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Autophagy, Protein Aggregation and Hyperthermia: A Minireview

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Abstract

Purpose—We aim to explore the role of macroautophagy in cellular responses to hyperthermia. Protein damage incurred during hyperthermia can either lead to cell death or may be repaired by polypeptide quality control pathways including: (1) the deterrence of protein unfolding by molecular chaperones and (2) proteolysis of the denatured proteins within the proteasome. A third pathway of protein quality control is triggered by formation of protein aggregates in the heat shocked cell. This is the macroautophagy pathway in which protein aggregates are transported to specialized organelles called autolysosomes capable of degrading the aggregates. The consequences for cell viability of triggering this pathway are complex and may involve cell death, although under many circumstances, including exposure of cells to hyperthermia, autophagy leads to enhanced cell survival. We have discussed mechanisms by which cells detect protein aggregates and recruit them into the macroautophagy pathway as well as the potential role of inhibiting this process in hyperthermia.

Conclusions—Directed macroautophagy, with its key role in protein quality control, seems an attractive target for a therapy such as hyperthermia that functions principally through denaturing the proteome. However, much work is needed to decode the mechanisms of thermal stress-mediated macroautophagy and their role in survival / death of cancer cells before recommendations can be made on targeting this pathway in combination with hyperthermia.

Keywords

Hyperthermia; protein aggregate; autophagy

(1) Introduction: Hyperthermia, protein quality and cell death

When cells are exposed to elevated temperatures, they are killed in a time-and temperature-dependent manner once a threshold thermal dose is achieved. This has led to the use of elevated temperatures in hyperthermia treatment of a range of cancers[1, 2]. Early studies established cell protein denaturation as the dominant target in thermal cell killing[3]. However, the manner of tumor cell death after hyperthermia appears to be complex. Hyperthermia can lead to the induction of intrinsic programmed cell death pathways such as apoptosis, cause death by mitotic catastrophe and may block cell proliferation by stimulating

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replicative senescence [4-6]. Initiating triggers for death in heat shocked cells include both (1) induction of physiological cascades and (2) thermal protein unfolding and aggregation [7]. Necrosis can also be observed at extremely elevated temperatures[8]. However, cells also possess homeostatic responses to decrease killing that involve cell cycle arrest and most notably the induction of a transcriptional program leading to the production of molecular chaperones / heat shock proteins (HSP) [1, 9]. Such molecular chaperones are highly effective at preventing toxic damage to proteins that lead to denaturation and aggregation and can lead to protein refolding[9]. Indeed it is well known that preconditioning cells with a priming heat shock leads to a heat-resistant, thermotolerant state in which cells express extremely elevated levels of HSP [10-12]

The responses of cells to protein damage and unfolding involve multiple metabolic pathways that lead to either: (1) salvation of cells through repair / dismantling of damaged proteins or (2) sacrifice of the cells through the triggering of programmed cell death[13]. Thermally unfolded proteins may be refolded in the cytoplasm by molecular chaperones. However, a second process may also be invoked in which the damaged proteins are broken down by the proteases embedded in the proteasome [13]. Protein aggregates also accumulate in cells during stress as well as in the etiology of protein aggregation disorders such Parkinson's and Alzheimer's disease. Such aggregates compromise cell survival and can lead to apoptosis and loss of cognitive function in neurodegenerative diseases [13]. As protein aggregates are too insoluble and excessively bulky for removal by either chaperone refolding or proteasomal degradation they are instead removed by a process known as *autophagy*. Autophagy was originally discovered as a component of the nutrient stress response and is described in more detail below (Figure 1). A number of contrasting autophagy mechanisms have been described, including macroautophagy, microautophagy and chaperone-mediated autophagy[14-17]. We will concentrate here on macroautophagy and its potential role in hyperthermia treatment of cancer. Macroautophagy was originally thought to be a pathway of programmed cell death in complex organisms and molecular determinants of this process could be observed in dying cells [18]. However, recent studies suggest that under many conditions, this process may be beneficial to stressed cells and promote survival [19]. Hyperthermia has been shown to induce macroautophagy under range of conditions and in most cases, triggering of this pathway was associated with increased cell survival and decreased programmed cell death[15, 20-25] . Indeed inhibition of macroautophagy with the specific inhibitor bafilomycin may increase the anti-tumor effects of hyperthermia [21]. Clearly then, macroautophagy may play a significant role in tumor cell responses to hyperthermia and much may be learned by a deeper appreciation of the mechanisms by which hyperthermia triggers this response.

