FEBS Letters 581 (2007) 3689–3694

Minireview

Extracellular heat shock proteins in cell signaling

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Received 12 March 2007; revised 6 April 2007; accepted 15 April 2007

Available online 25 April 2007

Edited by Robert Barouki

Abstract Extracellular stress proteins including heat shock proteins (Hsp) and glucose regulated proteins (Grp) are emerging as important mediators of intercellular signaling and transport. Release of such proteins from cells is triggered by physical trauma and behavioral stress as well as exposure to immunological "danger signals". Stress protein release occurs both through physiological secretion mechanisms and during cell death by necrosis. After release into the extracellular fluid, Hsp or Grp may then bind to the surfaces of adjacent cells and initiate signal transduction cascades as well as the transport of cargo molecules such as antigenic peptides. In addition Hsp60 and hsp70 are able to enter the bloodstream and may possess the ability to act at distant sites in the body. Many of the effects of extracellular stress proteins are mediated through cell surface receptors. Such receptors include Toll Like Receptors 2 and 4, CD40, CD91, CCR5 and members of the scavenger receptor family such as LOX-1 and SREC-1. The possession of a wide range of receptors for the Hsp and Grp family permits binding to a diverse range of cells and the performance of complex multicellular functions particularly in immune cells and neurones.

Keywords: Extracellular; Heat; Shock; Protein; Secretion; Receptor

1. Introduction

Mammalian stress proteins appear to have been derived from prokaryotic ancestors that evolved to solve problems in protein folding [1]. Eukaryotic descendents of these proteins retain these properties and tend the tertiary and quaternary structures of a large group of intracellular proteins [2]. The properties of these proteins in stress involve the direct maintenance of protein structure (chaperoning) as well as the regulation of death pathways. In the heat shock response for instance, elevated temperatures trigger massive synthesis of heat shock proteins that fold heat-denatured proteins and block caspase dependent apoptosis, permitting repair and thwarting death [3]. Extreme exposure to heat overwhelms the capacity of intracellular stress proteins and triggers death [3]. In addition to the intracellular response, stress also triggers the release of proteins into the extracellular spaces [4–6]. Indeed stress proteins such as heat shock proteins (Hsp) 27, 60, 70, 90 and 110 and glucose regulated proteins (Grp) 78, 94, 170 and calreticulin are released from cells in a variety of circumstances and interact with adjacent cells or in some cases enter the bloodstream (Table 1). We will discuss here mechanisms by which such proteins are released from cells and the cell surface structures involved in recognizing them through autocrine loops, by adjacent cells or at distant sites.

A variety of cell types secrete stress proteins, including neuronal cells, monocytes, macrophages, B cells and tumor cells of epithelial origin [7–9]. This suggests that stress protein release is a fairly widespread phenomenon and may be implicated in a number of physiological or pathological events. Furthermore, it appears that some cell types may be adapted for specialized secretion of stress proteins into the blood stream and Hsp70 is released from brown fat tissue into the circulation in response to behavioral stress [10]. In addition, Hsps are released from cells undergoing necrosis after extremes of heat stress or other toxic treatments [11,12]. Extracellular Hsp60 and hsp70 may indeed be physiological alarm signals for cell trauma. Just as Hsp70 release is common to multiple cell types, the ability to bind stress proteins is also shared by many cell types including many cells of the haemopoietic lineage, neuronal cells, vascular and other epithelial cells [13–15].

2. Extracellular stress proteins and neuronal cells

The first reports of stress protein release involved hsp70 secretion released from neuronal cells along with hsp110 and actin [16]. Indeed further studies indicate that Hsp70 can be released from glial cells and then taken up by adjacent neurons [4,17]. The rationale for this altruistic behavior between different neuronal cells appears to be that many neurons have a deficit in terms of hsp expression due to inadequate hsp gene transcription [18] (Fig. 1). The neuronal cytoplasm in the region of the synapse may thus have a deficit in Hsp levels due to a twofold cause: decreased de novo synthesis of Hsp that characterizes such cells and the long axonal distances along which the Hsp must be transported to reach the synapse [18]. One solution to this problem might be to synthesize Hsp in glial cells adjacent to the synapse, release them into the extracellular fluid for uptake by neuronal receptors [17,19]. The functions of such donated Hsps are not known but probably include increasing the chaperoning power of the neurone and protection from programmed cell death which can accompany neurotransmission [20].

