

RESEARCH ARTICLE

Ebb-and-Flow of Macroautophagy and Chaperone-Mediated Autophagy in Raji Cells Induced by Starvation and Arsenic Trioxide

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Abstract

Autophagy is crucial in the maintenance of homeostasis and regenerated energy of mammalian cells. Macroautophagy and chaperone-mediated autophagy (CMA) are the two best-identified pathways. Recent research has found that in normal cells, decline of macroautophagy is appropriately parallel with activation of CMA. However, whether it is also true in cancer cells has been poorly studied. Here we focused on cross-talk and conversion between macroautophagy and CMA in cultured Burkitt lymphoma Raji cells when facing serum deprivation and exposure to a toxic compound, arsenic trioxide. The results showed that both macroautophagy and CMA were activated sequentially instead of simultaneously in starvation-induced Raji cells, and macroautophagy was quickly activated and peaked during the first hours of nutrition deprivation, and then gradually decreased to near baseline. With nutrient deprivation persisted, CMA progressively increased along with the decline of macroautophagy. On the other hand, in arsenic trioxide-treated Raji cells, macroautophagy activity was also significantly increased, but CMA activity was not rapidly enhanced until macroautophagy was inhibited by 3-methyladenine, an inhibitor. Together, we conclude that cancer cells exhibit differential responses to diverse stressor-induced damage by autophagy. The sequential switch of the first-aider macroautophagy to the homeostasis-stabilizer CMA, whether active or passive, might be conducive to the adaption of cancer cells to miscellaneous intracellular or extracellular stressors. These findings must be helpful to understand the characteristics, compensatory mechanisms and answer modes of different autophagic pathways in cancer cells, which might be very important and promising to the development of potential targeting interventions for cancer therapies via regulation of autophagic pathways.

Keywords: Chaperone-mediated autophagy (CMA) - macroautophagy - arsenic trioxide - serum deprivation - Raji cells

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Introduction

Autophagy is a highly-regulated cellular process that modulates the targeting and degradation of intracellular components -dysfunctional organelles and macromolecules-in lysosomes (Banjerdpongchai and Khaw-on, 2013) Under basal cellular conditions, this continuous fine adjustment contributes to the maintenance of balance between protein synthesis and degradation and plays an important role in cellular energy homeostasis and proper cellular functioning (Morimoto and Cuervo, 2009). In addition to this role in basal homeostasis, autophagy is also essential for cellular adaptation to microenvironmental changes and cellular response to intracellular or extracellular stimulation (Levine and Deretic, 2007).

According to the mechanism that mediates the delivery of cytoplasm components to lysosomal compartment for degradation, three types of autophagy in mammals have

been identified, namely macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Cuervo and Wong, 2014). Among them, macroautophagy and CMA are the best-characterized autophagic process in mammalian. In macroautophagy, aggregated proteins and dysfunctional organelles are secluded from the rest of the cytosol by the limiting membrane which elongates and seals to form autophagosome, then autophagosome matures and fuses with the lysosome to degrade its contents by lysosomal hydrolases (including proteases, glycosidases, nucleases and lipases) (Nakatogawa et al., 2009; Saumya and Chandravati, 2012). Macroautophagy has been suggested to function as a tumor suppressor to some degree by contributing to the maintenance of intracellular homeostasis. The different phases of macroautophagy are orchestrated by a set of autophagy-related proteins (Atg). Among these Atg proteins, two major conjugation events, the Beclin-1/ phosphatidylinositol kinase type III (Class III PI3K) complex and the Atg8 (LC3 in mammals)-

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phosphatidyl ethanolamine complex mediate the initiation of macroautophagy (Ohsumi and Mizushima, 2004; Niu et al., 2014), so the formation of autophagosomes can be blocked by class III PI3K inhibitors- 3-methyladenine (3-MA).

