Chapter 3
Extracellular Heat Shock Proteins as Stress Communication Signals

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Abstract Intercellular communication is a fundamental process necessary to maintain homeostasis and to mount an orchestrated response to stress. Although heat shock proteins (HSP) play a critical role by participating in the repair of damaged products as a result of the stress in the intracellular milieu, it is now evident that they play an alternative role when they escape from the cells and are placed in circulation, participating in a systemic stress response. Extracellular HSP appear as signaling molecules involved in intercellular communication during stress conditions. They have been found to modulate the function of many target cells. Moreover, extracellular HSP have been detected in several biological fluids, particularly from patients suffering from a large number of maladies. Extracellular HSP are released by many cell types and by several mechanisms, including passive dissemination after necrosis and active export by a nonclassical secretory pathway. Among several potential mechanisms for the export of HSP, their release associated with extracellular vesicles has gained increasing support. The appearance of extracellular vesicles containing HSP emerges as a new form of cellular communication during stress conditions directed at avoiding the propagation of the insult.

Keywords Heat shock proteins · Cellular communication · Extracellular vesicles · Stress · Signaling

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3.1 Introduction

Cellular communication is a major physiological event that is crucial to maintain homeostasis. In this regard, unicellular organisms use chemical gradients to synchronize their metabolism and growth. Plants release volatile compounds as signals to coordinate development, attract pollinizing insects, and repel predators. Multicellular organisms send signals to their counterpart cells to regulate metabolism, growth, and stress response. Intercellular communication is particularly important in tissue homeostasis, in which a synchronized propagation of signals among cells is required to adapt to changes in nutrients and other environmental factors. For example, hepatocytes within the hepatic acinus are interconnected by various mechanisms of communication to modulate their response to changes in the delivery of nutrients, such as glucose and oxygen. Cellular communication is also critical for an efficient response of the immune system. Thus, the communication between T, B, and antigen-presenting cells is necessary to orchestrate the adaptive immune response. Similarly, macrophages, dendritic cells, and neutrophils secrete cytokines and chemokines in response to infection and injury to promote an initial response to the insult. Therefore, a coordinated intercellular communication is vital to preserve normal physiological conditions and mount a sound response to stress.

3.2 Types of Cellular Communication

Cells communicate by a variety of mechanisms. The most common is via soluble molecules that are placed in the extracellular environment or in circulation directed at interacting with adjacent or distant cells. A typical example of this type of communication is when hormones and cytokines are released by a certain type of cell and captured by another via specific receptors. The ligand-receptor interaction triggers a signal transduction pathway within the plasma membrane or within internal compartments directed at activating the right response to the external stimuli. In other cases, cells communicate via surface contact molecules, such as adhesion proteins, resulting in cellular synapses [1]. A great example is the immune synapse between antigen presenting cells and lymphocytes [2]. Cells in close proximity can also interact via exchanging surface molecules by the direct transfer of plasma membrane portions, which is known as trogocytosis, by membrane tethers, or by nanotubes [3]. A very important form of cellular communication is via the transfer of low-molecular-weight metabolites via gap junctions. Gap junctions are larger channels or pores formed by similar proteins within the plasma membrane of adjacent cells that allow the passage of ions (e.g., calcium), nucleotides (e.g., ATP), and other small molecules in a regulated process, creating a network of signals across the multicellular environment [4].

An alternative mechanism for cellular communication is mediated by the release of membrane vesicles into the extracellular medium. These extracellular vesicles

(ECV) contain surface molecules, lipids, and cargo (e.g., proteins, nucleic acids, carbohydrates, and ions). The critical aspect of ECV is that they contain a large number of molecules within a very small volume [5–7]. These ECV are captured by target cells delivering the cargo, such as signaling proteins or microRNAs, which can modulate the function of the receiving cell. More importantly, the target cell senses a multiplicity of different molecules simultaneously, which is likely to result in a synergy of information. In other words, different components within ECV could concurrently activate various cellular pathways. Moreover, the concentration of a ligand within a small vesicle (e.g., 100 nm in diameter) is theoretically calculated in the millimolar range, which is much larger than the circulating concentration of any hormone. ECV could also travel long distances, delivering information to very distant cells. The fact that ECV are formed by membrane-encapsulated macromolecules assures the protection of the cargo from external environmental factors, such as circulating proteases and RNAses. The final stage for communication via ECV requires the recognition by the target cell that it could be mediated by various mechanisms. For example, ECV may contain surface molecules that are recognized by specific receptors on target cells acting as "zip codes." In addition, ECV could be taken by cells in a non-receptor-mediated process, such as macropinocytosis, or they could fuse with the plasma membrane delivering the cargo into the cytosol.