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0014-5793/$32.00
doi:10.1016/j.febslet.2007.04.044

Table 1
Intracellular and extracellular properties of HSP and GRP

Chaperone	Protein function	
	Intracellular	Extracellular
Hsp27	Chaperone anti-death	Anti-inflammatory
Hsp60	Chaperonin	Pro-inflammatory
Hsp70	Chaperone anti-death	Immunoregulatory pro-inflammatory neuronal agonist
Hsp90	Chaperone evolutionary modulator	Pro-immune
Hsp110	Chaperone co-chaperone	Pro-immune
grp78	ER chaperone	Anti-inflammatory
Grp94	ER chaperone	Immunoregulatory
Grp170	ER chaperone	Pro-immune

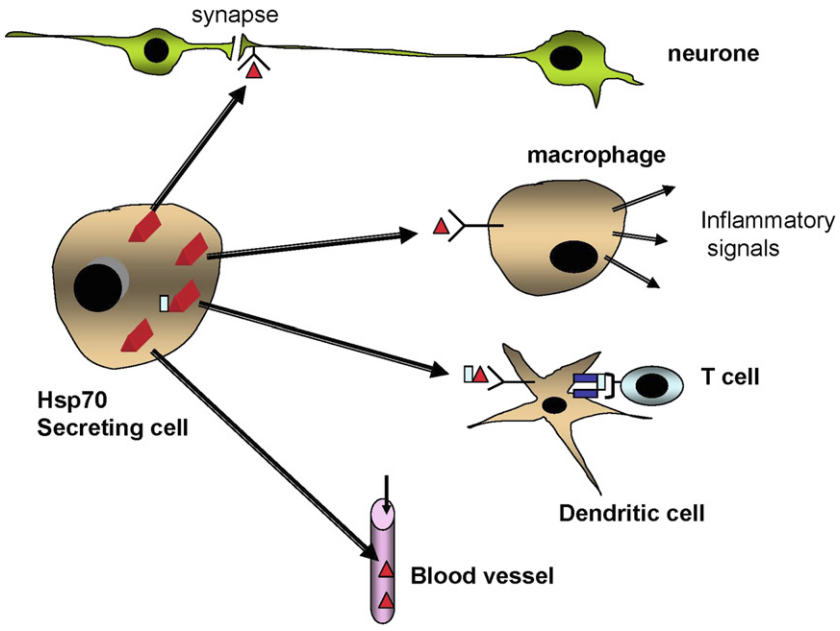

Fig. 1. Hsp70 is released from cells and may interact with a wide range of target cells. Hsp70 may be released by active secretion mechanisms or from cells undergoing necrosis. The resulting extracellular Hsp70 (red triangles) may then interact with neuronal cells, monocytes or macrophages or enter the circulation. Hsp70 may also be released conjugated to antigenic peptides (blue rectangles) and hsp70–peptide complexes are taken up by antigen presenting cells such as dendritic cells. Such peptides may then be transferred to major histocompatability class I molecules (dark blue rectangles) through process known as *cross-presentation*, and such MHC I–peptide complexes can be recognized by CD8+ T lymphocytes leading to T cell activation. Similar properties have been shown for other Hsp. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

There are also reports indicating the Hsp release into circulation following behavioral stress or the trauma associated with extreme exercise [10,21]. Hsp70 levels increase in the circulation of experimental animals and human subjects after these physiological stresses although the cellular source and physiological functions of such Hsp70 are not clear [10,21]. It has been suggested that brown fat tissue is the source of much of such Hsp70 [22].

