lysosome-endosome pathway, in which Hsp70 is translocated into the lysosome lumen via ATP-binding cassette (ABC) transport-like system and further transported outside cells via the endocytic process (40). Other studies have suggested that Hsp70 is released via secretory-like granules (41). However, the most accepted mechanism for the release of Hsp70, as well as other hsp, is via extracellular vesicles (ECV) (42). These vesicles are derived from the plasma membrane by various processes including membrane blebbing, which is known as ectocytosis and the derived vesicles coined ectosomes. Alternatively, ECV could be produced by endocytosis, which causes the formation of multiple vesicle bodies that are released into the extracellular medium, producing vesicles with the same topology as the plasma membrane and are named exosomes (42, 43). Regardless of the origin of the vesicles, there is compelling evidence that hsp can be detected within these vesicles (Table 2). The main difference between these studies is the location of hsp within the vesicles, which has been proposed to be in the lumen or the membrane of the ECV. The presence of hsp in the lumen of ECV likely results from the protein being trapped into the intracellular space of the vesicle during its formation since these proteins are very abundant in the cytosol (42). The argument against this idea is that proteins in the vesicle lumen may not be able to interact directly with target cells. Thus, it should be assumed that ECV may burst, releasing the cargo and allowing the hsp, now free in solution, to interact with cells. Alternatively, it is possible that ECV fuse with the membranes of cells, releasing the lumen content into the cytosol. A different idea is that hsp are associated with the membranes of ECV, which has been shown for several hsps including Hsp70 (18, 44, 45) and Hsp60 (46, 47). The presence of hsp on the membrane (surface) of ECV is important since it explains the specific interaction with target cells, probable by a process mediated by surface receptors. Moreover, the possibility that multiple copies of the hsp are present within a single ECV suggests that its biological activity may be enhanced dramatically. In fact, the concentration of Hsp70 within ECV has been calculated in the mM range, which is a concentration that is unlikely to be reached by soluble proteins (42). In support of this idea, it has been shown that the specific activity of Hsp70 associated with ECV is over 250 fold more elevated in the process of activating macrophages than the protein in solution, free of membranes (18). Hsp90, which also lacks a secretory signal that justifies the transport via the ER-Golgi compartment, has been proposed as being exported via exosomes (48–50) or by direct translocation across the plasma membrane (30).

We have hypothesized that the interaction of Hsp70 with membranes is the first step in the active secretion of this protein (42). The presence of Hsp70 on the surface of cells has been well documented by several groups (42, 51). Indeed, the presence of other hsp on the cell surface has been widely shown by many investigators under different physiological and pathological conditions (42). The constitutive member of the cytosolic Hsp70 family, Hsc70 (HSPA8), was observed on the bile duct membrane (52). Grp78, also known as BIP, has been detected on the cell surface (53). The mitochondria Hsp60 has been reported on the surface of human T cell-lines (54), endothelial cells (55, 56), as well as in the liver and spleen of mice (57). Hsp90 was initially detected on the cell surface of tumor cells (58) and subsequently in multipotential mesenchymal precursor cells (59), human neuroblastoma cells (60, 61), human monocytes, (62). Grp94 was observed on the plasma membrane of sarcoma cells (63). The initial observation for the interaction of Hsp70 with lipids came from Hightower and Guidon's work that found extracellular Hsp70 associated with fatty acids (12). Arispe and De Maio (64) showed that the addition of pure Hsc70 (HSPA8) to an artificial lipid bilayer resulted in the detection of cationic conductance activity, which was very stable and regulated by nucleotides. These observations were extended to Hsp70 (18). Further studies have demonstrated that both Hsc70 and Hsp70 display a high specificity for phosphatidylserine as compared with many other lipids (65, 66). In addition, Hsp70 appeared to interact with phospholipid bis(monoacylglycero)phosphate (67), which is also negatively charged, suggesting that the interaction with lipids is electrostatic. A domain containing a stretch of positively-charged amino acids at the C-terminus of Hsc70 was found to mediate the interaction of this protein with endosomes, which was confirmed by site directed mutagenesis (68).

Although the evidence for the active secretion of hsp from "healthy" cells is notable, it cannot be discarded that, under other circumstances, hsp are released into the extracellular medium or plasma after cell necrosis. Indeed, the concentration of Hsp70 released after necrosis can be potentially very high. Basu et al. have proposed that 1 g of necrotic tissue could release up to 200 μg of Hsp70 in the extracellular medium (13). In this regard, expression of Hsp70 has been observed after ischemia/reperfusion injury (I/R), which resulted in a necrotic focus (69). Assuming that the average amount of Hsp70 is in the order of 10^7 molecules per stressed cell (70), and there is an average of 3×10^8 hepatocytes per rat liver (71), a 1/30 hepatic ischemia/reperfusion injury could potentially result in the release of 10^{14} molecules of Hsp70, which is approximately 120 μg. If the total blood volume of a rat is approximately 8 ml, the circulating concentration of Hsp70 could be as high as 15 μg/ml, in theory. This massive amount of Hsp70 in circulation could have detrimental consequences. For example, Hsp70 has been shown to induce cell death (65, 66), which has long been recognized as a secondary effect of Hsp70 over-expression in cells (72).