In contrast to the vesicle-mediated substrate delivery of macroautophagy, CMA gives stringent affinity and selectivity for cytosolic soluble proteins, but this pathway cannot degrade organelles (Park and Cuervo, 2013). All substrate proteins for CMA contain a pentapeptide motif biochemically related to KFERQ in their amino acid sequence (Chiang and Dice, 1988). These substrates are selectively recognized by virtue of their KFERQ sequence and targeted to lysosomes membrane through the direct interaction with a cytoplasmic chaperone protein (Hsc70), a 70-kDa heat shock cognate protein. Hsc70 is a predominant member of the family of chaperones and co-chaperones, which not only recognizes the KFERQ sequence in the CMA substrates and forms substrate-chaperone complex, but also facilitates substrate unfolding which is conducive to the substrate translocation into lysosome (Dice, 2007). This CMA targeted substrate-chaperone complex binds to lysosomes through interaction with the lysosome-associated membrane protein type 2A (LAMP-2A) as a receptor for CMA substrates and translocates the substrate across the lysosomal membrane (Kaushik et al., 2006). LAMP-2A is one of the three splice variants of lamp2 gene, it is a single-span membrane protein with a very heavily glycosylated luminal region and a short (12-amino acid) C-terminus tail exposed on the surface of the lysosomes, where substrate-chaperone complex binds and the binding of substrate-chaperone complex to the cytosolic tail of LAMP-2A is limiting for this pathway (Cuervo et al., 2003). Recent work has demonstrated that the number of LAMP-2A molecules almost linearly with CMA activity under different pathologic and physiologic conditions, and changes in the expression level of LAMP-2A are used by the cells to rapidly upregulate and downregulate the activity of CMA (Cuervo and Dice, 2000).

Some original research indicated that both macroautophagy and CMA were maximally activated successively rather than simultaneously under different stress conditions such as serum removal in cultured cells, prolonged starvation in animals, and exposure to toxic compounds or oxidative stress (Massey et al., 2006). These results suggest that the different types of autophagic pathways are not completely independent. On the contrary, their functions and activations are coordinated as a part of the overall control for intracellular degradation. There may be a cross-talk among the different autophagic pathways that allows them to compensate each other. The blockage or deficiency of one autophagic pathway may lead to activation of another. For example, during starvation, macroautophagy is first triggered and with the persistence of starvation, cells switch from this bulk degradation to CMA. This autophagic cross-talk has been shown to be essential for shielding cells from the harmful effects in stress responses and certain pathogenic conditions and exist universally in normal tissue cells (Massey et al., 2008; Rodriguez et al., 2013). On the basis

of previous research in normal cells, here, we speculate that autophagic cross-talk also present in the cancer cells to deal properly with different stressors, such as serum deprivation and exposure to toxic compounds. By far, very little research has been done in this area.

Materials and Methods

Reagents

Arsenic trioxide (As₂O₃), 3-methyladenine (3-MA), MTT, RPMI 1640 medium and monodansylcadaverine (MDC) were purchased from Sigma (St. Louis, MO, USA); Fetal bovine serum (FBS) was from Sijiqing Biotechnology (Hangzhou, China); The antibodies to Hsc70, Beclin-1, p62, LC3 and β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA); The antibodies to LAMP-2A was purchased from Abcam Ltd (USA).

Cell line and cell culture

Burkitt's lymphoma Raji cells was purchased from Shanghai Institutes of Biochemistry and Cell Biology (Shanghai, China). Raji cells were cultured in RPMI-1640 medium with 10% (v/v) FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in the presence of 95% air, 5% CO₂ with medium changes every 2 days. Cells in the mid-log phase were used in the experiments.

Morphological features of macroautophagy

Cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1mol/L phosphate buffer (pH 7.4), followed by 1% OsO₄. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under JEM1230 transmission electron microscopy (JEOL, Japan). Images were acquired digitally from a randomly selected pool of 10-15 fields under each condition.

