DIFFERENTIAL EFFECTS OF ENDOPLASMIC RETICULUM STRESS-INDUCED AUTOPHAGY ON CELL SURVIVAL

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Running Title: ER stress induced autophagy in mammalian cells

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Autophagy is a cellular response to adverse environment and stress but its significance in cell survival is not always clear. Here we show that autophagy could be induced in the mammalian cells by chemicals, such as A23187, tunicamycin, thapsigargin and brefeldin A, that cause endoplasmic reticulum stress. Endoplasmic reticulum stress-induced autophagy is important for clearing polyubiquitinated protein aggregates and for reducing cellular vacuolization in HCT116 colon cancer cells and DU145 prostate cancer cells, thus mitigating endoplasmic reticulum stress and protecting against cell death. In contrast, autophagy induced by the same chemicals does not confer protection in a normal human colon cell line and in the non-transformed murine embryonic fibroblasts, but rather contributes to cell death. Thus the impact of autophagy on cell survival during endoplasmic reticulum stress is likely contingent on the status of cells, which could be explored for tumor-specific therapy.

Endoplasmic reticulum (ER) is critically involved in protein metabolism. Normal ER function is required for the correct folding of many proteins and their post-translational modifications, such as glycosylation and disulfide bond formation. ER stress is induced by the disturbance of the environment in the ER lumen, such as the calcium homeostasis or the redox status, or by the disturbance of ER function, such as glycosylation and transportation to Golgi complex (1). The typical chemicals that induce ER stress include A23187 and thapsigargin, both of which disturb the calcium homeostasis; tunicamycin, which suppresses glycosylation; and brefeldin A, which inhibits transportation to the Golgi complex (1,2). Thus these chemicals cause protein folding dysfunction and the accumulated misfolded/unfolded proteins induce ER stress. ER stress is frequently observed in pathological conditions where protein misfolding is caused by genetic mutations either in the molecule to be processed or in the machinery processing the folding (3,4).

The major protective and compensatory mechanism during ER stress is the unfolded protein response (UPR) (1,5), which leads to translational attenuation and selective upregulation of a number of bZip transcription factors (1,5). UPR serves multiple functions, including the assistance of protein folding via the up-regulated ER protein chaperones and the enhanced degradation of misfolded proteins via the upregulation of molecules involved in the ER associated degradation pathway (1,5). However, if the stress is excessive, the compensatory mechanisms may not be able to fully sustain ER function and ER de-compensation could lead to cell death (2,6). It is not clear whether there are other mechanisms that can regulate ER stress.

Macroautophagy (referred as autophagy hereafter) is mainly responsible for the degradation of long-lived proteins and subcellular organelles (7-9). Autophagy is frequently activated in response to adverse environment or stress (10-13), and has been shown to be involved in many physiological and pathological processes.
(8,14). However, whether autophagy serves a protective or detrimental role is controversial (15-17). While some studies indicate that autophagy is responsible for the non-apoptotic cell death (13,15,17-19), others indicate that autophagy is protective against cell death (12,20-24). The condition under which autophagy may be pro-survival or pro-death is not clear.

In the current study, we found that autophagy could be activated by the classical ER stress inducers in mammalian cells. However, autophagy alleviates ER stress and reduces cell death in cancer cells but not in non-transformed cells. This unique feature may be explored for certain types of cancer therapy in which ER stress constitutes a major cause of cell death.

**EXPERIMENTAL PROCEDURES**

*Reagents*--The following antibodies were used: Anti-Atg6/Beclin 1 (BD Biosciences), anti-Atg8/LC3B (25), anti-Atg5 (26), anti-ubiquitin (Santa Cruz), anti-β-actin (Sigma), and anti-GAPDH (Chemicon). All chemicals were from Sigma or Invitrogen.