(2) Molecular determinants of macroautophagy in stress

Macroautophagy is classically triggered by nutrient stress and is induced when the major repressor of autophagy, the nutrient sensing protein kinase mTOR becomes inhibited and mRNA translation is stopped [14, 15] (Figure 1). This sets in motion the macroautophagy cascade and permits the cell to digest the proteins, lipids and DNA trapped in the autophagosome permitting liberation of nutrients for short-term survival of the cells. The pathway of macroautophagy, deduced originally in studies on yeast and *C elegans* is shown

in Fig. 1. Inhibition of mTOR leads to dephosphorylation of the multiprotein ULK1 kinase complex, the gateway step in starvation-mediated autophagy [26, 27]. Although the exact targets of the ULK1 kinase complex are not clear, its dephosphorylation leads to the activation of the Beclin1 / PI-3 kinase (III) complex, a step that initiates macroautophagy by generating highly phosphorylated phosphoinositide domains in membranes thus permitting assembly of autophagy proteins into vesicles built from lipids derived from the Golgi [28]. Open autophagophore structures are thus generated which can then be further processed into the double-membrane enclosed autophagosome in which protein aggregates can be packaged. Autophagosomes then fuse with intracellular lysosomes and the protease repertoire of the lysosome can be employed to digest the protein aggregates[14]. One important factor in regulated macroautophagy is the ubiquitin-like protein atg-8 (also known as LC-3 /GABARAP) [15, 29]. LC-3 is found attached to the internal and external membranes of autophagosomes and the internal membranes of autolysosomes [29]. Such LC3, when processed to its active form LC3-II can be detected and bound by the proteins p62/SQSTM1 and NBR1. These proteins can also recognize ubiquinated proteins and aggregates as well as LC-3 in the autophagosome [30, 31].

In addition to nutrient deprivation, cell stresses can also trigger macroautophagy. Such stress-induced macroautophagy was originally thought to be a pathway of programmed cell death after stress and indeed can be triggered simultaneously with apoptosis through common intermediates such as Bcl2 family proteins and p53 [32-34]. These stress-mediated pathways generally intercede at the Beclin1 step defined in nutrient stress macroautophagy (Fig 1). Much recent work indicates that stress-induced macroautophagy can however be cytoprotective and may moderate the effects of pro-apoptotic signaling [18, 35]. One way in which macroautophagy can increase cell survival is through the resolution of intracellular protein aggregates within the autolysosome. As mentioned above, cells can resolve denatured / damaged proteins by refolding them with their batteries of molecular chaperones or by degrading such polypeptides through the proteasome [13]. However, these two processes are inefficient in dealing with protein aggregates as the pore of the proteasome is occluded by bulky protein aggregates [13]. Hyperthermia leads to the accumulation of a large fraction of polyubiquinated, aggregated proteins in cells [36, 37]. Protein aggregates can however be resolved after becoming ubiquinated on multiple sites after interaction with the ubiquitin ligase parkin [30, 38] (Figure 2). Aggregates marked by polyubiquination can then interact with the motor protein dynein and histone deacetylase 6 to form *aggresomes* that are retrotransported within the cell to the microtubule organizing center (MTOC) adjacent to the nucleus [30, 39]. The aggresomes can then become enclosed in autophagosomes at the MTOC. It is thought that the protein p62/SQSTM1 may play a key role in this process, based on its ability to bind both UBL domain protein LC-3 and the polyubiquinated proteins in the aggresome through its UBA domain [30]. It can thus function as the “missing link” between protein aggregation and macroautophagy. Interestingly, p62/SQSTM1 becomes consumed along with its polyubiquinated client proteins within the autolysosome [14, 30]. Sorting of proteins targeted for degradation to the proteasome or autophagosome involves the nature of the binding site on ubiquitin; K-48 linked polyUb chains are in general recognized by proteasome receptors while K-63 linked chains are targeted to autophagy. The complexities of this process are described in a recent minireview[40].

