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Review

Hsp70 interactions with membrane lipids regulate cellular functions in health and disease

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A B S T R A C T

Beyond guarding the cellular proteome the major stress inducible heat shock protein Hsp70 has been shown to interact with lipids. Non-cytosolic Hsp70 stabilizes membranes during stress challenges and, in pathophysiological states, facilitates endocytosis, counteracts apoptotic mechanisms, sustains survival pathways or represents a signal that can be recognized by the immune system. Disease-coupled lipid-associated functions of Hsp70 may be targeted via distinct subcellular localizations of Hsp70 itself or its specific interacting lipids. With a special focus on interacting lipids, here we discuss localization-dependent roles of the membrane-bound Hsp70 in the context of its therapeutic potential, particularly in cancer and neurodegenerative diseases.

1. Introduction

The stress-inducible heat shock protein 70, HSPA1A or Hsp70.1 (Hsp70 hereafter) [1] is expressed at low or undetectable levels in unstressed, healthy cells. Upon different stresses its expression is rapidly induced through mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and stress-activated protein kinase (SAPK) signaling cascades, which activate heat shock factors (HSFs) [2–5]. Hsp70 restores the balance of the cell's proteome by assisting in refolding of denatured proteins. Importantly, Hsp70 is frequently upregulated in disease states, including cancer. The tumor microenvironment, where cells are subjected to free radicals, acidosis, hypoxia and nutrient deprivation, and where high levels of mutant proteins are present, causes stressful conditions challenging cancer cells [5]. The resultant high levels of Hsp70 in various cancer cells [6,7] enhances cell growth, suppresses senescence and confers resistance to stress-induced apoptosis [8].

Hsp70 is commonly known as a cytosolic molecular chaperone that translocates to the nucleus upon stress conditions [9]. However, it has been documented that Hsp70 also localizes to the luminal side of the endosomal-lysosomal system [10] and to the plasma membrane [11,12], as well as to the extracellular space [13] in pathophysiological states, such as cancer. Importantly, the unusual localization of Hsp70 is associated with a series of tumor specific functions such as counteracting lysosomal membrane permeabilization (LMP) and subsequent lysosome-dependent cell death [14] or immunomodulatory and invasion promoting roles of cell surface and extracellular Hsp70 [15]. Given that normal cells do not show these specific features, Hsp70 unusually localized in endosomes, lysosomes and at the extracellular side represents therapeutically targetable functions.

In fact, membrane association and lipid interactions have also been reported for several other members of the ubiquitous heat shock protein family, e.g. small heat shock proteins, Hsp60, Hsp70 and Hsp90, in different organisms [16–20]. As an indication of a functional interplay between Hsps and membranes, expression of Hsps is controlled by the physical state of the membrane through activation of the Rac1-mediated heat shock response [21–26]. Following specific lipid changes, membrane reorganization and interaction of Hsps with cellular membranes stabilize membrane structure and function during stress challenges [27–30]. Membrane-controlled initiation and stopping of the heat shock response has led to the concept of regulating heat shock protein expression by modulating the membrane's lipid phase through

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"membrane lipid therapy" [31,32]. The heat shock protein co-inducer hydroximic acid derivatives, such as Bimoclomol and BGP-15, are small multi-target molecules that intercalate into membranes and stabilize their lipid rafts by modulating membrane composition and structure [33,34]. Several studies have shown beneficial effects of BGP-15 on various disease models [35]. It is noted that such Hsp co-inducer compounds potentiate the response to a pre-existing stress without exhibiting effects in nonstressed environments. Dihydropyridine derivatives, another recently explored family of Hsp co-inducers, such as LA1011 and LA1044, improve the spatial learning and memory functions in wild type mice, and eliminate neurodegeneration by increasing dendritic spine density and reducing tau pathology and amyloid plaque formation in APPxPS1 double mutant mouse model of Alzheimer's disease [36,37]. Recently it was shown that binding of these dihydropyridines to Hsp90 compromises Hsp90's chaperone activity [36], which consequently induces the heat shock response in diseased cells. Furthermore, xenohormetic plant compounds with a general beneficial effect on animals also induce Hsp expression, and therefore have been applied for the treatment of neurodevelopmental delay [38]. Further modulators of Hsp expression with respect to neurological diseases have been described elsewhere [39,40].

2. Membrane crossing and post-translational modifications of HSP70

Despite the high therapeutic potential of Hsp70 – membrane interaction, the mechanism by which Hsp70, lacking a leader sequence, is capable of crossing the endosomal-lysosomal or the plasma membrane is not well understood. In vitro studies with reconstituted protein-lipid systems have unraveled a specific interaction between Hsp70 and phosphatidylserine (PS) [41,42] and proposed that Hsp70 oligomers generate pores in the cell membrane [43]. PS indeed confers a negative charge to the cytosolic leaflet of the plasma membrane and also to the endosomal membrane, allowing the recruitment of proteins with strong or moderate positive charges, respectively [44]. More recently, it has been shown that a cluster of positively charged Lys and Arg residues (R533 to K601/K597) anchor Hsc70/Hsp70 to the endosomal membrane, which enables entry of Hsc70/Hsp70-cargo complexes to endosomes through microautophagy [45]. Interestingly, this lipid interacting region has been identified to be important for other functions as well. Hsp70 is composed of a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD), which are connected by a linker (Fig. 1A). The linker domain (aa 384-397) and a fraction (aa 557-641) of the helical lid subdomain (HLS) of SBD, which overlaps with the lipid interacting region (R533, R535, K569, K573, K589, K597 of human Hsp70), are involved in oligomerization [46,47] (Fig. 1B). More specifically, Morgner et al. identified Lys rich regions throughout the whole molecule, but mostly in the SBD (K108-K561/569), that direct Hsp70 monomers in an antiparallel orientation [48]. T504, K561, K568, K569 and K507, K512, K526 residues of the SBD in ATP and ADP bound state allow not only dimerization but also interaction with the co-chaperones Hsp40, Hsp90, HopGR and client proteins (Fig. 1C). Importantly, phosphorylation and acetylation of these residues stabilize protein-protein interactions and, therefore, they are likely to also affect lipid interactions of this region. Further, trimethylation of K561 of the Hsp70 family members by METTL21A methyltransferase alter the affinity of Hsp70 towards monomeric and fibrillar α-synuclein [49], and phosphorylation and methylation of HLS residues including K561 and Y611 are necessary for proper ubiquitination by E3 ubiquitin ligase CHIP [50]. Hsp70 is ubiquitinated at 12 out of its 39 Lys residues including K561 [51]. Hot-spots of phosphorylation in Ssa1, the yeast homologue of Hsp70, at T36-S38 and T492-S495-T499 are important for normal growth and survival [52]. A large number of multiple post-translational modification sites point to a combinatorial code for a specific function [53]. Overlapping patterns of motives and post-translational modifications, in particular in the SBD, imply tight regulation of interrelated or interfering Hsp70 functions such as substrate or lipid binding (Fig. 1). To dissect the impact of post-translational modifications on Hsp70 localization and function necessitates further in-depth studies using subcellular fractions that can then be rendered to a specific function.