*Plasmids, siRNA and transfection*--One to two micrograms of GFP-LC3B (rat) (25) was transfected into 2x10⁵ cells using Effectene according to the supplier's protocol (Qiagen). Alternatively, MEFs and CCD-18Co cells were infected with Ad-GFP-LC3B (human) for 24-48 hours before analyzed. siRNA (0.24 μM) were transfected into 1x10⁵ cells using Oligofectamine (Invitrogen) for 48 hours before analysis. siRNAs against the following human genes were used: Atg6/Beclin1 (5’-GGUCUAAGACGUCCAACAA-3’); Atg8/LC3B (5’-GAAGGCGCUUACAGCUCAA-3’).

*Cell culture and microscope*--HCT116 Bax-positive and Bax-negative cell lines (27) were maintained in McCoy’s 5A with the routine supplements. DU145 cell lines were maintained in DMEM with routine supplements. Wild type and Atg5-deficient MEFs were immortalized through SV40 large T over-expression and cultured in DMEM with standard supplements (11). The non-immortalized human colon cell line CCD-18Co was purchased from ATCC (CRL-1459™) and cultured in DMEM with standard supplements. All cell lines were maintained in a 37°C incubator with 5% CO₂. Cells (2x10⁵ per well) were seeded into 12-well plates. After 24 hours, cells were treated as indicated in the figure legends.

For electron microscopy, cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), followed by 1% OsO₄. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1016CX electron microscope. To examine the distribution of GFP-LC3B, cells were observed under a fluorescence microscope and digital images were acquired for analysis (SPOT, Diagnostics Instruments). To examine and quantify cellular vacuolization, digital phase-contrast images were recorded.

*Analysis of cell death*--Cell death was determined using propidium iodide staining (1 μg/mL) and quantified by digital microscopy. Apoptotic cells with condensed or fragmented nuclei were quantified after Hoechst 33342 (5 μg/mL) staining. Caspase activities were measured using 30 μg of proteins and 20 μM of fluorescent substrates (Ac-DEVD-AFC, AC-IETD-AFC and Ac-LEHD-AFC for caspase-3,-8 or -9, respectively). The fluorescence signals were detected by a fluorometer (Tecan GENios) at 400 nm/510 nm (excitation/emission), as previously described (28).

*Immunoblot assay*--This is essentially performed as previously described (28). Cells were washed in PBS and lysed in RIPA buffer. Forty micrograms of protein was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were stained with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

**RESULTS**

*Induction of macroautophagy by ER stress inducers in mammalian cells*--A characteristic feature of autophagy activation is the conversion of the Atg8/LC3B from the unconjugated form (LC3B-I) to the phosphatidylethanolamine-conjugated form (LC3B-II) (25,29). The mammalian Atg8/LC3B is first cleaved by Atg4 to expose the conserved Gly¹²⁰ and then conjugated to phosphatidylethanolamine via a ubiquitination-like reaction mediated by Atg7, a ubiquitin-activating enzyme (E1)-like protein, and Atg3, a ubiquitin carrier protein (E2)-like protein.
process is also affected by another conjugation system, Atg5-Atg12-Atg16, and Atg6/Beclin 1 (25,29). The non-cleaved un-conjugated form of Atg8/LC3B (LC3B-I) is in the cytosol while the cleaved, conjugated form (LC3B-II) targets to the autophagosomal membrane (25).

The ER stress inducer A23187 could illicit a strong formation of the lipidated LC3B-II in a dose-dependent manner in a colon cancer cell line, HCT116 (Figure 1A), independent of the presence of the pro-apoptotic molecule, Bax, which is critical to apoptosis induction in this cell line (see below). Other ER stress inducers, including tunicamycin (TM), thapsigargin (TG) and brefeldin A (BA) could also induce such an accumulation (Figure 1B and data not shown). This conversion could be suppressed by the autophagy-inhibiting agent, 3-methyadenine (3-MA) (12,13,30) (Figure 1B) or by the specific siRNA against Atg6/Beclin 1 or Atg8/LC3B (data not shown), suggesting the specificity of the phenomenon caused by the ER stress inducers. Consistently, cells expressing GFP-LC3B showed a transition of the GFP-LC3B signals from the diffusive cytoplasm pattern to the punctated membrane pattern following the application of the ER stress inducers (Figure 1C-D), suggesting the formation of autophagic vacuoles. Similarly, GFP-LC3B puncta could be suppressed by 3-MA or a specific siRNA against Atg6/Beclin 1 (Figure 1D-E). Electron microscopic study indicated that a significant amount of autophagic vacuoles were present in HCT116 as well as in DU145, a prostate cancer cell line, following the treatment of A23187, TM, TG or BA (Figure 2). Both double-membrane and multi-membrane structures with different intracellular contents could be observed.