(3) Heat shock triggering of macroautophagy

The next question is- how are the protein aggregates that are generated during hyperthermia sensed by the cell and targeted to the macroautophagy pathway? It has been shown that formation of protein aggregates can be induced by physiological stimuli. The stress-induced kinase MEKK1 leads to the nucleation of protein aggregates and can itself initiate the aggregation cascade [41]. MEKK1 was found in the same insoluble fractions as aggregated proteins suggesting formation of complexes. MEKK1 may thus form part of the "aggregation sensor".

It has also been shown that molecular chaperones can mediate the formation of the autophagosome. Heat shock protein 70 (Hsp70) and Hsp90, although contributing to a process known as chaperone-mediated autophagy, do not appear to play key roles in stress-induced macroautophagy (reviewed [13, 17]). By contrast the small HSP (HSPB) family appear to play key roles in macroautophagy (Figure 2). HspB8 forms a complex with the Hsp70 co-chaperone BAG3 that can prevent protein aggregate formation, increase the levels of LC3-II and stimulate macroautophagy [42, 43]. This complex is distinct from Hsp70-BAG3 complexes and binding to HspB8 involves dedicated domains within the BAG3 protein distinct from the Hsp70 binding site [43]. It seems likely that HspB8-BAG3 complexes could thus target aggregated proteins to the autophagosome and stimulate macroautophagy. Interestingly, BAG3 inhibits Hsp70-mediated proteasomal degradation and may thus bias traffic of aggregates away from the proteasome and towards the autophagosome [44, 45]. More recently, a role for other members of the HSPB family have been discerned and HspB7 has been shown to be highly effective in preventing the toxicity of protein aggregates through the macroautophagy pathways [46, 47]. In contrast to HspB8, HspB7 appears to accomplish this without increasing the rate of macroautophagy and may utilize basal autophagy processes in resolution of aggregates. It should be noted that many of the experiments quoted here involve forced expression of aggregation-prone mutant proteins in cells rather than heat shock itself and it remains to be fully discerned how similar these processes are.

It is currently unclear how stress-induced aggregation is coupled to macroautophagy. It is possible that the presence of aggregated proteins might be detected by HSPB family molecules through their molecular chaperone properties, bound and delivered to the autophagosome. Such a mechanism would be homologous to the role of Hsp70 in sensing denatured proteins, coupling to the ubiquitin-E3 ligase CHIP and delivery of polyubiquinated, denatured proteins to the proteasome for digestion[48]. In each case a molecular chaperone is used to bind disordered proteins and deliver them to a pathway of destruction. This illustrates the versatility of the HSP in detecting protein unfolding and coupling denaturation to other cellular processes such as proteasome function and macroautophagic degradation generally through association with co-chaperones. It will be interesting to determine whether other enzymatic activities can be thus coupled to protein unfolding through molecular chaperones. One interesting possibility is the scaffold protein TTC5/STRAP that can bind Hsp90 as well as histone acetylases and other molecules active in transcriptional regulation [49, 50].

In addition to being detected by HSP, stress-induced aggregates may be sensed and polyubiquinated by ligases such as parkin, packaged into aggresomes and delivered to autophagosomes by p62, or a combination of mechanisms may be involved [39] (Fig. 2). Much remains to be learned regarding the interplay between the multiple arms of the stress response, the relative role of molecular chaperones or aggresomes. As mentioned above, members of the BAG family of co-chaperones may be involved in interplay between the various pathways as they can influence transcription of the HSP cohort, target Hsp70-CHIP complexes to the proteasome and promote interaction of HspB8 with the macroautophagy pathway[45, 51]. It is also unclear how the stress kinase MEKK1 is coupled to the response to protein aggregation [41]. MEKK1 is a high molecular weight protein kinase that possesses ubiquitin ligase activity as well as kinase function and these properties could be involved in interaction with protein aggregates [52].