3. Immune and inflammatory effects of extracellular Hsps

Extracellular stress proteins of the Hsp and Grp families have powerful effects on the immune response [23–25]. These stress proteins interact with the immune response in a number of contexts. During exposure to many pathogens, prokaryotic Hsp are released at high levels and are dominant antigens in the immunological responses to such pathogens [26,27]. Mammalian cells express endogenous stress proteins to high levels after trauma or exposure to bacteria or bacterial proteins [28]. Such stress proteins can be pro-inflammatory and lead to cytokine transcription and release [29,30]. In addition, stress proteins can act as stimulants of the adaptive immune response through their ability to bind antigenic peptides during antigen processing [31]. When such stress protein–peptide complexes are released from dead and dying cells they bind to receptors on antigen processing cells and antigens can be delivered to MHC class I molecules on the surfaces of such cells through a process known as antigen cross-presentation (Fig. 1) [32,33]. Such interactions form the basis for molecular chaperone based anti-cancer vaccines. Hsp–peptide complexes extracted from tumors can stimulate a specific CD8+ T cell mediated immune response in the tumor bearing host [32,33]. The potency of such vaccines has been ascribed to the ability of stress proteins to stimulate both the innate and adaptive arms of the anti-tumor immune response (Fig. 1) [24].

Stress proteins can also be anti-inflammatory and such interactions are noted specifically in inflammatory diseases.

Diseases such as RA can be triggered by cross reactive T cells which recognize common epitopes in mammalian and highly immunogenic prokaryotic Hsp [34,35]. It was speculated that the close degree of conservation in the sequences of these stress proteins might trigger an autoimmune response to mammalian stress proteins. Interestingly however, application of the corresponding mammalian Hsp suppresses the pro-inflammatory responses to bacterial Hsp epitopes and leads to remission of inflammatory diseases [36]. Heat shock proteins can thus be both profoundly immunostimulatory or immunosuppressive, depending on context [34,37].

4. Mechanisms of Hsp70 release

Hsp70 is not secreted by the classical pathway; its sequence encodes no secretion leader signal and inhibitors such as brefeldin A that antagonize transport through the ER-Golgi system do not inhibit its release. However, a number of non-canonical pathways for release of "leaderless" proteins exist. In some cases release of leaderless proteins involves cell lysis and this may occur both in pathological conditions that give rise to necrosis or in physiologically regulated release of cytokines [38]. Hsp70 for instance is released when tumor cells undergo necrotic death, presumably when cell membranes become compromised [11]. It has also been suggested that Hsp70 may be released and enter the blood stream under a number of pathological conditions that lead to widespread cell death [21]. In addition, a second pathway involves release of intracellular proteins in secretary vesicles [39]. This mechanism has been observed in macrophages stimulated to release IL-1β by LPS and extracellular ATP [39]. Stress proteins such as Hsp27, Hsp70, Hsc70 and Hsp90 can apparently be released within the lumen of "exosomes" through such a pathway when B cells for instance are exposed to heat shock [8]. The postulation of vesicles or exosomes as a source of extracellular Hsp70 requires that these structures should rupture or lyse on entering the extracellular microenvironment [39]. A third secretion pathway involves the entry of the leaderless protein into secretary lysosomal endosomes, migration of these organelles to the cell surface and release of the contents of the endolysosome into the extracellular space [40]. Again this pathway has been observed in IL-1β secretion by macrophages stimulated with LPS and ATP [41,42]. Indeed hsp70 has recently been shown to be secreted from tumor cells and macrophages by this pathway [6]. Fever range conditions have been shown to induce Hsp70 to enter endolysosomes and be secreted in an extracellular ATP-dependent manner [6]. Study of these processes is still in its infancy and further studies are required to determine the favored pathways for Hsp70 release by neuronal and immune cells.