Disturbing ER homeostasis or function causes the accumulation of misfolded proteins, which in turn induce ER stress (1-5). Limiting the protein influx into the ER network could reduce the stress level and thus autophagy induction. Indeed, treatment of cells with the general transcriptional inhibitor, actinomycin D, or the translational inhibitor, cycloheximide, suppressed the formation of LC3-II (Figure 3A) and the translocation of GFP-LC3B to autophagic vacuoles (Figure 3B). Together, these data strongly indicate that a diverse array of chemicals could activate macroautophagy via the induction of ER stress.

Autophagy protects cancer cells from ER stress and cell death—We then examined the significance of autophagy in the regulation of ER stress. In HCT116 and DU145 cells, ER stress inducers could cause cellular vacuolization to different degrees (Figure 4A-C). These vacuoles represented dilated ER lumens under stress based on electron microscopic examination (data not shown). Notably, suppression of autophagy with either 3-MA or a specific siRNA against Atg6/Beclin 1 (Figure 4B-C) or Atg8/LC3B (data not shown) increased the percentage of cells with cytoplasmic vacuolization, suggesting an enhanced level of ER stress.

The accumulated misfolded proteins in the ER lumen are normally degraded through ER associated degradation pathway via the proteasomes following ubiquitination (31). Thus an accumulation of polyubiquitinated proteins could indicate the level of misfolded proteins and therefore the level of ER stress. Indeed both A23187 and TM could elevate the level of polyubiquitinated proteins in HCT116 cells and DU145 cells (Figure 4D-E). Ubiquitinated protein aggregates could be also readily detected in treated cells (Figure 4F-G), which suggested that the accumulation of these proteins exceeded the capacity of proteasomes for degradation (32). Importantly, suppressing autophagy by 3-MA or a specific siRNA against Atg6/Beclin 1 (Figure 4D, F, G) led to further increases in polyubiquitinated protein aggregates. Taken together, it seems that autophagy can function to reduce ER stress based on its effects on cellular vacuolization and polyubiquitinated protein accumulation.

As the level of ER stress could correlate with the extent of cell death, we examined whether autophagy induced in these cancer cells could have any effects on ER stress-induced cell death. Apoptosis in HCT116 cells is heavily dependent on the presence of Bax (27). The syngeneic Bax-deficient cells derived from the parental Bax-positive cells require more potent stimuli to go apoptosis through the alternate Bak-mediated mechanism (33). In the regular dose ranges where ER stress and autophagy could be activated, Bax-positive HCT116 cells were much more sensitive to A23187, TG, TM or BA induced apoptosis than the Bax-deficient HCT116 cells (Figure 5A). Suppression of autophagy with either 3-MA (data not shown) or a specific siRNA against
Atg6/Beclin 1 or Atg8/LC3B (Figure 5B-C) led to increased apoptosis in the Bax-positive HCT116 cells. The activities of both effector caspases and the ER stress-specific caspase-4 were enhanced (Figure 5D-E). Interestingly, under this condition a significant amount of Bax-deficient HCT116 cells also became apoptotic with enhanced caspase activation (Figure 5), perhaps reflecting an elevated level of death stimulation when autophagy was suppressed. Similar enhancement in cell death could be also observed in the Bax-deficient DU145 cells (data not shown). These observations suggest that autophagy can protect against cell death in cancer cells likely by curtailing the level of ER stress and therefore the potency of death stimulation.