MDC fluorescence staining

The autophagic vacuoles were labeled with MDC by incubating cell growth on cover-slips with 0.05 mmol/L MDC in PBS at 37°C for 1 h. After incubation, cells were washed three times with PBS and immediately analyzed by fluorescence microscopy using an IX81 inverted microscope (Olympus, Japan). Cell number was counted to normalize the measurement, and the percentage of MDC incorporation in cells was calculated.

Western blot analysis

At the end of the designated treatments, Raji cells were lysed in RIPA lysis buffer (Beyotime, P0013B) with 1mM PMSF. Equal amounts of protein was separated by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% nonfat milk, the membrane was probed with primary antibodies (anti-Beclin-1, anti-LC3-I, II, anti-Hsc70, anti-LAMP-2A, anti-p62 and anti- β -actin) at recommended concentration. Then, the membranes were incubated with the IRDye800CW or IRDye700DX conjugate secondary antibodies (LI-COR, USA). Antibody coated protein bands of immunoblot were visualized by an Odyssey Double-color infrared-laser imaging system (LI-COR, USA).

Statistical analysis

All data represent at least three independent experiments and are expressed as means \pm SD. Statistical analysis was performed using Student's t-tests and SPSS 13.0. An association was considered significant when the exact significant level of the test was $p < 0.05$.

Results

Macroautophagy and CMA were successively activated in the case of nutrition deprivation

To gain insights into the activities of macroautophagy and CMA and their chronological sequence under stressors, we cultured Raji cells in serum free medium for 0, 3, 6, 9, 12 hours respectively and detected the fluorescence intensity of MDC using fluorescence microscope to judge the macroautophagic activity. As shown in Figure 1, the fluorescence intensity of MDC gradually increased in the first few hours of, reached the peak in 6 hours group, and then gradually declined to baseline in 12 hours group.

We then confirmed the macroautophagic activity or flux by detecting the expression of macroautophagic related proteins (including LC3-II, P62 and Beclin-1). As shown in Figure 2A, The expression of LC3-II and Beclin-1 peaked around 3-6 hours after serum withdrawal, and following gradually decreased to the basal levels. However, P62 as a macroautophagy substrate, could be used to monitor autophagic flux. In theory, the expression levels of p62 inversely correlate with macroautophagic activity. Our research showed that the average level of P62 was significantly lower than that of the controls, reduced by approximately 60% around 3-6 hours. As the starvation

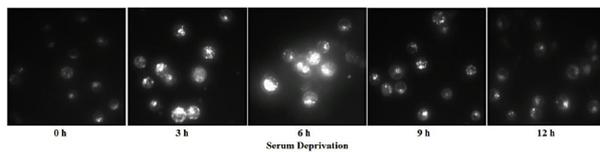


Figure 1. Fluorescence Micrographs of Starvation-induced Raji Cells. The fluorescence intensity of MDC in Raji cells was detected by microscope (original magnification $\times 100$) after Raji cells were cultured in serum free medium for 0, 3, 6, 9, 12 hours, respectively

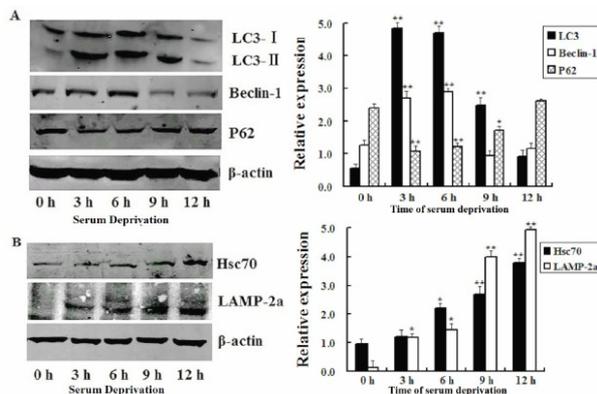


Figure 2. Macroautophagy and CMA-related Protein Expressions in Starvation-induced Raji Cells. Raji cells were cultured in serum free medium for 0, 3, 6, 9, 12 hours, respectively. The expressions of LC3-I/II, Beclin-1, P62 (A), Hsc70 and LAMP-2A (B) were determined by Western blot. * $p < 0.05$; ** $p < 0.01$, vs control group.

time prolonged, the expression of P62 protein increased in turn, and came close to the level of the comparison group at 12 hours. The above results confirmed that the macroautophagy activity was obviously enhanced in the early stages of starvation, and then decreased progressively. Thus macroautophagy was a “fast but fleeting” response to starvation.