(4) Downstream of stress signaling after hyperthermia- apoptosis or macroautophagy, life or death?

It is apparent that stress produces a number of effects on protein homeostasis that may compete in order to repair stress induced protein damage or lead to apoptosis. Pro-death pathways include stress-kinase mediated apoptosis as well as apoptosis due to accumulation of unrepaired protein aggregates [7]. In addition, heat stress triggers the HSF1-mediated stress response as well as the activation of small heat shock protein HspB1 through the p38MAPK-signaling pathway each of which process leads to inhibition of the death pathways and enhanced folding of the proteome [53, 54]. Macroautophagy may be another component in this stress response network with its unique ability to target protein aggregates for degradation by lysosomal proteases. Indeed it will be a challenge in the coming years to understand how the various arms of the stress response are coordinated in order to promote cell survival.

(5) Macroautophagy and cancer treatment

Macroautophagy plays a somewhat ambiguous role in responses of tumor cells to some cancer therapies. This pathway may be triggered by some therapeutics in tumor cells harboring mutations that inhibit the caspase-dependent apoptosis pathway [55]. Indeed in such cells, macroautophagy has been described as the *programmed death type II pathway*, a default mechanism that permits cell death in the absence of the classical apoptosis cascade[19]. However, in many contexts macroautophagy constitutes a prosurvival pathway, particularly in the acidic and hypoxic tumor microenvironment [56]. Most of the available studies on hyperthermia suggest that autophagy reduces the cytotoxic effects of the therapy and may be a potential target in combination therapies[21-24]. Autophagy can be inhibited by chemicals such as 3-methyladenine, hydroxychloroquine or bafilomycin A1 or reduced by targeting members of the autophagy-related gene family (*ATG5*, *ATG6* (Beclin1), *ATG10* and *ATG12*), by siRNA approaches [19]. Combining inhibition of macroautophagy with hyperthermia by one of these approaches may thus be a promising approach to explore.

In conclusion therefore, directed macroautophagy, with its key role in protein quality control, seems a particularly attractive target in terms of a therapy such as hyperthermia that

functions principally through denaturing the proteome[57]. However, much work is needed on the mechanisms of thermal stress-mediated macroautophagy and its role in survival / death of cancer cells before recommendations can be made on targeting this pathway in combination with hyperthermia.