**Autophagy promotes cell death in non-transformed cells treated with ER stress inducers**—To determine the significance of autophagy in ER stress-induced cell death in different types of cells, we took the advantage of the availability of immortalized murine embryonic fibroblasts (MEFs) that are Atg5 deficient (11). All four ER stress inducers could induce GFP-LC3B punctation and the lipidated LC3B-II formation in the MEFs in an Atg5-dependent manner (Figure 6A-B). Electron microscopic examination of these cells indicated that there was an Atg5-dependent accumulation of intracellular autophagic vacuoles (Figure 6C). However, treatment of ER stress inducers did not induce cellular vacuolization in the MEFs (Figure 7A), no matter whether Atg5 is expressed or not. Furthermore, in contrast to what was observed in HCT116 and DU145 cells, deficiency of autophagy in MEFs led to reduced apoptosis. Thus Atg5-deficient MEFs were much less susceptible to apoptosis and caspase activation induced by A23187, TG, TM or BA than the wild type MEFs (Figure 7B-D). Consistently, reintroduction of Atg5 into the Atg5-deficient MEFs restored their sensitivity to the cytotoxic effects of ER stress (Figure 7E).

To support the hypothesis that this pro-death character of autophagy may be related to the non-cancerous status of the MEFs, we examined a non-immortalized normal human colon cell line (CCD-18Co), which would be more comparable to HCT116 colon cancer cells. Treatment of this cell line with A23187 or TM induced autophagy that could be suppressed by 3-MA (Figure 8A). However, 3-MA co-treatment did not lead to increased cell death, but rather resulted in reduced cell death (Figure 8B-D), consistent with the observations in MEFs. Interestingly, these treatments did not induce noticeable cellular vacuolization in this cell line, either. Taken together, these observations in the non-transformed cells are in stark contrast to those in HCT116 and DU145 cells, suggesting that the role of ER stress-induced autophagy in cell survival is contingent on the status of the cells and can be different in cancer cells and in non-transformed cells under a given set of stimuli.

**DISCUSSION**

Induction of macroautophagy by ER stress—As the major organelle processing post-translational modifications and supporting correct protein folding, ER also possesses the mechanisms to retro-transport proteins that fail to be modified and/or folded properly (31). These proteins are then ubiquitinated and degraded by proteasomes. This ER-associated degradation pathway is important for maintaining ER homeostasis. Accumulation of the unfolded/misfolded proteins, such as that due to the use of A23817, TM, TG, or BA, can lead to ER stress. The UPR is a classical mechanism that cells mount to relieve ER stress, which aims to reduce overall cellular protein synthesis, assists protein folding and promotes ER-associated degradation (1,5). Now we show here that autophagy is activated in response to ER stress in the mammalian cells and could be another mechanism regulating ER stress and the outcome. Notably, ER stress could also induce autophagy in the yeast (34), indicating that this response is evolutionarily conserved.

How ER stress leads to the activation of autophagy is not quite understood. Transcriptional upregulation of certain Atg genes, such as Atg12 (24) and Atg8/LC3B (data not shown) have been observed and could be important. Although the majority of cellular protein synthesis is shut down during ER stress, a selected group of proteins, such as the bZIP transcription factors ATF4 and CHOP, are activated downstream of eIF2α kinase, which is an important component of the UPR (1,5). These transcription factors could be involved in the autophagy activation. Indeed, eIF2α has been found to be important for
autophagy induced by the pathogenic polyglutamine repeats (24), viral infection and starvation (35). Other UPR response pathways, such as those mediated by Ire1 and ATF6 could be also involved. While other activation mechanisms may also be required for a full activation of autophagy, our data strongly indicate that signals from ER stress are critical for the triggering of autophagy, which would require further characterization in the future.

The significance of autophagy in ER stress and ER stress-induced cell death--In the cancer cells, autophagy helps to alleviate ER stress and subsequently the cell death. How autophagy mitigates ER stress is not completely clear at this moment. Our observations indicate that autophagy is important for the clearance of ubiquitinated unfolded/misfolded proteins and therefore reduces ER stress induced by these molecules. This notion is also supported by the finding made in mice with conditional deletion of Atg5 or Atg7 in the central nervous system, which leads to the accumulation of polyubiquitinated proteins in the neurons (22,23). In addition, it has been found that misfolded proteins, such as the mutant α1-antitrypsin Z protein and the pathogenic polyglutamine repeats could all induce autophagy in addition to ER stress, independent of their cellular locations (24,36-38). While promotion of autophagy enhances the clearance of the mutant molecules and reduces the toxicity, inhibition of autophagy results in the opposite (21,24,39,40). Thus autophagy can be protective against ER stress in a number of circumstances including cancer cells.