To determine the change of CMA activity and its relationship with macroautophagy in cultured cells during the various stages of serum deprivation, western blot was adopted to detect the expression levels of Hsc70 and LAMP-2A proteins. As the main CMA components, they participated in substrate targeting, unfolding, and translocation. Our results showed that CMA and macroautophagy were not synchronously but successively activated in the case of serum deprivation. That is, the expression of Hsc70 and LAMP-2A weren't obviously increased along with the macroautophagy activity enhancement in the early of starvation. On the contrary, their expression gradually increased with declining macroautophagy activity after being starved for more than 6 hours, and reached maximum around 12 hours (Figure 2B).

In summary, these results supported the idea that macroautophagy and CMA were maximally activated during different stages of serum deprivation. Activation of these two pathways was often sequential.

As₂O₃-mediated changes of macroautophagy and CMA in Raji cells

Aside from serum deprivation, we further investigated the activities of both macroautophagy and CMA in Raji cells when exposed to toxic compound As₂O₃ which has been shown to induce growth arrest in many different cancer cell lines. We treated Raji cells with As₂O₃ (3 μ mol/l) for 24 and 48 h, and observed the formation of autophagosomes and the fluorescence intensity of MDC using transmission electron microscope and fluorescence microscope, respectively. The expression of LC3-II, Beclin-1 and P62 were detected by western blot. Our results showed that the number of autophagosomes and

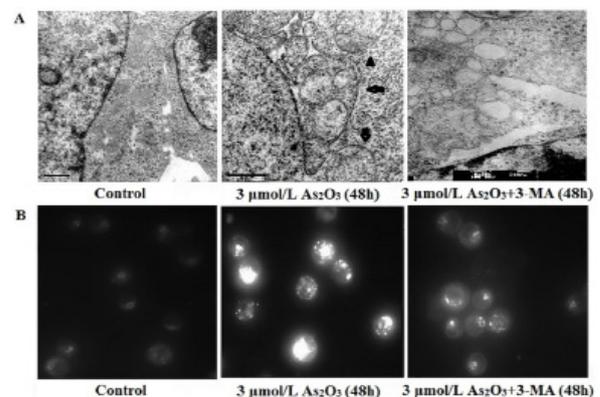


Figure 3. Effect of As₂O₃ Alone or with 3-MA on the Activity of Macroautophagy of Raji Cells. (A) The number of macroautophagic vacuoles (\rightarrow) in Raji cells were detected by transmission electron microscopy (original magnification $\times 30,000$). (B) The average fluorescence intensity of MDC was analysed by fluorescence microscopy (original magnification $\times 100$).