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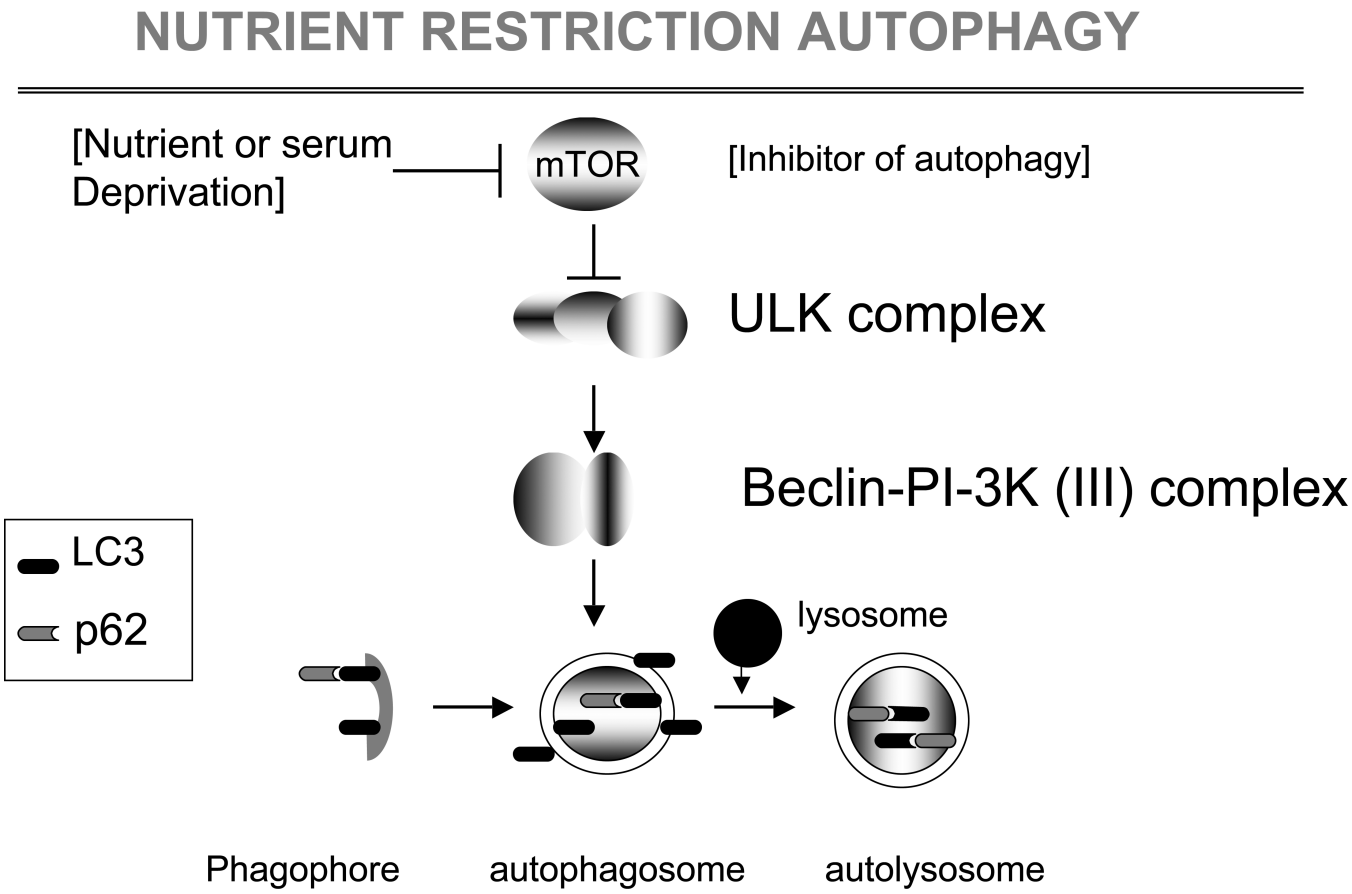

Figure 1. Nutrient restriction autophagy is triggered when the kinase mTOR is inhibited

mTOR inhibition leads to relief of repression of the ULK kinase cascade and formation of a Beclin1 / PI-3K (III) complex that triggers assembly of the phagophore and autophagosome. LC3 is induced and activated to the LC3-II form by conjugation to phosphatidylethanolamine during autophagy. LC3-II stimulates the formation of autophagosome and can bind to the protein aggregate binding protein p62/SQSTM1. Protein aggregates conjugated to p62 and LC3-II are then degraded when the autophagosome becomes associated with lysosomes to form the autolysosome.