3.3 Extracellular HSP as Communication Signals

Heat shock proteins (HSP) were first discovered as part of the cellular response to elevated temperatures, initiated by the discovery of the heat shock response by Ritossa [8], followed by the identification of HSP by Tissières et al. [9]. Subsequent studies showed that HSP correspond to a large family of proteins expressed after a variety of stress conditions [10, 11]. Various homologs to the stress-inducible HSP were identified afterward participating in normal basic cellular processes, including folding of newly synthesized polypeptides, translocation of polypeptides across subcellular compartments, assembly of macromolecule complexes, stabilization of receptors, and signal transduction [11, 12]. The capability of folding denatured proteins or stabilizing protein complexes gave rise to their denomination as molecular chaperones [13]. Various HSP belong to particular families that are classified according to their molecular weight, for example the Hsp70 family, which has a molecular weight of 70 kDa, is composed of four members: Hsp70 (the stress inducible form), Hsc70 (the constitutive cytosolic form), Mit70 and Grp78 (both constitutive forms located in the mitochondria and endoplasmic reticulum, respectively). Recently, a new nomenclature for HSP has been proposed [14], displayed in Table 3.1.

Although the most recognized function of HSP is as molecular chaperones in the cytosol and other subcellular compartments, they have been found outside cells. The first observations regarding the presence of HSP in the extracellular environment was made on Hsp70 by studies of Tytell et al. [15] and Hightower and Guidon

Table 3.1 Classification of HSP

Family name	Common name	New name
HSP 100	HSP105	HSPH1
	HSP110	HSPH2
	Grp170	HSPH4
HSP90	HSP90α	HSPC2
	HSP90β	HSPC3
	Grp94	HSPC4
HSP70	HSP70(HSP72)	HSPA1
	HSC70(HSP73)	HSPAB
	Grp78(BIP)	HSPA5
	Utp70 (Grp75)	HSPA9
HSP40	HSP40 (Dnaj)	DNAJB1
Small HSP	αA-Crystallin	HSPB4
	αB-Crystallin	HSPB5
	HSP25	HSPB1
	HSP27	HSPB2
	HSP20	HSPB6
	HSP22	HSPB8
Chaperonins	GloEL (HSP60)	HSPD1
	GloES	HSPE1

[16]. These pioneer findings were followed by more recent observations documenting the presence of Hsp70 in the extracellular medium in a variety of conditions (reviewed by De Maio) [6]. Today, practically all members of the HSP family have been detected outside cells. Thus, Hsp90α (HSPC3) was identified as a secreted oxidative stress-induced factor by vascular smooth cells [17]. Hsp90β (HSPC4) was reported released by osteosarcoma cells [18]. Grp75 (HSPA9) or mortalin, which is a mitochondrial chaperone protein, has been shown to be released after complement treatment of cells [19]. Grp78 (HSPA5) and Grp94 (HSPC4), which are endoplasmic reticulum (ER) residents, have been found in the extracellular space [20–22]. HSP60 (HSPD1) has been detected in circulation of patients suffering from various conditions [23]. Hsp25/27 (HspB1) was observed in the extracellular environment of astrocytes [24]. Finally, a large member of the HSP family, Grp170 (HSPH4), has also been detected outside cells [25].

Extracellular HSP are secreted by a variety of cell types and captured by others. The function of extracellular HSP has not been associated to their chaperone activity, which is not surprising since it requires cochaperones and nucleotides for the function. On the contrary, extracellular HSP act as signaling molecules involved in the communication between cells, inducing an array of activities. For example, Hsp70 (HSPA1) secreted by parenchymal cells has been shown to induce a robust activation of macrophages [26–28], dendritic cells [29], and natural killer cells [30, 31]. Extracellular Hsp70 has also been shown to modulate the response of monocytes to endotoxin [32, 33], activate chemotaxis [34], and phagocytosis [35–37].