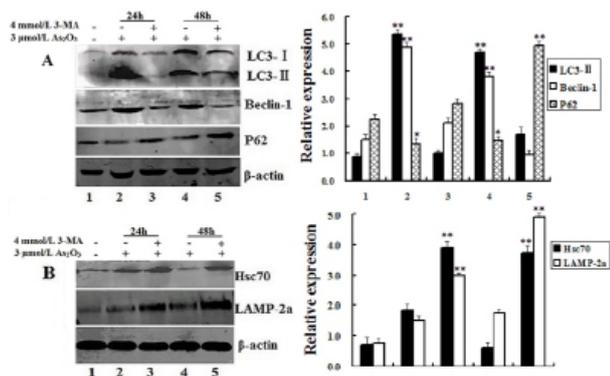


Figure 4. Effect of 3-MA on Macroautophagy and CMA-related Protein Expressions in As_2O_3 -treated Raji Cells. Raji cells were treated by 0 $\mu\text{mol/L}$ As_2O_3 (1), 3 $\mu\text{mol/L}$ As_2O_3 for 24 h (2), 3 $\mu\text{mol/L}$ As_2O_3 + 3-MA for 24 h (3), 3 $\mu\text{mol/L}$ As_2O_3 for 48 h (4) and 3 $\mu\text{mol/L}$ As_2O_3 + 3-MA for 48 h (5), respectively. The expressions of LC3-I/II, Beclin-1, P62 (A), Hsc70 and LAMP-2A (B) were detected by Western blot. * $p < 0.05$; ** $p < 0.01$, vs control group.

MDC-positive fluorescent points (Figure 3), as well as the expressions of both LC3-II and Beclin-1 in As_2O_3 group were significantly higher than those in the control group, and the level of autophagic substrate P62 protein was obviously decreased (Figure 4A) ($p < 0.05$). However, As_2O_3 just caused the negligible increase in the expression levels of Hsc70 and LAMP-2A during this process (Figure 4B).

Overall, the results suggested that macroautophagy and CMA couldn't be activated simultaneously in Raji cells when exposed to As_2O_3 . Macroautophagy was a quick and intense stressful response of the cell against the stimulation from the outside or inside of it. However, CMA was a sluggish and gentle response.

Pharmacological blockage of macroautophagy Activated CMA

To better illustrate the cross-talk between macroautophagy and CMA in toxin-induced Raji cells, we investigated the effect of blocking macroautophagy on the activation of CMA in Raji cells when exposed to As_2O_3 . In the course of studying, we used 3-MA (4 mmol/l), one of the most widely pharmacological inhibition of macroautophagy, to pretreat Raji cells for 4 hours, and then exposed to 3 $\mu\text{mol/L}$ As_2O_3 for another 24h and 48h. The results showed that the number of autophagosomes and MDC-positive fluorescent points (Figure 3), as well as the expressions levels of both LC3-II and Beclin-1 in As_2O_3 combination with 3-MA group were significantly lower than those in As_2O_3 alone group. However, compared with As_2O_3 alone group, the level of P62 protein was significantly increased in the combination group (Figure 4A).

In order to further corroborate the relationship between macroautophagy and CMA, we detected the expression of Hsc70 and LAMP-2A proteins in Raji cells after macroautophagy was inhibited by 3-MA. As shown in Figure 4B, compared with As_2O_3 alone group, the expressions of both Hsc70 and LAMP-2A in As_2O_3 combination with 3-MA group were significantly strengthened, and the relative quantitative expression of

Hsc70 at 24 and 48 h was increased by about 2.1-fold and 6.2-fold, respectively. In the similar way, the 1.5-fold and 2.8-fold expressions were gained for LAMP-2A at 24 and 48 h, respectively.

Overall, the above results confirmed that, in toxin-induced cancer cells, the blockage of macroautophagy could lead to the activation of CMA to take over its homeostasis-maintaining actions.

Discussion

Autophagy is an evolutionarily highly conserved catabolic process that degrades cytoplasmic contents (literally "eats itself") to maintain cellular homeostasis during cellular stress or starvation (Zhang et al., 2013). Studies have revealed that a basal level of autophagy acts as housekeeping functions, and a deficiency of autophagy will result in the accumulation of dysfunctional organelles and abnormal proteins, which leads to disorder of cellular function or cell death (Bejarano, 2010; Du et al., 2013).