Paradoxically, while disturbing ER homeostasis and/or functions by the chemicals could also illicit autophagy in the primary colon cells, suppression of autophagy does not enhance but reduce cell death. In addition, suppression of autophagy induced by the same chemicals in the immortalized, but non-transformed MEFs by deletion of Atg5 also reduces cell death. These data suggest that autophagy can contribute to ER stress-induced cell death in different scenarios, which may dependent on cellular status under a given stimulation. However, the exact mechanisms affecting such a different outcome are yet to be determined. In the context of current experiment system, we speculate that the differential role of autophagy in cell survival in the cancer cells and non-transformed cells may be related to how well the ER stress is compensated. Both MEFs and CCD-18Co might be better compensated in response to the stimulation. One hint for this assumption is that treatment of ER stress inducers did not cause cellular vacuolization and inhibiting autophagy did not seem to promote vacuolization in these cells (Figure 7A, Figure 8D). While autophagy could be still involved in clearing misfolded proteins, its degradation capability might turn to be “too extensive” for the better compensated cells, in which autophagy may lead to excessive consumption of by-stander normal cellular constituents and therefore contribute negatively to cell survival. Future work will be directed to seek experimental evidence to test this hypothesis. However, our current work has indicated the importance of understanding the underlining mechanism and pointed out a potential benefit of such an understanding that the differential effect of autophagy in cancer cells and non-transformed cells may be explored for tumor-specific therapy.

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FOOTNOTES

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1The abbreviations used are: 3-MA: 3-methyadenine, ActD: actinomycin D, BA: brefeldin A, CHX: cycloheximide, ER: endoplasmic reticulum, MEF: murine embryonic fibroblasts, TM: tunicamycin, TG: thapsigargin, UPR: unfolded protein response.

FIGURE LEGENDS

**Figure 1. Induction of autophagy by ER stress inducers.** (A) Bax-positive and Bax-deficient HCT116 cells were treated with different doses of A23187 for 24 hours followed by immunoblot analysis. (B) Bax-deficient HCT116 cells were treated with A23187 (2.5 μM), TM (5 μg/mL), or TG (0.5 μM) for 24 hours with or without 3-MA (10 mM). Immunoblot analysis was performed on total lysates. (C) Bax-deficient HCT116 cells stably expressing GFP-LC3 (a-d) were treated for 24 hours with vehicle control (a), TG (0.5 μM, b), A23187 (2.5 μM, c), or TM (5 μg/mL, d). Alternatively, Bax-positive HCT116 cells stably expressing GFP-LC3B (e-f) were transfected with a negative siRNA (e) or a specific siRNA against Atg6 (f) for 48 hours before treated with A23187 for 24 hours. Images were digitally recorded to indicate the change of GFP-LC3B distribution pattern. (D) Bax-deficient HCT116 cells stably expressing GFP-LC3B were treated for 24 hours as in B. Percentages of cells with punctated GFP-LC3B pattern were then determined. (E) Percentage of cells with punctated GFP-LC3B pattern was determined in GFP-LC3B-expressing Bax-positive and Bax-deficient cells treated with a negative siRNA (Neg) or a specific siRNA against Atg6 for 36 hours followed by A23187 or TM for 24 hours. The insert shows the immunoblot analysis of Atg6 in HCT116 Bax-negative cells receiving the designated siRNA.