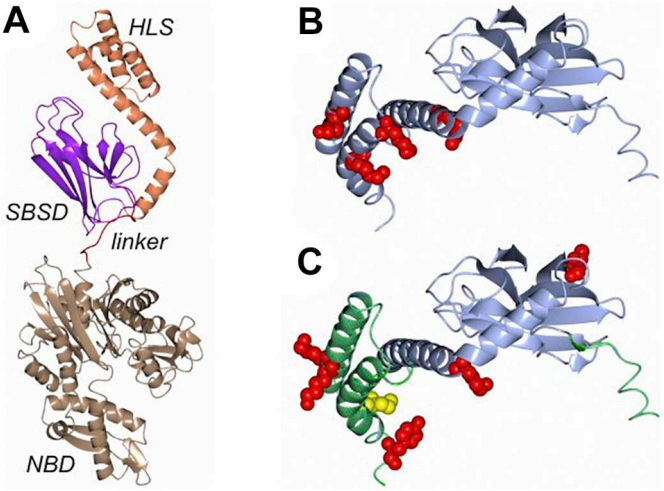

Fig. 1. Lipid interacting and post-translational modified regions of Hsp70 **(A)** Full length crystal structure and domains of Hsp70 shown for the prokaryotic Hsp70 DnaK (PDB: 2KHO). Hsp70 has an N-terminal nucleotide binding domain (NBD: pale brown), a short linker region (red) that couples to the substrate binding domain (SBD) consisting of a substrate-binding subdomain (SBSD: purple) and a helical lid subdomain (HLS: coral). **(B)** Residues of the "lysine-arginine cluster" interacting with the lipid phosphatydilserine (PS) (PDB: 4PO2 of the linker and SBD of human Hsp70). Positively charged R533, R535, K569, K573, K589, K597 shown in red are proposed to specifically bind to PS at the cytoplasmic leaflet of endosomes, allowing Hsp70-cargo entry to endosomes via autophagy [45]. **(C)** Example residues that are post-translational modified (PTM) and functionally relevant (PDB: 4PO2 of the linker and SBD of human Hsp70). Regions involved in oligomerization of Hsp70 are shown in green. Further residues that are exposed to PTMs are shown (in red) as relevant for Hsp70 dimerization and client protein interaction (T504, K561, K568, K569 and K507, K512, K526), E3 Ub ligase CHIP interaction (K561, Y611). Different PTMs of K561 (in yellow) were found to be important for substrate interaction, oligomerization, client and self-ubiquitination, cell growth and survival. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. HSP70 trafficking and tumor invasion

Cell surface, endosomal, lysosomal and extracellular pools of Hsp70 are interconnected in a highly dynamic fashion (Fig. 2). Plasma membrane-bound Hsp70 enters the endosomal route via clathrin dependent and independent mechanisms, and a fraction of internalized protein is recycled back to the surface. When excess Hsp70 is present in the cell, Hsp70 is further trafficked to late endosomes and lysosomes [54]. Cytosolic Hsp70 may also enter the *endo*-lysosomal system via an autophagic mechanism as implicated above [45]. Importantly, Hsp70 is resistant to proteolytic cleavage (and is, hence distinguishable from its cargos which are destined for lysosomal degradation) thus allowing it to exert its anti-apoptotic role. A large body of evidence describes Hsp70 present in both membrane-bound and soluble forms in the endo-lysosomal system, which are released by multivesicular bodies [55–58] and secretory lysosomes [54,59], respectively. These mechanisms not only supply plasma membrane bound Hsp70, but also result in a considerable amount of exosomal membrane-bound or soluble Hsp70 which has immunomodulatory potential.

Upregulated expression levels of Hsp70 is a diagnostic measure in several cancers, indicating increased cancer cell proliferation, 'clinical stage', or 'increased grade' together with shorter overall survival

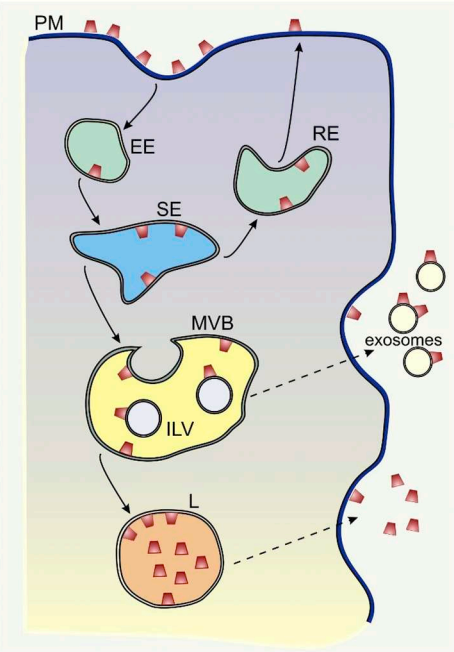

Fig. 2. Intracellular trafficking and secretion of Hsp70 Hsp70 (bucket symbol) is bound to the extracellular leaflet of the plasma membrane (PM). Surface Hsp70 is internalized to early endosomes (EE), a fraction of which is recycled back to the PM through sorting and recycling endosomes (SE, RE). Provided sufficient intracellular Hsp70, internalized Hsp70 is further trafficked to late endosomes/multivesicular bodies (MVB), where BMP enriched intraluminal vesicles (ILV) are formed with Hsp70 attached to the membrane. Fusion of MVBs with the PM exposes Hsp70 at the cell surface and releases exosomes containing Hsp70. Hsp70 may further be targeted to lysosomes (L), which upon lysosomal exocytosis expose Hsp70 at the cell surface and release their soluble Hsp70 content to the extracellular space. Mechanisms of membrane crossing and supply of Hsp70 to the endolysosomal system are not known, but autophagy and direct membrane crossing mechanisms have been implicated.

[60–63]. Given the correlation between excess Hsp70 levels and its lysosomal, cell surface and extracellular appearance (Fig. 3), unusual localization of Hsp70 appears to be an attractive target for therapeutic interventions. These targets include but may not be limited to lysosomal membrane-bound Hsp70, which protects against lysosome-dependent cell death [10,14,64], and plasma membrane- bound Hsp70, which promotes invasion [15,65] and endocytosis [47,66]. These features that would give rise to survival benefit for cancer patients may provide unique possibilities to fight tumor progression and metastasis. Moreover, surface localized and extracellular Hsp70 serve as potent stimuli for the innate immune system and can therefore be exploited as an effective adjuvant therapy [67,68]. These targets and their therapeutic potential are detailed in the following sections.

4. Plasma membrane bound and extracellular HSP70

Global cell surface protein profiling of membranes of tumor and normal cells revealed a tumor-specific, plasma membrane localization of a variety of different Hsps [12,69,70]. Although lacking a classical consensual transmembrane sequence, Hsp70 also has been found on the cell surface [12,69,71] and in the extracellular milieu of intact tumor cells [72–74]. Membrane localization of Hsps appears to be restricted to malignantly transformed cells [12,69,75,76], bacterial/viral/fungal/parasite-infected cells and spermatogenic cells [77–79]. In normal cells, Hsp70 is only found inside the cell but not on the plasma membrane. Therapeutic interventions such as radiochemotherapy, Hsp90 inhibition and hyperthermia have been found to further increase the levels of cytosolic and membrane-bound Hsps [12,15,80] in tumor cells. The presence of Hsp70 in the extracellular milieu of viable cells [81–83] is currently explained by an alternative lysosomal/endosomal pathway (Fig. 2), which does not involve the classical ER-Golgi compartment [59]. These findings concur with those from Asea and colleagues who demonstrated that drugs which perturb ER-Golgi transport, including monensin and brefeldin A, do not influence membrane expression and release of Hsp70 [84].