Macroautophagy and CMA, as two different autophagic pathways, are different in the chronological order in which they occur, the type of cargo preferentially degraded, and their mechanisms that mediate the delivery of cargo to the lysosomal compartment. Despite so many differences, they are deeply connected and crucial in the maintenance of intracellular homeostasis and guarantees continuous renewal of proteome and organelles (Morimoto and Cuervo, 2009; Banjerdpongchai and Khaw-on, 2013). So they are necessary for cell growth, proliferation, differentiation and information transfer. Recent research has found that normal cells respond to decline or inhibition of macroautophagy by up-regulation of the CMA pathway, and macroautophagy is always prior to CMA while CMA lasts longer than macroautophagy (Massey et al., 2008; Rodriguez et al., 2013). These suggest the existence of cross-talk between the two stress-related autophagic pathways. However, it has been rarely reported about whether this phenomenon also exists in cancer cells. This study detected the activities of both macroautophagy and CMA in cultured Burkitt lymphoma Raji cells when facing serum deprivation and toxic compound As_2O_3 with the objective to elucidate whether some cross-talk existed between macroautophagy and CMA in cancer cells. The results showed that, in starvation-induced Raji cells, macroautophagy was quickly activated during the first hours of serum deprivation, which peaked in around 3-6 hours. If nutrient deprivation persisted, macroautophagic activity gradually decreased to the baseline level. However, this decline in macroautophagy was concomitant with a progressive increase in CMA activity which eventually up to maximum at 12 hours. This sequential switch from macroautophagy to CMA might award higher levels of selectivity when deciding the cellular cargo that could be degraded to obtain the amino acids required for cellular energy metabolism and renewal of proteome and organelles under these conditions. This asynchronous and coordinating mechanism of activation strongly supported that there must be intermolecular interactions between macroautophagy and CMA. For example, it was possible that activation of macroautophagy during the first hours of

starvation providing a first peak of free amino acids for the synthesis of essential proteins involved in defense against nutritional stress. As starvation persisted, the gradually increase of CMA activity prevented the degradation of important proteins while needless ones were preferentially degraded, and provided a second peak of amino acids to synthesize proteins.

Similarly, in As₂O₃-induced Raji cells, the macroautophagy activity was also significantly increased, but CMA activity wasn't rapidly enhanced until macroautophagy was inhibited by 3-MA. These results further proved that the blockage of macroautophagy led to the activation of CMA. However, what was the difference from nutritional deprivation was that the duration of macroautophagy increased to 24-48 h rather than a few hours. Why the cancer cells were differentially responsive to toxic compound As₂O₃? We speculated that As₂O₃ violently damaged Raji cells and produced a large number of dysfunction organelles and abnormal conformational proteins, which caused the excessive activation and prolongation of macroautophagy to maintain homeostasis and get rid of harmful cargo and repair the injured cells. The plethora of trapped cargo arrested the timely decline or stop of macroautophagy and resulted in smother or delay of the subsequent CMA activation. At this point, if macroautophagy was forcibly blocked by 3-MA, CMA might be passively activated to complete subsequent cleaning of harmful cargo. Figuratively in a word, macroautophagy was first-aiders or fire-fighters, while CMA was the stabilizers of homeostasis. The actively or passively synergistical switch from macroautophagy to CMA might be conducive to the adaption of cancer cells to miscellaneous intracellular or extracellular stressors. This is just a speculation and more research is needed to confirm the hypothesis and explore the molecular players in the cross-talk between CMA and macroautophagy.

In summary, the results of this research confirm a previously little-known cross-talk between macroautophagy and CMA in lymphoma Raji cells facing different stressors, and these findings must be helpful to understanding the characteristics, compensatory mechanisms and answer mode of different autophagic pathways in cancer cells, which may be very important and promising to the development of interventions targeting regulate one form of autophagy for potential targeting therapeutic purposes in human diseases, such as cancer or neurodegenerative disorders (Kon et al., 2011; Saha, 2012; Xilouri et al., 2013). Up-regulation of one autophagic pathways after the failure of another particular form of autophagy can be the basis for future therapeutic interventions to preserve normal cellular function in these pathologies.

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