The arguments presented above suggest that Hsp70 could be released by two possible mechanisms: passive (necrosis) or active (secretion). For active secretion, Hsp70 is likely associated with membranes within ECV (membrane-bound), whereas for the passive process Hsp70 is likely to be in solution or soluble (membrane-free). These possibilities were investigated in various *in vivo* models. Male rats that underwent regional hepatic I/R by total blockage of the blood supply to the median liver lobe (30 min), whereas blood flow was

preserved in the rest of the liver (69), displayed a level of Hsp70 in plasma in the order of 60.3 ng/ml, which was more elevated than in sham-operated rats (0.02 ng/ml) and non-manipulated control animals (non-detectable). The plasma levels correlated with induction of Hsp70 in the ischemic liver (detected by Western blotting). In contrast, no extracellular Hsp70 was detected in urine samples taken after I/R. The presence of Hsp70 in plasma was also investigated after total body thermal stress or heat shock (HS) at 42°C for 10 min as previously described (73). The level of Hsp70 in the plasma samples of thermally stressed animals was 99.4 ng/ml as opposed to non-stressed controls (0.04 ng/ml, p=0.0002 Student t-Test). Hsp70 was also detected in bronchoalveolar lavage fluid after HS (2.52 ng/ml), which was significantly higher than in non-stressed rodents (0.99 ng/ml, p=0.004 Student t-Test). After both conditions, I/R or HS, ECV were isolated from plasma by differential centrifugation and the content of Hsp70 compared with supernatant of the sample corresponding to the membrane-free fraction. ECV derived from thermally stressed rats contained Hsp70 (30.9 ng/ml), whereas no detectable Hsp70 was found in samples from non-stressed rats (p=0.01). The content of Hsp70 in the ECV fraction corresponded to 20% of the total concentration in the sample (Table 3). In contrast, all Hsp70 detected in plasma samples after I/R was in the membrane-free fraction (Table 3). These observations suggest that extracellular Hsp70 could be released by both, cell necrosis (I/R) and active secretion, the latter via ECV.

Clinical relevance of extracellular hsp

The number of clinical conditions presenting Hsp70 in plasma samples is increasing, including the top killers in the Western world: cancer, cardiovascular disease, diabetes, and trauma (42). Major attention has been directed at the effect of extracellular hsp on the immune system in which they act as signaling molecules. Indeed, Hsp70 has been shown to induce the activation of macrophages, monocytes, dendritic cells, and natural killer cells (42). Extracellular Hsp70 has also been reported to increase microbicidal capacity and chemotaxis of neutrophils (74, 75), phagocytosis (76), and modulate the response of monocytes to endotoxin (77). Moreover, extracellular Hsp70 has been associated with both immunostimulatory and immunosuppressive activities (78). However, the mechanisms involved in the modulation of the response of cells of the immune system are still unknown. Extracellular hsp have also been shown to modulate the activity of cells from other biological systems. Thus, extracellular Hsp70 has been reported to affect cardiomyocyte contractile dysfunction (79) and increase the growth of tumor cells and resistance to apoptosis (80). Glial cells have also been reported to release Hsp70, conferring protection to neighboring neurons (81). Extracellular hsp have been observed to interact with amyloid β peptides, which are involved in the pathology of Alzheimer's disease (82, 83). Moreover, Hsp70 and Hsp90 inhibited amyloid β aggregation *in vitro* conditions (84). Extracellular Hsp70 has been revealed to protect Schwann from hydrogen peroxide induced apoptosis (85). Exogenous α-crystallin, a small hsp, has been shown to induce neuroprotection during acute inflammation in mice (86). Extracellular Hsp25 (HSPB1) also resulted in reduced cardiotoxicity induced by doxorubicin (87). Extracellular Hsp90 has been reported to activate cancer cell motility (88), migration, and metastasis (60, 89). It has also been associated with neuronal motility (90) and wound healing (91, 92). Extracellular Hsp90 has been shown to transport antigens from the outside to the cytosol, resulting in cross-

presentation (93). In addition, extracellular Hsp60 has been demonstrated to modulate the immune system (19, 94). Other studies have proposed that Hsp60 induced apoptosis cardiac myocytes (95). Grp170 has also been demonstrated to modulate the innate immune system (37).