5. Membrane anchorage of HSPS in tumor cell membranes

Approximately 15 to 20% of the total cellular Hsp70 is found on the plasma membrane of some tumor cells [85]. Since neither high-salt conditions nor changes in the extracellular pH affect the Hsp70 membrane expression density on tumor cells, it is unlikely that Hsp70 is bound to proteinous cell surface receptors [86]. Already in 1989, Hightower and Guidon noted that Hsp71/Hsp73 could bind fatty acids and suggested possible direct interactions with membrane lipids [87]. Further on it has been proposed that Hsps accumulate in glycosphingolipid and cholesterol-rich microdomains (CRMs) [83,88–90]. CRMs were originally defined as regions within the plasma membrane that are enriched in cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins and some other acylated proteins [91,92]. As super-resolution cell imaging techniques are now suitable for investigating membrane lipid domains [93,94] these early findings should be revisited. A more recent effort has confirmed strong binding of Hsp70 to cholesterol and sphingomyelin domains in model membranes, and importantly high resolution atomic force microscopy revealed nano-domain size (up to 200 nm in diameter) of Hsp70 clusters on the cellular membrane (see Fig.4). These results may point to possible Hsp70-membrane lipid platforms formed [47]. Glycosphingolipids that are enriched in tumor cell membranes, provide neoplastic and normal stem cell markers with immunogenic potential [95]. However, glycosphingolipid-mediated immunoreactivity is often limited by a cholesterol-induced reorientation of glycosphingolipid head groups in a parallel rather than perpendicular conformation, which in turn hinders their recognition by the immune system [95]. Therefore, one could assume that cholesterol depletion by methyl-beta-cyclodextrin might improve immunogenicity of tumor cells. A comparative lipidomic analysis of the glycosphingolipid content

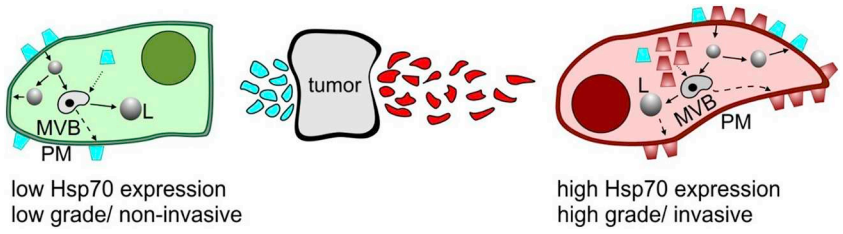

Fig. 3. Model for excess Hsp70-mediated tumor invasion Low grade tumor cells with lower levels of intracellular Hsp70 (left side, bucket symbol used) also express low levels of Hsp70 in the *endo*-lysosomal system and at the cell surface, which correlate with a non-invasive phenotype. Contrary, high grade tumor cells often with high levels of intracellular Hsp70 (right side) express high levels of Hsp70 in the endo-lysosomal system and at the cell surface, as well as displaying an invasive phenotype. Anti-apoptotic effects of cytosolic or lysosomal Hsp70 and the tumor-promoting effect of surface Hsp70 are involved in facilitating tumor invasion as reviewed in [63]. Blue and red symbols correspond to basal (low level) and excess Hsp70, respectively. For trafficking routes refer to Fig. 2. MVB (late endosome, multivesicular body), L (lysosome), PM (plasma membrane). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

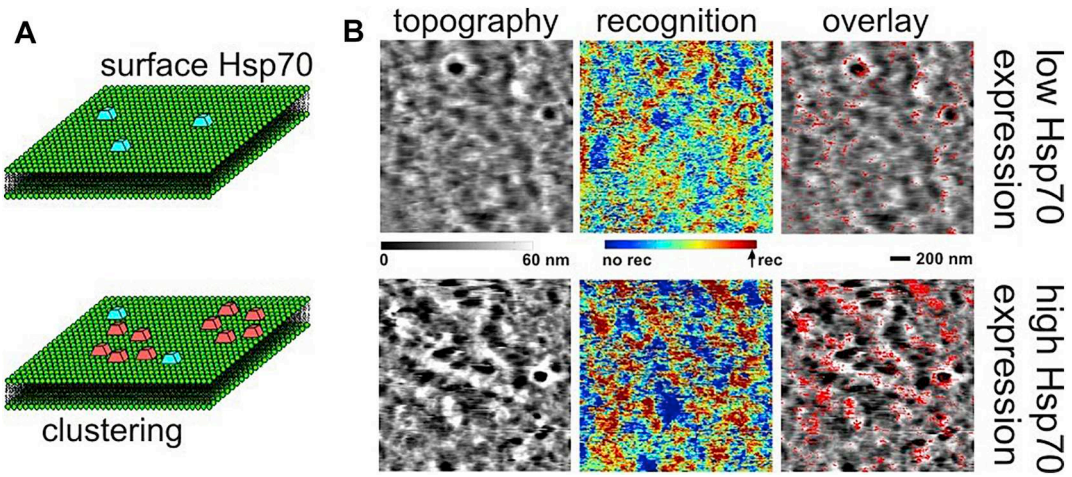

Fig. 4. Hsp70 clustering at the tumor cell surface Hsp70 forms larger size of nano-domains in the cell membrane of tumor cells expressing higher level of intracellular Hsp70. **(A)** Model of plasma membrane-bound Hsp70, where blue and red symbols correspond to basal (low level) and excess Hsp70, respectively. **(B)** Topography, atomic force microscopy recognition and overlay images. Note that only red pixels above the recognition threshold (rec) are shown in overlay images. These areas are found Hsp70 positive [47]. Data are displayed with courtesy of Dr. Lilia Chtcheglova and Prof. Peter Hinterdorfer, Johannes Kepler Univertity, Linz, Austria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed significantly greater amounts of globotriaosylceramide Gb3 [96] in tumor cells with a high compared to a low Hsp70 membrane expression. Gb3 is a receptor for Verotoxin [97,98] and AB5-Shiga toxin, an enterotoxin produced by Shigella dysenteria and enterohemorrhagic *Escherichia coli*. It is frequently found in the plasma membrane of germinal center B cells and Burkitt's lymphoma cells and solid tumors [99–104] but it is not present in most normal cells. Staining of Gb3 and Hsp70 on the plasma membrane of Hsp70-positive tumor cells revealed their co-localization. Moreover, cholesterol depletion results in a loss of Hsp70 from the plasma membrane of tumor cells [85]. Previous work by Lingwood et al. has demonstrated that Hsp70 also binds to 3`-sulfogalactolipids via its ATPase domain (NBD) (Fig. 1A) [105]. Based on binding patterns of antibodies that detect different epitopes of Hsp70 in the ATPase and the C-terminal substrate binding domain, the orientation of Hsp70 in Gb3 containing membrane domains appears to support the above result. Together with the finding that recombinant Hsp70 specifically interacts with artificial lipid vesicles containing Gb3, this supports the hypothesis that Gb3 might be one of the tumor-enriched lipid components that enables the integration of Hsp70 in the plasma membrane of tumor cells [85].