They also could modulate antigen presentation [36]. *Mycobacterium tuberculosis*-derived DnaK has been shown to polarize macrophages to M2-like phenotype [38] and induce anti-inflammatory response [39]. Recently, Hsp70, Hsp90 (HSPC), and Hsp40 (DNAJB1) have been proposed to promote protein homeostasis in distant cells [40]. Moreover, extracellular Hsp70 has been associated with both immunostimulatory and immunosuppressive activities [41]. Extracellular Hsp90 has been shown to transport antigens from the outside to the cytosol, resulting in cross-presentation [42]. Small HSP are also secreted by cells and modulate the immune system [43]. Hsp90α was detected outside cells participating in wound reepithelialization and healing [37, 44, 45]. Extracellular Hsp70 has been shown to affect cardiomyocyte contractile dysfunction [46], and increase tumor growth and resistance to apoptosis [46]. Exogenous Hsp70 appeared to disrupt gap junction communication between human microvascular endothelial cells [47].

Extracellular HSP may be recognized by a variety of cell surface targets [48]. The list of potential receptors for extracellular HSP is large, including CD91 [49, 50], CD40 [13, 51], Scavenger receptor A [52], Lox 1 [53], mannose receptor [54], and even the β-subunit of ATP synthase [55]. Recently, Hsp70 was shown to bind to Siglec-5 and Siglec-14, which are Ig-superfamily lectins on mammalian leukocytes that recognize sialic acid-bearing glycans [56]. Some lipids have also been proposed as targets for HSP, such as sphingolipids [57, 58], phosphatidylserine [59, 60] and phospholipid bis(monoacylglycero)phosphate [61]. In general, it appears that HSP have a large appetite for molecules, raising the possibility that a single receptor model may not be correct.

3.4 Extracellular HSP in Pathological Conditions

Extracellular HSP has been associated with several clinical conditions, following their detection in various biological fluids (Table 3.2). In addition, antibodies against HSP have been found in the serum of a variety of patients [23, 62]. For example, circulating levels of Hsp70 and Hsp60 or their antibodies have been proposed as a risk factor for coronary heart disease [63–65]. Similarly, Hsp60 has been detected in circulation of individuals suffering from cardiovascular diseases [66, 67]. Extracellular Hsp25 has been shown to reduce cardiotoxicity induced by doxorubicin [68]. Hsp70 has been reported to be present in the serum of patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [69]. Hsp27 has been detected in the serum of patients with chronic pancreatitis and pancreatic carcinoma [70, 71]. Hsp60 has also been observed in the saliva and serum of patients with type 2 diabetes mellitus [72] and Hsp70 in patients presenting diabetic ketoacidosis [73].

Extracellular HSP have been associated with infection and other pathologies. Thus, extracellular Hsp70 has been identified following acute infection in humans [86]. Hsp70 and Hsp60 were found in wound fluids at the site of soft tissue injury [83]. Moreover, the presence of Hsp70 in circulation has also been linked with improved survival of critically ill patients [80–82]. In other studies, Hsp70 was

Table 3.2 Heat shock proteins in clinical conditions

Pathology	HSP	Reference
Heart disease	Hsp70, Hsp60	[66] [63] [74] [75] [64] [65] [46]
Cancer	Hsp70, Hsc70	[76] [77] [78] [69]
Liver cirrhosis	Hsp70	[69]
Hepatitis		[69]
Pancreatitis	Hsp27	[71] [70]
Diabetes	Hsp60, Hsp70	[79] [73] [72]
Trauma	Hsp70, Hsp60	[80] [81] [82] [83]
Ischemia–reperfusion injury	Hsp70	[75] [84] [85]
Infection	Hsp70	[86] [87]
Preeclampsia	Hsp70	[88] [89]

found to be released from human fetal membranes after exposure to *E. coli* [87]. Hsp70 has also been detected in normal and pathological pregnancies, including preeclampsia [88, 89]. Moreover, Hsp70 has been observed in amniotic fluid [90]. Circulating Hsp70 has been detected after extenuating exercise [91–93]. Finally, extracellular Hsp70 was present in the blood of experimental rodent models of diabetes [94], sepsis [95], and ischemia–reperfusion injury [85].