5. Hsp70 uptake mechanisms

Stress proteins including Hsp, Grp and calreticulin interact with arrange of receptors on target cells (Fig. 2). These include the oxidized LDL binding protein CD91/LRP found on antigen presenting cells and other cell types [43,44]. It was in fact proposed that CD91 could be the common receptor for all immunogenic Hsp, including Hsp 60, 70, Gp96 and calreticulin

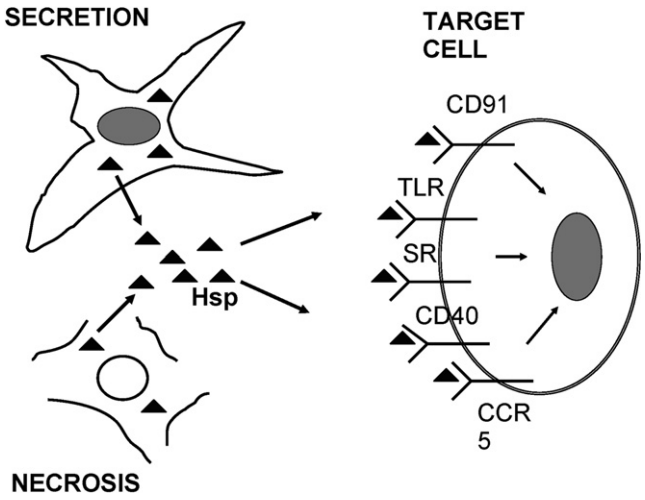

Fig. 2. Release of stress proteins from cells and binding to cell surface receptors on the donor cells, adjacent cells or distant cells. Hsp or Grp reach the extracellular microenvironment through active SECRETION or passive release after NECROSIS. The stress proteins can be recognized by an array of receptors on the TARGET CELL. Cells may have one or a number of receptors, which may be arrayed in parallel as here or hierarchically with binding to one receptor influencing another.

[43]. However, its role as a direct high/medium affinity Hsp binder is still not clear. Theriault et al. examined the ability of Hsp70 in free solution to bind cells with, or without CD91 expression and observe minimal differences [15]. In addition, overexpression of substrate binding domains of CD91 in cells deficient for Hsp70 binding does not restore Hsp70 binding [15]. Hsp70 binding may thus involve low affinity interactions or be indirect. In addition, CD40 can function as a Hsp70 receptor [45]. CD40 is a member of the *tumor necrosis factor* receptor family and plays a major in antigen presenting cell maturation through binding to its counter receptor on activated T cells (CD40L) [46]. The studies of Lehrner et al. showed that mycobacterial Hsp70 can bind avidly to CD40, a finding which may have major implications suggesting the ability to activate APC an cause the release of CC cytokines CCL3, CCL4 and CCL5 [45]. It has also been reported that human Hsp70 binds to CD40 [47]. However, subsequent studies indicated that when CD40 is overexpressed in previously null cells, they fail to acquire hsp70 binding capacity [15]. The exact role of CD40 as a direct binder for mammalian Hsp70 is thus still in the balance. However, Millar et al. showed an essential role for CD40 in Hsp70 stimulation of antigen presenting cells indicating its importance at one of the stages in the pathways of antigen presentation and T cell stimulation [48]. As major *pattern recognition receptors* (PRR), the Toll Like receptors sparked the interest of number of groups and instigated investigations of TLR as Hsp receptors. The TLR couple exposure to prokaryotic cell derived danger signals such as bacterial LPS, lipopeptides and CpG DNA, to intracellular signal transduction and transcription pathways that include the NF-κB and interferon response factor pathways [49]. There are now at least 11 members of the TLR, most of which have not been tested as Hsp receptors [49]. However, at least two TLR members function as Hsp receptors and can couple the binding of Hsp60, Hsp70 and gp96 to NF-κB activity [30,50–54]. In addition, the cell surface

protein CD14 that couples LPS exposure to TLR4 activation is also required for Hsp70 induction of the cytokines TNFα, IL-1β and IL6 [50]. Again, however, some studies suggest that these interactions between hsp70 and TLR are likely not exerted through the direct binding of Hsp70 to CD14, TLR2 or TLR4, as null cells that stably express Cd14, TLR2 or TLR4 do not bind avidly to hsp70 [15]. Thus, low affinity interactions may be involved in TLR activation by Hsp or an indirect mechanism, involving Hsp binding to a primary receptor that secondarily activates TLR signaling may be invoked. However, previous studies have shown that TLR activation by hsp70 requires the internalization of the Hsp70; simple experiments using TLR gene overexpression in Hsp null cell lines may thus be inadequate to assess direct Hsp–TLR binding [55]. Another receptor that can directly bind to mycobacterial Hsp70 is the chemokine receptor CCR5; this finding may have considerable significance for signaling cascades induced by Hsp [56].