Apart from the glycosphingolipid Gb3, Hsp70 has been found to interact with artificial lipid bilayers in the presence of phosphatidylserine (PS) [41,43]. The group of DeMaio has shown that the interaction of Hsp70 with PS is largely based on the negative charge of phospholipids [106]. PS residing in liposomes enables the insertion of Hsp70 into the lipid bilayer and thereby can form higher molecular weight oligomers that facilitate ion conductance in artificial lipid bilayers [107]. Assuming that PS serves as the natural binding partner for Hsp70 in vivo, a higher PS content would be expected in Hsp70 membrane-positive tumor sublines. In non-stressed cells, PS is predominantly found on the inner membrane layer, whereas, upon stress PS can switch to the outer membrane leaflet, where it can be determined by a specific cell surface staining using the Ca^{2+}-dependent phospholipid binding protein Annexin A5. PS on the outer membrane leaflet is considered as an early marker for apoptotic cell death in many cell types where it acts as an “eat-me” signal for macrophages [108]. However, in the case of tumor cells PS can also be present on the outer membrane leaflet of viable, therapy-resistant, hypoxic cells [109]. It appears that under non-stressed conditions, Hsp70 predominantly resides in membrane clusters whereas following stress Hsp70 often co-localizes with PS outside these clusters. In line with this, atomic force microscopy combined with antigen specific recognition of surface Hsp70 demonstrated that plasma membrane bound Hsp70 forms large clusters and rings potentially surrounding endocytic sites in the cell membrane at higher intracellular and cell surface Hsp70 concentrations (Fig. 4). Shown in both the cell membrane and reconstituted systems clustering was found to depend on the ability of Hsp70 to oligomerize, and larger nano-domains (above 70 nm in diameter) of surface Hsp70 correlated with its ability to facilitate endocytosis in cancer cells [47,66].

6. Immunological role and therapeutic exploitation of membrane-bound and extracellular HSP70

Significant amounts of membrane-associated Hsp70 are often indicative of highly aggressive tumors, metastatic potential and resistance to therapy [11,65,110]. However, Hsps with molecular weights ranging from 70 to 90 kDa also elicit protective anti-tumor immune responses if expressed on the plasma membrane or in the extracellular milieu. Previous work of Multhoff and colleagues reported that in the presence of interleukin-2 (IL-2), plasma membrane-bound Hsp70 acts as a tumor-specific recognition structure for natural killer (NK) cells pre-activated with Hsp70 protein [11,111,112] or a peptide derived thereof (TKD) [113]. In contrast, resting NK cells of tumor patients are unable to kill Hsp70 membrane-positive tumor cells. Since the induction of the cytolytic activity of NK cells with TKD/IL-2 is dose-dependent and saturable, it has been assumed that the stimulation of NK cells with Hsp70 peptide might be mediated via receptors. Blocking experiments revealed that the C-type lectin receptor CD94 in combination with the activatory co-receptor NKG2C as well as other activatory receptors such as the homodimeric receptor NKG2D and natural killer receptors (NKp30, NKp44, NKp46, NKp80) can act as mediators of the interaction of NK cells with Hsp70 membrane-positive tumor cells [114–117]. Following binding of these NK cell receptors to membrane-bound Hsp70, the production and release of the serine protease granzyme B and perforin is initiated which, in turn, results in apoptotic cell death of the tumor cell [56,118]. Even in the absence of perforin, granzyme B has been found to interact with membrane-bound Hsp70 on tumor cells. Following binding and uptake of granzyme B into tumor cells via Hsp70-mediated endocytosis, apoptosis can thus be induced [119]. It

remains a matter of debate how granzyme B induces tumor cell apoptosis after *endo*-lysosomal transfer via an Hsp70 pathway.

Depending on the Hsp profile of the lipid surface of actively released exosomes derived from tumor cells [56,120] either stimulatory or inhibitory NK-mediated immune responses can be elicited. In the presence of immunogenic peptides that are chaperoned by extracellular Hsps also adaptive immune responses can be initiated following peptide cross-presentation via antigen presenting cells [121,122]. Another mechanism whereby extracellular Hsp70 might be able to stimulate tumor cell death is the complex formation of the innate immunity protein Tag7 with Hsp70. It has been shown that the interaction of the Tag7-Hsp70 complex with TNFR1 triggers the activation of RIP1-kinase, an increase in intracellular concentration of Ca^{2+} and an activation of calpains, a family of Ca^{2+} dependent cytoplasmic cysteine proteases, which result in the permeabilization of lysosomal membranes [123]. The lysosome-induced release of cathepsins B and D can depolarize mitochondrial membranes and induce ROS production which eventually initiates tumor cell necroptosis [123]. In contrast to tumor cells, Hsp70 which is released by normal human monocytes in response to granulocyte monocyte-colony stimulating factor (GM-CSF) can prevent the formation of gap-junction intercellular communication between capillary cells and monocytes, and thus could affect inflammation and tumor growth [124]. An anti-inflammatory cardioprotective effect could be shown by plasma exosomes expressing CD63, CD81 and Hsp70 derived from healthy donors [125]. This protective effect has been found to be dependent on Hsp70/Toll-like receptor 4 (TLR4) interactions and an activation of kinases that stimulate Hsp27. In summary, depending on the source of the releasing cell type (tumor or normal cells) and the micromilieu (e.g. hypoxia [126]) Hsp-bearing exosomes can exert contradictory immunological responses.

Patients with highly aggressive tumors have elevated levels of serum exosomes, which regulate cell-cell communication by transferring molecules such as cytosolic proteins (including Hsps), lipids, microRNAs and mRNAs [127]. Hsp70 membrane-positive tumor cells secrete exosomes carrying Hsp70 on their membranes [56]. Extracellular as well as membrane-bound Hsp70 fulfil dual functions of mediating therapy resistance [65] and playing pivotal roles in antitumor immune responses [11]. Hsp70 membrane-positive tumor cells have been found to be significantly more susceptible to the lysis of Hsp70-peptide and IL-2 activated NK cells as compared to their Hsp70 membrane-negative counterparts [11,12]. At present the capacity of ex vivo TKD/IL-2-stimulated NK cells to kill autologous tumor cells is being tested in a clinical phase II trial in patients with non-small cell lung cancer after radiochemotherapy [128,129].

Furthermore, surface Hsp70 positive exosomes derived from tumor cells have been found to stimulate the migratory and cytolytic capacity of NK cells [56]. In line with this finding, an intratumoral injection of recombinant Hsp70 into patients with glioblastoma has been shown to induce an increased cytolytic activity of NK cells and a cytokine shift towards a T helper 1 (Th1)-mediated immune response in preclinical models [130] and a pilot study in human patients [131]. Apart from recombinant Hsp70 protein that interacts with membrane Hsp70 through its oligomerization domain [132], the serine protease granzyme B has been found to interact with membrane Hsp70 on tumor cells. Following binding and Hsp70-mediated recycling endosomes, granzyme B induces tumor-specific apoptosis via perforin-independent pathway [119]. Regarding these findings EGFR targeting granzyme B which is overexpressed in NK cells has been found to enhance tumor apoptosis [133]. The presence of perforin oligomers induces a rapid plasma membrane flip-flop of phospholipids that facilitate the translocation of granzyme B across plasma membrane bilayers [134]. HS-1 associated protein X-1 (HAX-1), a protein that is involved in the maintenance of the mitochondrial membrane potential also serves as a target for granzyme B. After granzyme B-mediated HAX-1 cleavage, the N-terminal part stimulates mitochondrial depolarization and subsequent lysosomal degradation [135].