The central nervous system has also been a target for extracellular HSP activity. For example, extracellular Hsp70 has been observed after brain and spinal cord ischemia [84]. Several small HSP, including Hsp20 and Hsp22, have been detected in closed proximity of amyloid β deposits in the brains of Alzheimer disease patients [96]. Moreover, they were found to block amyloid β aggregation in vitro [96, 97]. Similar observations regarding inhibition of amyloid β aggregation have been made for Hsp70 and Hsp90 [98] and Hsp40 [99]. Moreover, HspB1 (Hsp25/27) was reported released by astrocytes in response to amyloid β exposure [24].

Extracellular Hsp70 has been shown to protect Schwann cells [100] and neurons [101]. Both αA-crystallin (HSPB4) and αB-crystallin (HSPB5) have been reported to protect astrocytes from various toxic agents [102].

3.5 Mechanisms of HSP Export

Extracellular HSP are released from at least two different sources. HSP are disseminated by a passive process secondary to cell lysis after necrosis [29, 85] or exported by an active mechanism independent of cell death, which could not be blocked by typical inhibitors of the ER-Golgi pathway, such as brefeldin A [16, 79]. The only exceptions are ER-resident HSP, Grp78 and Grp94, which are already in place within the classical secretory pathway. In contrast, the majority of HSP lack the consensus signal required for secretion via the ER-Golgi pathway. Therefore, they are likely exported by an alternative mechanism that has been named the nonclassical or unconventional secretory pathway. Many proteins besides HSP use this pathway for export, including interleukin-1β, high-mobility group box 1, galectin 1 and 3, and fibroblast growth factor 1 [103].

The major argument against the active export of HSP is that these proteins are localized in the cytosol. In order to reach the extracellular environment, they need to cross the plasma membrane. Thermodynamically, the passing of a protein across a lipid membrane results in a less favorable change of free energy [104]. Therefore, it should not be a spontaneous process. In spite of the prior assumption, there is substantial evidence that Hsp70s can spontaneously get inserted into lipid bilayers. Indeed, our pioneering work showed that Hsc70 got inserted into artificial lipid bilayers opening ion conductance pathways [105]. This observation has been confirmed by others [106] and extended to Hsp70 [28]. Additional studies showed that both Hsp70 and Hsc70 interact with liposomes resulting in their aggregation in a process that was concentration dependent and requiring the presence of nucleotides [4]. Moreover, Hsp70 have been demonstrated to get spontaneously and selectively inserted into phosphatidylserine (PS) liposomes, forming high molecular mass oligomers [107]. The interaction of Hsp70 with PS liposomes has been confirmed by others [108]. Similarly bacterial Hsp70 (DnaK) also gets inserted into liposomes, but the translocation was not lipid specific and only forms dimeric forms within the membrane [109]. These observations suggest that HSP, at least Hsp70, could move spontaneously from the cytosol into the plasma membrane. Indeed, Hsp70 has been extensively reported to be present in the plasma membrane of transformed cells [110, 111]. The presence of Hsp70 on the plasma membrane was resistant to acid washes indicating that it was actually inserted into the lipid bilayer [28, 57]. Therefore, the question that emerges is whether Hsp70 could also spontaneously come out from the lipid membrane outside the cells. Although this option has not been demonstrated experimentally, it may be an interesting possibility to explain the extracellular release of this protein. Other mechanisms that have been proposed for the active secretion of HSP include the lysosome–endosome pathway, in which the protein translocates into

the lysosome lumen via ATP-binding cassette (ABC) transport-like system and is further released outside cells via the endocytic process [112]. This pathway has also been proposed for the secretion of IL-1β, which also moves from the cytosol to outside the cell without passing through the ER-Golgi pathway [113]. Other studies have suggested the release of Hsp70 via secretory-like granules [114].