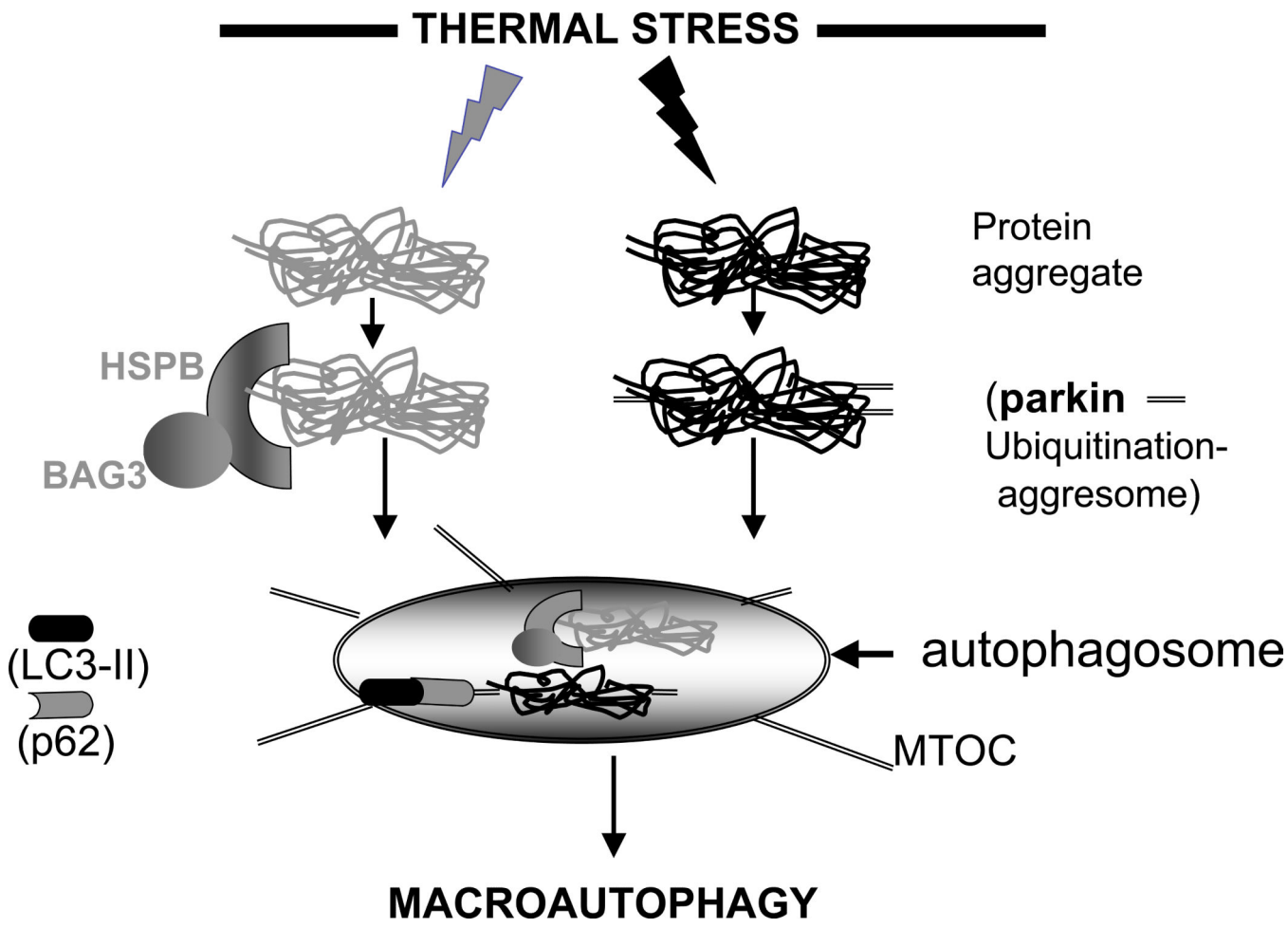

Figure 2. Triggering of the macroautophagy response by stress induced protein aggregates

At least two pathways may be involved in sensing the presence of aggregates and directing them to the autophagosome. In the gray pathway, aggregates are sensed / bound by HSPB / BAG3 complexes and directed to the autophagosome. In the black pathway, aggregates are recognized by the ubiquitin E3 ligase parkin that sees aggregated proteins, polyubiquinates them and leads to the formation of the aggresome. Aggresomes are translocated to the autophagosome located at the microtubule organizing center (MTOC). The polyubiquitin chains on the aggresome are then recognized by p62 which interacts with LC3 and triggers autophagy. Responses to stresses such as hyperthermia may involve a combination of the two pathways.