7. HSP70 as a regulator of lysosomal lipid catabolism and membrane stability

As discussed above, ample amounts of Hsp70 are found on the surface of cancer cells [11,136]. Since high endocytic activity being a characteristic of cancer cells, it is, therefore, not surprising that their lysosomal membranes also contain this protein [59,137]. More surprisingly and contrary to most other proteins ending up in the lysosomal lumen, Hsp70 is capable of resisting lysosomal hydrolases and of remaining functional in this hostile environment [10,14]. The resistance to hydrolysis is likely due to the effective, pH-dependent anchorage of Hsp70 to the lysosomal membranes via its high-affinity binding to bis(monoacylglycero)phosphate (BMP, lysobisphosphatidic acid), an anionic phospholipid abundant in lysosomes [14]. BMP accumulates predominantly in the membranes of intraluminal vesicles (ILV) of the endolysosomal system, and is critical for the formation of ILVs [138]. Fluorescence spectroscopy-based analyses of BMP-Hsp70 interactions suggest that BMP attaches to both the ATP- and the substrate-binding domain of Hsp70 in an extended conformation with acyl chains inserting into hydrophobic crevices within Hsp70 [139]. This anchorage is expected to cause a stringent orientation of Hsp70 on the membrane surface and to induce a transition of its substrate-binding domain into an intermediate conformational state, which may be essential to retain substrate interactions within the hydrophobic bilayer interior. The functionality of lysosomal Hsp70 is supported by accumulating data showing that not only Hsp70 expressed in cells, but also extracellularily added recombinant Hsp70 taken up by endocytosis and accumulating in lysosomes, regulates lysosomal lipid catabolism and lysosomal membrane integrity [14,137,140–149]. As discussed below, these cytoprotective, lysosomal functions of Hsp70 open new possibilities to inhibit and promote cell death in the treatment of various degenerative diseases and cancer, respectively.

8. Lysosomes and lysosome-related disorders

Lysosomes are cytosolic vesicles that function as cellular recycling stations, where over 50 acid hydrolases digest all major macromolecules of the cell to breakdown products available for metabolic reutilization [150]. Additionally, they serve as major endocytic, Ca^{2+} signaling and more recently as metabolic hubs that sense the nutrient availability and translate it to appropriate signaling pathways [151–154]. Lysosomal membranes can be divided into the limiting membrane and any internal membranes of ILVs [155]. These differ significantly in their function and composition. The internal membranes are the sites of lipid degradation. As previously mentioned, they are characterized by high levels of an anionic phospholipid, BMP, whose negative charge serves as a docking site for positively charged domains of lysosomal lipases (e.g. acid sphingomyelinase) or their co-factors (e.g. saposin) [155]. At the same time, the limiting membrane serves as a barrier that inhibits lethal leakage of lysosomal hydrolases into the cytosol while controlling the proper exchange of ions and the export of metabolites [150]. Heavily glycosylated luminal tails of lysosomal-associated membrane proteins (e.g. LAMP-1 and LAMP-2) form a protective glycocalyx shield to the inner face of the membrane [156], and numerous channel-forming proteins transport ions and metabolites across the lysosomal membrane [154,157].

Deficiency or malfunction of various lysosomal hydrolases or their co-factors, transport proteins or membrane proteins leads to chronic, often lethal, lysosomal storage disorders that affect many organs, most critically brain [158,159]. In addition to the classic lysosomal storage disorders that are the most common cause of childhood neurodegeneration [160], milder lysosomal dysfunction may contribute to pathologies of more common human diseases, such as neurodegeneration [160–162]. Moreover, lysosomal hyper-activation has recently emerged as a hallmark of metastatic cancer [163–165]. Although lysosomal storage disorders can be of mutation origin in over 50 different

lysosomal or lysosome-regulating genes, also the accumulation of storage material and the resulting dysfunction of lysosomes results in overlapping tissue pathology and clinical symptoms, with cell death and neuronal loss being marked features in critically ill patients. Loss of lysosomal membrane integrity and release of lysosomal hydrolases to the cytosol can be acutely lethal to cells. As the primary point of no return in a wide variety of cell death cascades [166–169], lysosomal leakage may, in turn, cause cellular and organ dysfunction developed during chronic lysosomal dysfunction. This view is supported by the demise of cells observed in samples from patients with some lysosomal storage disorders [14,170–172], and cancer cell death following lysosome-targeting therapies (reviewed in [168,173]). Notably, cancer cells either overexpressing Hsp70 or treated with recombinant Hsp70 are significantly protected against lysosomal leakage and subsequent cell death, whereas those depleted of Hsp70 undergo spontaneous lysosomal membrane permeabilization, or become more susceptible to lysosome-disruptive stimuli [14,137,140–147,174–176].

9. Lysosomal membrane integrity is regulated by HSP70

Maintenance of the lysosomal membrane integrity is of utmost importance for cellular homeostasis and survival. Yet, our knowledge on the mechanisms regulating lysosomal membrane permeability is only beginning to emerge. Among the emerging lysosomal membrane destabilizers are certain lipids and reactive oxygen species [167,169], both of which can be regulated by Hsp70. Sphingomyelin, arachidonic acid and possibly high concentrations of sphingosine promote lysosomal leakage, cell death and enhanced pathology in cells and tissues from lysosomal storage disease patients [14,147,175,177–179]. The ability of Hsp70 to stabilize lysosomal membranes has been largely attributed to its ability to enhance sphingolipid catabolism in the lysosomes through its high-affinity binding to BMP [14,148,175]. As discussed above, this anionic phospholipid is an essential cofactor for lysosomal sphingolipid catabolism [180]. Via its negative charge, it tethers several sphingolipid-degrading enzymes to the internal lysosomal membranes where their substrates are located, thereby increasing their activity and protecting them from lysosomal degradation. The high-affinity association of Hsp70 and BMP, which protects Hsp70 from lysosomal degradation as discussed above, also facilitates the BMP binding of sphingolipid-degrading enzymes and, in so doing, further enhances their activity and inhibits their degradation [14,139,148]. The lysosomal membrane-stabilizing effect of Hsp70 may rely in particular on its enhancing effect on the enzyme acid sphingomyelinase that hydrolyses sphingomyelin to ceramide and phosphocholine (Fig. 5). Hsp70-induced conversion of ILV-sphingomyelin to ceramide counteracts lysosomal aggregation and membrane permeabilization, which are hallmarks of stress-induced cell death and may contribute to cellular pathophysiology in some lysosomal storage disorders [14,170,175,181]. The mechanism by which accumulating ceramide stabilizes lysosomes remains largely unknown. Nevertheless, levels of very long chain ceramide species ($C_{24:0}$, $C_{24:1}$, $C_{24:2}$) were significantly increased in Hsp70 transgenic mouse embryonic fibroblast (MEF) cells as compared to their controls [14]. While short to long chain ceramides are frequently considered as mediators of cellular death, very long chain ceramide species may protect membrane integrity and confer survival benefit on cells [182–184]. It is possible that ceramides generated in the lysosome eventually influence other cellular membranes, therefore affecting lysosomal stability indirectly as well [185]. If the plasma membrane integrity should be severely impaired lysosomal ASM, facilitated by Hsp70 may be also exposed to the cell surface, where ceramide-enriched platforms seal the membrane. This is achieved by conical shaped ceramides capable of inducing membrane invaginations hence facilitating vesicle budding and fission [186]. Increased concentration of lysosomal ceramide counteracts aggregation of lysosomes with other intracellular vesicles and membranes, and perhaps strengthen the lysosomal limiting membranes by its ability to alter membrane properties [187] Interestingly, Hsp70 could enhance also the catabolism of several less abundant sphingolipids [148], whose role in the maintenance of lysosomal membrane integrity remains to be studied. Of special interest is the enzyme galactosylceramidase, whose loss of activity results in accumulation of galactosylsphingosine (a.k.a. psychosine) that disrupts lysosomal pH [188], possibly destabilizing the lysosomal membranes by interaction with the pH sensitive ion channel TDAG8 [189]. Finally, it should be also noted that Hsp70-facilitated sphingomyelin degradation and concomitant ceramide formation allows a Niemann-Pick C2 (NPC2) mediated cholesterol egress from the lysosome [190,191], which is expected to affect membrane integrity and cell survival in multiple ways.