A well-accepted mechanism for the export of HSP is their association with ECV [6]. These vesicles are derived from the plasma membrane by various processes. Protuberances or blebs can be formed in the outer side of the plasma membrane by a process of ectocytosis, resulting in the release of large vesicles known as microvesicles (>1 μm) particles or smaller vesicles named ectosomes (about 100 nm). Alternatively, ECV could be produced by endocytosis of the plasma membrane-forming endosomes. The membrane of these endosomes invaginates toward the lumen resulting in the formation of new vesicles included within a large vesicle that has been named multiple vesicular bodies X. The vesicles inside the multiple vesicular bodies have the same topology of the plasma membrane. When these multiple vesicular bodies fuse with the plasma membrane the vesicle content within the lumen are released outside the cell. ECV derived from this process are known as exosomes [5, 6]. There is extensive evidence that HSP are present within different ECV that is summarized in Table 3.3. The detection of HSP within ECV has primarily been made by mass spectroscopic analysis and, in some cases, confirmed by Western blotting. Since HSP are mainly present in the cytosol, their localization within ECV was assumed to be in the lumen as a result of trapping these proteins during the formation of the vesicles. However, it has been proposed that the composition of ECV is not random but rather a very selective process [6, 115]. Other observations have shown that HSP are located within the membranes of ECV, as in the case of Hsp70 [28, 31, 116], Hsp90 [117], and Hsp60 [118, 119]. The presence of HSP on the membrane (surface) of ECV is important because it may explain a specific interaction with target cells, most likely by a process mediated by surface receptors. On the contrary, the potential biological role of HSP within the lumen of ECV is less evident, which should require the fusion of the vesicles with the plasma membrane or by the burst of ECV liberating the cargo within the extracellular milieu. The presence of HSP on the membrane of ECV has led us to postulate that insertion into the lipid bilayer is the first step in the secretion of this protein [6]. Additional observations have shown that the export of Hsp70 and Hsc70 within ECV was blocked by the reduction of membrane cholesterol levels [79, 120]. Indeed, Hsp70 within ECV was resistant to nonionic detergents, such as Triton X-100, suggesting that the protein is within lipid rafts in the vesicles [28]. In this regard, several studies have shown that Hsp70 is present within lipid rafts of cells [28, 79, 121, 122].

Although the evidence for the active secretion of HSP from living cells is well established, it cannot be ignored that, under other circumstances, HSP are released into the extracellular medium after cell necrosis. Indeed, the concentration of HSP70 released after necrosis could be potentially very high [29]. In this regard, expression of HSP70 has also been observed after ischemia–reperfusion (I/R) injury, which resulted in a necrotic focus [85].

Table 3.3 Detection of HSP in ECV

HSP	Cells	Reference
HSP70	Dendritic cells	[123]
HSP90	Dendritic cells	[124]
HSP70	Peripheral blood mononuclear cells	[125]
HSP70, HSC70, HSP27, HSP90	B cells	[126]
HSC70/HSP70	Reticulocytes	[127]
HSP70, HSP90, Grp78	Hepatocytes	[128]
HSP60	Cardiac myocytes	[118]
HSP70	Pancreas carcinoma Colon carcinoma	[31]
HSP70	Hepatoblastoma	[28]
HSP70	Thymolymphoma Mammary carcinoma Colon carcinoma	[116]
HSP90	Glioblastoma Fibroblastoma Mammary gland adenocarcinoma	[129]
HSP90, HSC70	Mesothelioma	[130]
HSP60	Cardiac myocytes	[118]
HSP60	Bronchial carcinoma Lung adenocarcinoma Erythroleukemia	[119]
HSP70	*Mycobacterium smegmatis* and *Mycobacterium avium*-infected RAW 264.7	[131]