The presence of Hsp70 in plasma has been identified as a risk predictor for acute coronary syndrome (96). Moreover, Hsp70 has been detected in circulation after pregnancy (97), hypertension (98), acute infection (99), brain and spinal cord ischemia (100) and extenuating exercise (101–103). The presence of Hsp70 in circulation has also been correlated with improved survival of critically ill patients (104–106). Circulating levels of Hsp60 (107) or Hsp70 (108, 109) or their antibodies have been proposed as a risk factor for coronary heart disease. In addition, extracellular levels of Hsp70 have been correlated with severity and survival after chronic heart failure (110). Hsp60 has also been detected in saliva and serum of type 2 diabetic patients (111), whereas Hsp70 has been found during diabetic ketoacidosis (112). Both Hsp70 and Hsp60 have been detected in circulation after soft tissue trauma (113). The levels of Hsp27 were increased in serum of patients with chronic pancreatitis and pancreatic carcinoma (35, 36).

CONCLUDING REMARKS

The presence of hsp in the extracellular medium has been a very controversial topic, probably due to the reduced understanding of the mechanism for the release of these proteins that lack a consensus secretory peptide signal. However, evidence accumulated during the last several years has demonstrated that the presence of extracellular hsp is not an artifact, but rather a novel biological event. Therefore, the role of extracellular hsp appears to be at the level of signaling or cellular communication rather than at the traditional chaperone activity. Indeed, there is no evidence that extracellular hsp could carry out their chaperone activity outside cells. Moreover, the requirement of co-chaperones and other factors for their intracellular function are unlikely to be necessary for their extracellular role as signaling molecules. Certainty, it is possible that we will encounter multiple roles for extracellular hsp, which will likely depend on the cellular milieu or type of target cell. A particularly interesting finding is the modulation of the immune system, which has been envisioned as a priming event or a novel form of cellular communication in the case of stress conditions. Indeed, the modulation of the immune system associated with ECV bearing hsp has been coined the Stress Observation System (SOS), which has the primary function of activating the immune system to avoid the propagation of the insults (42). Another variant to add flavor to the role of extracellular hsp is the observation that they could be derived from both healthy and necrotic cells. Moreover, extracellular hsp can be detected in two “flavors:” membrane-bound or membrane-free, depending on their origin. It is likely that these two variants of extracellular hsp could play alternative roles in the modulation of the immune system or other systems. Finally, the detection of extracellular hsp, which is a very improbable observation that is against the conventional wisdom, is changing the biology of the stress response just as it was challenged 50 years ago by Ritossa‘s discovery.

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TABLE 1

Classification of hsp

Family Name	Common Name	New Name
Hsp100	Hsp105	HSPH1
	Hsp110	HSPH2
	Grp170	HSPH4
Hsp90	Hsp90α	HSPC2
	Hsp90β	HSPC3
	Grp94	HSPC4
Hsp70	Hsp70 (Hsp72)	HSPA1
	Hsc70 (Hsp73)	HSPA8
	Grp78 (BIP)	HSPA5
	Utp70 (Grp75)	HSPA9
Hsp40	Hsp40 (Dnaj)	DNAJB1
Small Hsp	αCrystallin	HSPB4
	Hsp25	HSPB1
	Hsp27	HSPB2
	Hsp20	HSPB6
	Hsp22	HSPB8
Chaperonins	GroEL (Hsp60)	HSPD1
	GroES	HSPE1

TABLE 2

Detection of hsp in ECV

Hsp	Cells	Reference
Hsp70	Dendritic Cells	Thery, et al. 1999 (44)
Hsp70	Colo357/CX2 Pancreas carcinoma Colon carcinoma	Gastpar, et al. 2005 (46)
Hsp70	PBMC	Lancaster and Febbraio 2005 (112)
Hsp70	Human hepatoblastoma	Vega, et al. 2008 (19)
HSP70	EL4 thymolymphoma Mammary carcinoma Colon carcinoma	Chalmin, et al. 2010 (47)
Hsp70	Mycobacteria-infected (M. smegmatis and M. avium) RAW 264.7	Anand, et al. 1999 (113)
Hsc70/Hsp70	Reticulocytes	Mathew, et al. 1995 (114)
Hsp70, Hsc70, Hsp27, Hsp90	B cells	Clayton, et al. 2005 (50)
Hsp70, Hsp90, Grp78	Rat hepatocytes	Conde-Vancells, et al. 2008 (115)
Hsp90	Dendritic cells	Chaput, et al. 2006 (116)
Hsp90	Human glioblastoma Human fibroblastoma Human mammary gland adenocarcinoma	McGready, et al. 2010 (52)
Hsp90, Hsc70	Mesothelioma	Hegmans, et al. 2004 (117)
Hsp60	Cardiac myocytes	Gupta and Knowlton 2007 (48)
Hsp60	Bronchial carcinoma Lung adenocarcinoma Erythro-leukemia	Merendino, et al. 2010 (49)

TABLE 3

Levels of membrane bound and free Hsp70 after I/R and thermal stress.

System	Stress	Membrane Bound (%)	Membrane Free (%)
HepG2	Heat shock	100	0
Liver	I/R	0	100
Whole body	Heat shock	20	80