In addition to regulating lysosomal lipid catabolism, Hsp70 may regulate lysosomal membrane stability by protecting the membranes from oxidative stress. Inside the lysosomes, iron and other chemically reactive metals (e.g. copper, zinc and cobalt) can generate reactive oxygen species through Fenton-type chemical reactions, which can lead to oxidization and destabilization of membrane lipids [192,193]. Interestingly, one of the common pathologies in various lysosomal storage diseases is the marked elevation of oxidative stress providing a possible mechanistic clue to the loss of lysosomal integrity and cell death occurring in these diseases [194–197]. Importantly, the well documented protective effect of Hsp70 against oxidative stress is preserved inside the lysosomes. In the case of photo-oxidation of acridine orange-loaded lysosomes, real-time high-resolution imaging has demonstrated that Hsp70 localized in the lysosomal lumen effectively protects lysosomal membranes and thereby mitigates their destabilization upon local oxidative stress [14]. Furthermore, Hsp70 is cytoprotective in other lysosomal oxidative stress models, including age-related macular degeneration of retinal pigment epithelium and lysosomal iron accumulation [10,143,176]. It remains to be studied, whether Hsp70 has a direct antioxidant effect or whether the protection of lysosomal membranes against oxidative stress is due to indirect effects, such as changes in the lipid composition of the membranes.

10. Therapeutic exploitation of lysosomal HSP70 function

After the initial discoveries of the role of Hsp70 in lysosomal membrane stability and lysosomal lipid catabolism [10,14], a number of recent publications have reported improved lysosomal enzyme activities and lysosomal function through the induction of heat shock proteins in various lysosomal storage disorders [148,149,198–201]. As a consequence, the induction of the heat shock response is emerging as an attractive therapeutic approach to treat these devastating diseases. The power of this approach is supported by recent data showing that recombinant Hsp70 can reverse lysosomal pathology in primary fibroblasts from eight different lysosomal storage disorders and has significant therapeutic effects on both substrate accumulation and neurological manifestations in murine models of three of them, i.e. Fabry, Sandhoff and Niemann-Pick type C diseases [148]. Notably, these therapeutic effects of recombinant Hsp70 can be recapitulated by oral administration of arimoclomol, a small molecule co-inducer of heat shock proteins, currently in clinical trials for Niemann-Pick disease type C [148]. It should be noted that the therapeutic effects of Hsp70 in lysosomal storage diseases are not confined to its direct effects in lysosomes, but are likely to depend also on the classic chaperone functions of Hsp70.

Contrary to lysosomal storage disorders and degenerative disease, where increased lysosomal Hsp70 activity appears to have a beneficial effect, the inhibition of lysosomal Hsp70 function is emerging as an attractive approach to treat cancer. Whereas the direct inhibition of Hsp70 in the lysosomal lumen may be technically challenging, the inhibition of its target, acid sphingomyelinase, can be easily achieved. In fact, over a hundred commonly used, FDA-approved drugs are functional inhibitors of this enzyme [202]. These drugs are characterized by a hydrophobic ring structure and a hydrophilic side chain with a

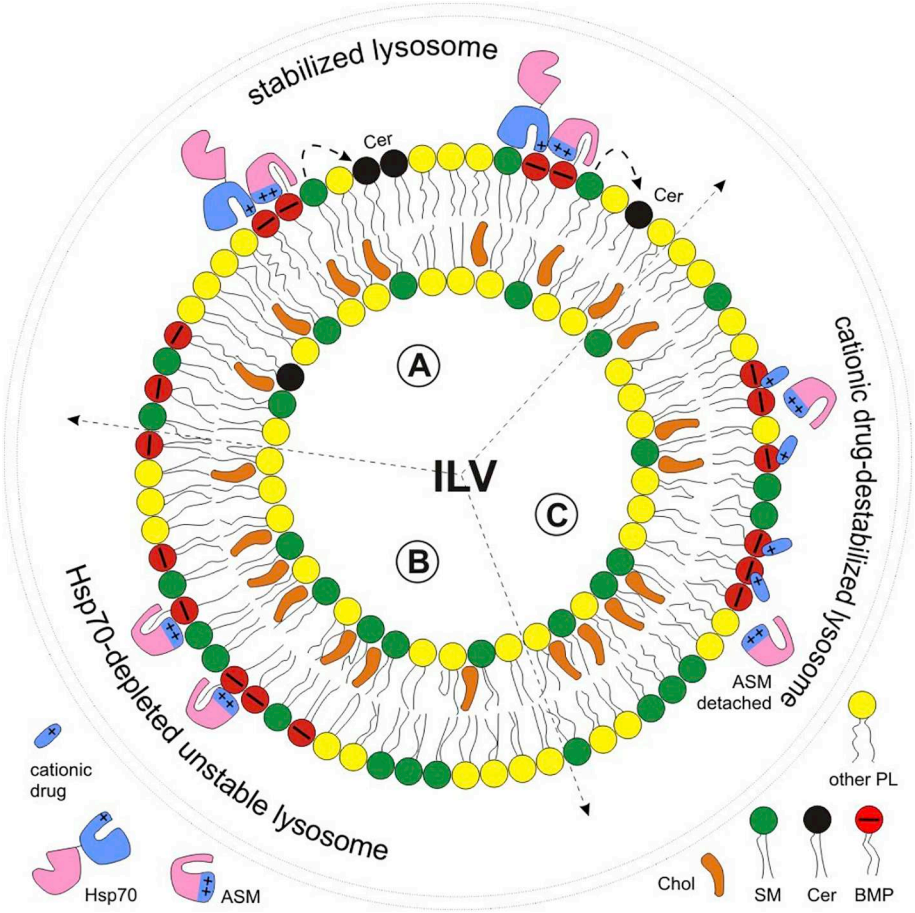

Fig. 5. Hsp70-mediated preservation of lysosomal membrane integrity Lysosomal membranes, in particular those of intraluminal vesicles (ILVs) are enriched in bis(monoacyl)glycerophosphate (BMP) of inverted conical shape that allows high curvature membrane formation. (A) Negative charge of the head group of BMP recruits acid sphingomyelinase (ASM) as well as Hsp70 to the membrane surface. ASM converts sphingomyelin (SM) to membrane stabilizing ceramide (Cer), which is largely dependent on Hsp70 bound to the luminal side of ILVs. Hsp70 dependent activation of ASM and other lysosomal lipases eventually changes the lipid composition of all cellular membranes. (B) Hsp70 depletion generates lysosome instability, triggering in turn lysosomal membrane permeabilization (LMP)-mediated cell death. (C) Alternatively, cationic lysosomotropic drugs neutralize the negative charge of bis(monoacyl)glycerophosphate (BMP), that ASM and Hsp70 are anchored to, therefore causing lysosomal instability, LMP and cell death. PL: glycerophospholipid, Chol: cholesterol, double circle: limiting membrane.