3.6 The Stress Observation System

There is clear evidence that cells secrete ECV during normal physiological conditions as well as after stress. We have argued that the composition of ECV reflects the physiological stage of the cell [6]. Thus, constitutive proteins are present in ECV derived from cells under normal conditions, such as CD9 and CD63, which belong to the family of tetraspanin proteins [132]. During stress conditions, ECV should reflect the insult type, such as the presence of stress-inducible HSP. Then, ECV are recognized by other cell types, in particular cells of the immune system, as part of an assessment of the stress conditions. If there is not stress, it is unlikely that there is a response. However, ECV during stress conditions are likely to activate the immune system to prepare a preemptive response directed at avoiding the propagation of the insult (Fig. 3.1). The process of sensing stress via ECV has been termed "Stress Observation System" [6]. Thus, ECV derived from macrophages infected with intracellular pathogens were observed to activate uninfected macrophages by a Toll-like receptor and myeloid differentiation factor 88 dependent mechanism [133]. They also induced polymorphonuclear leukocyte recruitment in lungs after

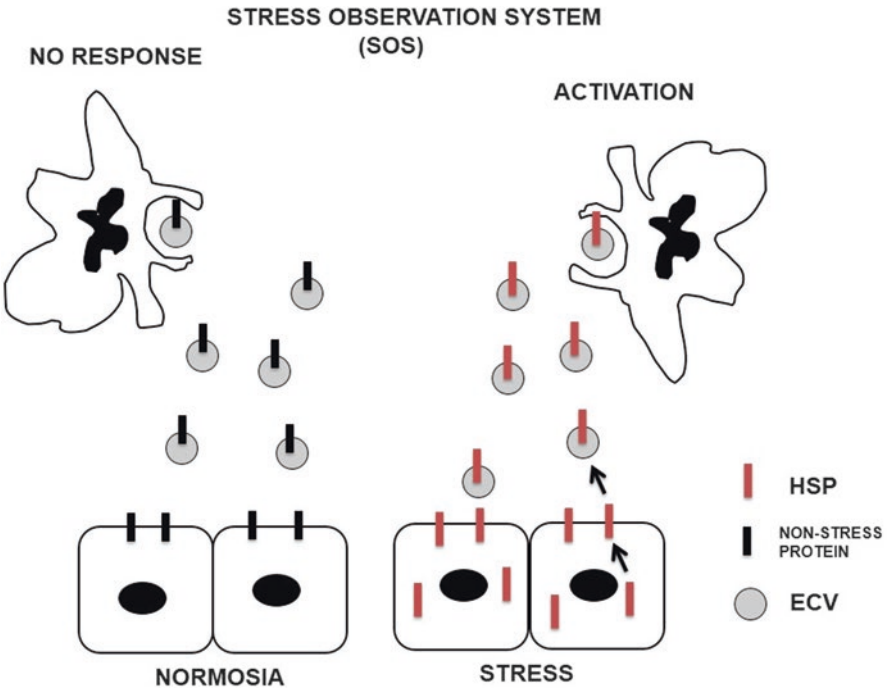

Fig. 3.1 During normal physiological conditions, cells release ECV containing markers for cellular homeostasis that when captured by immune cells do not trigger any response. However, the composition of these ECV changes after stress, resulting in a signal for the immune system to mount an appropriate response directed at mitigating the insult.

intranasal delivery [133]. Moreover, ECV containing Hsp70 isolated from mycobacteria-infected cells induced an inflammatory response in macrophages [134]. ECV containing Hsp70 on their surface displayed a robust and specific activation of macrophages, which was higher than the same concentration of recombinant Hsp70 in solution [28]. Finally, Hsp70-positive ECV were also found to stimulate the cytotoxic capacity of NK cells [31].

3.7 Conclusions

HSP appear to display a different role in the extracellular environment than the well-characterized function as molecular chaperones. Extracellular HSP emerge as new signaling molecules involved in intracellular communication. The presence of extracellular HSP has been detected in biological fluids from individuals suffering from a large number of illnesses. Therefore, they are likely to become biomarkers of various disease conditions. Extracellular HSP are released by many cell types and by at least two main mechanisms, including the passive dissemination after necrosis

or an active export process independent on cell death, named the nonclassical secretory pathway. Extracellular HSP come in various flavors. Thus, they can be found in a soluble form within biological fluids, trapped in the lumen of ECV or exposed to the surface of these vesicles in a membrane-bound fashion. Finally, HSP associated with ECV appear to be part of a mechanism directed at both alerting the immune system to the presence of an insult and avoiding the propagation of stress.

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