Scavenger receptors (SR) constitute another family of PRR. The SR is receptors for modified forms of lipoproteins including oxidized and acetylated low-density lipoproteins (oxLDL and acLDL). The SR family are subdivided into eight different sub-classes (A–H) and most receptors belonging to this family are expressed on the surface of APC [57,58]. It has been shown that Hsp70 can interact with at least three members of the SR family including LOX-1, SREC-1 and FEEL-1/CLEVER-1 [15,59,60]. Hsp70 can both be bound at high affinity by these SR and internalized [12,59]. Both Hsp90 and Hsp60 can also bind to LOX-1 [59]. In addition, Gp96/Grp94 and calreticulin show significant affinity to Scavenger Receptor-A (SR-A) and SREC-1 and are internalized by this receptor [61]. Extracellular calreticulin uptake can also be mediated by SREC-1 but apparently not by LOX-1 [61,62]. Clearly members of the SR, which are expressed widely in a range of cell types may play and important role in stress protein binding and internalization [59,60]. Besides its functional relationship to other SR family members, LOX-1 shares structural homology to type V c-type lectin family members such as Dectin-1, NKG2D and CD94 [63]. Previous studies have demonstrated that another type V c-type lectin CD94 binds to Hsp70 but not to type II c-type lectin DC-SIGN [15,64]. Although of the c-type lectins, Dectin-1 shares the greatest structural homology with LOX-1, Hsp70 binding was not detected in CHO over-expressing Dectin-1. However, a significant affinity was seen between Hsp70 and C type lectins found in NK cells such as NKG2D and to a lesser extent the heterodimer CD94/NKG2A. Binding to these c-type lectins was selective in that other family members overexpressed in CHO cells, including DC-SIGN, CLEC-1 and CLEC-2 showed minimal Hsp70 association (J. Theriault and S.K. Calderwood, unpublished). Thus, a number of c-type lectins can serve as receptors for Hsp70 although the conserved *c-type lectin domain* was not the sole determinant required for Hsp70 interaction, as indicated by Hsp70 binding to unrelated SR such as SREC-1 that do not contain such a domain. C-type lectins may play a significant role in interaction of Hsp70 expressing tumor cells with NK cells [65].

This perplexingly large group of receptors may reflect the large and heterogeneous group of stress proteins often with radically different cellular effects (Table 1). Indeed, we have found that even between quite closely related members of the Hsp70 family there are differences in interactions with individual SR members (J. Theriault and S.K. Calderwood, unpublished). In addition, stress proteins likely recognize different receptors on different cell types [15]. The multiplicity of receptors may also indicate specialization for individual functions: receptors such as the TLR, CD40 and CCR5 may be adapted for transmembrane signaling while CD91 and SR may play more important roles in internalization of Hsp (Fig. 2).

6. Conclusions

Secretion of *heat shock proteins* and *glucose regulated proteins* thus extends the reach of the stress response into the extracellular microenvironment. Such proteins escape the plasma membrane through both active secretion and passive loss from necrotic cells. Once in the extracellular milieu, the stress proteins can increase stress resistance after binding to stress-sensitive recipient cells such as neurones, can signal tissue destruction and danger to inflammatory cells and aid in immunosurveillance by transporting intracellular peptides to distant immune cells.

Acknowledgements: We acknowledge the support of the Department of Radiation Oncology at Beth Israel Deaconess Medical Center, Boston. We thank out colleagues Kishiko Ogawa and M. Abdul Khaleque for sharing their thoughts and Rong Zhong for managing the laboratory. These studies were supported by Grants 5RO1CA047407 and 3RO1CA094397.

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