cationic amine group. In the acidic pH of lysosomes, the basic amine groups are protonated resulting in an up to 1000-fold accumulation [203]. The incorporation of such cationic amphiphilic drugs into membranes in the lysosomal lumen neutralizes the negative membrane charge and inhibits the function of several lysosomal lipases, including acid sphingomyelinase [204]. Thus, they have exactly the opposite effect to lysosomal Hsp70 (Fig. 5). Importantly, cancer cells are especially sensitive to the accumulation of sphingomyelin [175,205,206], which may explain why these functional inhibitors of acid sphingomyelinase display selective cytotoxicity towards transformed cells both in vitro and in various cancer models in mice [175,207–212]. Their putative efficacy in cancer treatment is further supported by a recent pharmacoepidemiological register-based cohort study showing a statistically significant association between cationic amphiphilic antihistamine use and reduced mortality among Danish cancer patients [212].

11. HSP70 disorder and lysosomal-mediated neuronal death

Lysosome-dependent cell death is characterized by the destabilization of its limiting membrane (Fig. 5) followed by the leakage of cathepsins from the lysosomal lumen into the cytoplasm [167,213–218]. Using the monkey experimental systems of transient brain ischemia, Yamashima et al. [219–221] formulated the ‘calpain-cathepsin hypothesis’ as a mechanism of programmed neuronal necrosis. They demonstrated that the lysosomal membrane of hippocampal CA1 neurons is disrupted by the activated μ-calpain after transient ischemia, which causes the release of lysosomal cathepsins B and L. Thereafter, the ‘calpain-cathepsin hypothesis’ has been confirmed, using a variety of experimental paradigms from *C. elegans* to rodents [222–225]. The role of lysosomal enzyme cathepsins in initiation and execution of the necrotic cell death program has become clear [149,166,167]. Moreover, the brain and neurons are regularly exposed to different kinds of acute and chronic environmental stresses. The brain contains high levels of polyunsaturated fatty acids and redox transition metal ions, especially iron. In spite of its high oxygen consumption, however, levels of lower molecular weight and enzymatic antioxidants are relatively low in the brain. Accordingly, the brain with poor antioxidant defense appears particularly susceptible to lipid peroxidation by reactive oxygen species [226]. Peroxidation of membrane lipids may show numerous effects such as increased membrane rigidity, decreased membrane-bound enzyme activity, altered membrane receptor activity, and altered membrane permeability. Therefore, it is not surprising that the role of lipid peroxidation has been widely investigated in the pathogenesis of a variety of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, Down syndrome [227].

Importantly, lipid peroxidation yields a variety of bioactive products and one of the most extensively studied examples is hydroxynonenal [228,229]. The most common source of hydroxynonenal is an endogenous one, when it is produced by peroxidation of membrane phospholipids or plasma low-density lipoproteins. Hydroxynonenal generation in the brain has been associated with exposure to drugs, ethanol or irradiation, and with ischemia or inflammation. In contrast, exogenous hydroxynonenal is generated during food processing, i.e., heating, especially deep-frying, of ω-6 vegetable oils (Fig. 6 A,B) [228]. Because of its chemical reactivity, hydroxynonenal can exert pleiotropic effects notably cell death. After the ischemia/reperfusion sequence in myocardial infarction, accumulated reactive oxygen species promote generation of hydroxynonenal, which disrupts actin cytoskeleton, alters Ca^{2+} homeostasis, and triggers cardiomyocyte cell death [230]. Hydroxynonenal induces signaling for apoptosis via both the Fas-mediated extrinsic and the p53-mediated intrinsic pathways [228,231]. Thus,

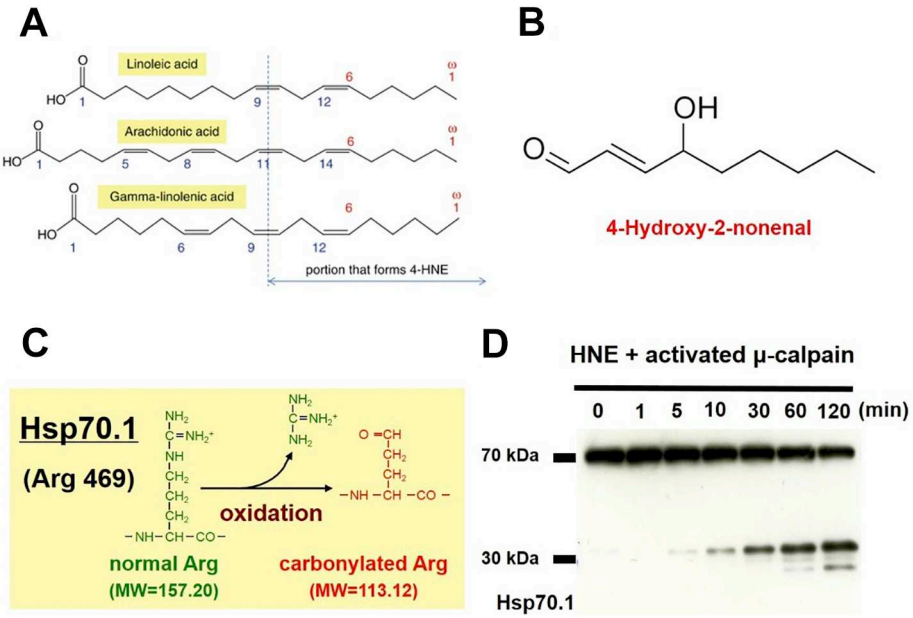

Fig. 6. Generation of hydroxynonenal (HNE), HNE-mediated carbonylation and calpain-mediated cleavage of carbonylated Hsp70 (A, B) Generation of hydroxynonenal (4-hydroxy-2-nonenal) from ω-6 polyunsaturated fatty acids such as linoleic, arachidonic and gamma-linolenic acids. (**C**) Carbonylation of Hsp70 occurs at the key site Arg469 in post-ischemic CA1 neurons. (**D**) Time-dependent μ-calpain-mediated cleavage of carbonylated-Hsp70 by hydroxynonenal (HNE) in monkey CA1 tissue. For further details see text.

hydroxynonenal can trigger β- cell apoptosis in the pancreas, and induce glucose intolerance and type 2 diabetes [232]. Since hydroxynonenal can impair Na^+/Ca^{2+} pumps and glucose and glutamate transporters by modifying membranes, the resultant ionic and energetic disturbances cause neuronal cell death [233,234]. However, the detailed mechanism of how hydroxynonenal can lead to cell death has been controversial until recently.

Accumulated data suggest dual roles of Hsp70 not only as a molecular chaperone for altered (misfolded/aged/damaged) proteins but also as a guardian of lysosomal integrity [14,235–237]. Hsp70 contributes to lysosomal stabilization (Fig. 5) by binding to the endolysosomal anionic phospholipid BMP, a co-factor essential for sphingomyelin catabolism [14]. Membranes of ILVs of the functioning lysosomes are characterized by abundant BMP [155,238]. Then, Hsp70-BMP binding enhances activity of acid sphingomyelinase, which mediates the sphingolipid degradation at the internal membrane in the acidic (pH 4.5) compartment to generate ceramide [180,239,240]. Ceramide protects lysosomal membrane integrity as discussed above [14,235,241] (Fig. 5A). Thus, in cases of Hsp70 depletion, not only failure of protein trafficking and degradation but also lysosomal destabilization or rupture may occur (Fig. 5B). In the monkey hippocampal CA1 neurons after transient ischemia, Oikawa et al. [242] previously found by proteomic analysis that Hsp70 can become an in vivo target of carbonylation by hydroxynonenal (Fig. 6C). Intriguingly, carbonylation of Hsp70 increased more than ten-fold in the post-ischemic CA1 neurons, compared to the non-ischemic controls. Subsequently, in the in vitro experiments, Hsp70 being carbonylated by hydroxynonenal was found to become susceptible to cleavage by activated μ-calpain (Fig. 6D) [243]. 'Calpain-mediated cleavage of carbonylated Hsp70' can lead to both autophagy failure and lysosomal destabilization with the resultant release of cathepsins and neuronal death [244].

Although neuronal death in Alzheimer's disease has been thought to be caused by the initial cerebral accumulation of amyloid β for half a century, it still remains enigmatic because the underlying mechanism of Alzheimer neuronal death due to amyloid β still remains unknown. Thus, research of this disease is moving away from the simple assumption of linear causality as proposed in the 'amyloid hypothesis'. Recently, hydroxynonenal has been shown to be involved in a great number of pathologies such as neurodegenerative diseases, metabolic diseases, and cancers [232]. Yamashima recently put forward a perspective view that the causative substance for Alzheimer neuronal death is actually ω-6 vegetable oil-derived hydroxynonenal [245].

Hsp70 is known to recycle altered proteins, stabilize lysosomal membranes and protect cells from diverse oxidative stresses. 'Hydroxynonenal-induced Hsp70 carbonylation' (Fig. 6C) followed by 'calpain-mediated cleavage of carbonylated Hsp70' (Fig. 6D) may be crucial for the execution of both ischemic and degenerative neuronal death. Calpain activation and Hsp70 disorder, combined together, at the lysosomal membranes may bring about programmed neuronal death by releasing hydrolytic cathepsin enzymes [219–221]. The pathway of cerebral ischemia and/or oxidative stresses, either acute (in case of stroke) or chronic (in case of degeneration) could result the following sequence of μ-calpain activation → excessive intake of ω-6 vegetable oils → increase of hydroxynonenal in the brain → hydroxynonenal-mediated Hsp70 carbonylation → activated μ-calpain-mediated cleavage of carbonylated Hsp70 → lysosomal membrane destabilization → cathepsin release → breakdown of the cell constitutive proteins, which may in turn represent a central role not only for ischemic neuronal death [221,236,244] but also for Alzheimer neuronal death [237,245].

A continuum of abnormalities of the lysosomal system can be identified in ischemic and Alzheimer neurons [160,246]. The common characteristic is that functional Hsp70 is indispensable for lysosomal autophagy that is crucial for the homeostasis of neurons. The control of protein turnover is particularly important in post-mitotic cells such as neurons, where accumulation of altered proteins may be highly detrimental to cell survival [247]. Neurons must maintain large volumes of membrane and cytoplasm, and continually traffic autophagy-related garbage long distances from distal ends of dendrites and axons back to the cell body where lysosomes are most active for catabolite clearance [248]. Hsp70 is the most structurally and functionally conserved chaperone that plays a principal role in the trafficking and degradation of altered proteins and their quality control for the cytoprotection of neurons under a number of different conditions [245]. Accordingly, in case of Hsp70 dysfunction, failure of lysosomal autophagy may occur, which leads to accumulation of autophagic vacuoles in both ischemic and Alzheimer neurons [246]. Since the proteotoxic stress in ischemia/reperfusion during stroke is severe, neurons die within hours or days after the insult. On the contrary, the proteotoxic stress in Alzheimer's diseases is extremely mild, and neurons can battle it for months or years, perhaps by raising pro-survival defenses such as Hsp70. Previous studies [249–253] indicated increased levels of protein oxidation in the Alzheimer brains, and suggested a possible involvement of hydroxynonenal-mediated protein carbonylation for the progression of

Alzheimer's disease. When sub-threshold levels of Hsp70 carbonylation are coupled with sub-threshold levels of calpain activation, for example, due to long-standing mild cerebral ischemia and/or amyloid β accumulation, programmed neuronal death becomes steadily significant year by year. Not only in cerebral ischemia but also in Alzheimer's disease, 'calpain-mediated cleavage of carbonylated Hsp70' may cause lysosomal membrane rupture/destabilization, which leads to the release of cathepsins into the cytoplasm, which can then trigger progressive neuronal death. Nowadays, researchers of Alzheimer's disease are gradually but steadily moving away from the classical amyloid hypothesis, and speculate that another age-related, disease-promoting factor and/or substance probably interact with the core mechanisms of the disease. Accordingly, targeting Hsp70 might be a promising strategy for both elucidating the mechanism of Alzheimer neuronal death as well as developing novel therapeutic agents for Alzheimer's disease where defects in lysosomal proteolysis and lipid accumulation have been observed [254].

12. Concluding remarks

It is now widely accepted that some of the heat shock protein molecular chaperones have additional biological functions over their basic role in cellular proteostasis, i.e. acting as 'moonlighting proteins'. The moonlighting Hsp70 has been emerging an important therapeutic target. However, efforts targeting essential chaperone activity or the interacting complexes of Hsp70 with proteins have not yet resulted in excellent specific and efficient drug candidates of low toxicity [255–257]. Drug design is hampered by the facts that Hsps are highly conserved [258] and that Hsp70, specifically Hsp70.1 has multiple functions. Hsp70 is in fact more than simply a cytosolic chaperone [19,63] and considered a major regulator of signaling pathways [8,259,260]. As reviewed here, non-cytosolic localization, membrane crossing and lipid interactions of Hsp70 are associated with membrane resistance, facilitation of endocytosis, counteracting apoptotic mechanisms and sustaining survival signaling at pathophysiological states. Unlike roles in the cytosol these unique functions may not only be targeted via Hsp70 itself or its interacting proteins, but also via specific lipids that either interact with or can be modulated by Hsp70. In order for rational drug design of membrane/lipid-mediated Hsp70 functions, we need further mechanistic and structural studies of Hsp70 membrane interactions and lipid modulation.

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