**Figure 2. Electron microscopic detection of autophagy vacuoles.** (A) Bax-deficient HCT116 (a-d) or DU145 (e-h) cells were treated with A23187 (2.5 μM, a, e), TG (0.5 μM, b, f), TM (5 μg/mL, c, g) or BA (2.5 μM, d, h) for 16 hours. Cells were then fixed and subjected to electron microscopy. The representative morphology of autophagic vacuoles is shown. Both double membrane and multi-membrane vacuoles are observed. Scale bar: 0.5 μm. (B) The number of autophagic vacuoles was quantified in Bax-deficient HCT 116 and DU145 cells. Data were presented as means±SD per 100 μm² area from different cells.

**Figure 3. Inhibition of gene transcription or protein synthesis suppresses ER stress-induced autophagy.** (A) Bax-negative HCT116 cells were treated with A23187, TM, TG or vehicle control (Ctr) in the presence or absence of CHX (10 μg/ml) or ActD (0.1 μg/ml) for 24 hours. Total lysates were prepared and subjected to immunoblot assay. (B) Bax-deficient HCT116 cells stably expressing GFP-LC3B were treated as in C. Percentage of cells showing punctated GFP-LC3B was determined (mean±SD).
Figure 4. Autophagy protects against ER stress and promotes degradation of poly-ubiquitinated proteins in cancer cells. (A) Bax-positive HCT116 cells were transfected with a negative siRNA (a, c) or a specific siRNA against Atg6 (b, d) for 36 hours. Cells were then treated with A23187 (2.5 µM, a, b) or BA (2.5 µM, c, d) for 24 hours before phase microscopy was conducted. (B) Percentages of cells with cellular vacuolization after indicated treatment were determined (mean±SD). (C) DU145 cells were treated with A23187 (2.5 µM) or BA (2.5 µM) in the presence or absence of 3-MA (10 mM) for 24 hours. Cells with vacuoles were determined by phase microscopy and quantified. (D) Bax-negative HCT116 cells were transfected with a negative siRNA (Neg) or a specific siRNA against Atg6 for 36 hours. Cells were then treated with vehicle control (Ctr), A23187 (A, 2.5 µM) or TM (5 µg/ml) as indicated for 24 hours. Total lysates were prepared and subjected to immunoblot assay with the indicated antibodies. (E) DU145 cells were treated with A23187 (2.5 µM), or TM (5 µg/ml) in the presence or absence of 3-MA (10 mM) for 24 hours. Total lysates were prepared and subjected to immunoblot assay with the indicated antibodies. (F) Bax-negative HCT116 cells were treated with vehicle control (Ctr), A23187 (A, 2.5 µM) or TG (0.5 µM) as indicated for 24 hours. Cells were then immunostained with an anti-ubiquitin antibody followed by Cy3-conjugated secondary antibody and counter-stained with Hoechst 33342. Arrows indicate the ubiquitin-positive protein aggregates. (G) Bax-negative HCT116 cells were transfected with a negative siRNA (Neg) or a specific siRNA against Atg6 for 36 hours. Cells were then treated as indicated for 24 hours before immunostained with an anti-ubiquitin antibody as in panel F. Cells with cytosolic ubiquitin positive aggregates were then quantified.

Figure 5. Suppression of autophagy enhances cell death in cancer cells. (A) Bax-positive and Bax-deficient HCT116 cells were treated with A23187 (2.5 µM), TM (5 g/ml), or TG (0.5 µM) for 24 hours. Percentage of apoptotic cells was determined by Hoechst 33342 staining. (B-E) Bax-positive and Bax-deficient HCT116 cells were transfected with a negative siRNA (Neg) or Atg6 or Atg8/LC3B-specific siRNA for 48 hours. Cells were then treated as in panel A. Percentage of apoptotic cells was determined by Hoechst 33342 staining (B). Overall cell death was determined by PI staining (C). Caspase-3 (D) and caspase-4 (E) activities were determined using Ac-DEVD-AFC or Ac-LEVD-AFC as the substrate, respectively, and expressed as the fold over the non-treated control.

Figure 6. Induction of autophagy by ER stress in MEFs. (A) Wild type (a-d) and atg5-deficient (e-h) MEF cells were first infected with Ad-GFP-LC3B for 48 hours and then treated with vehicle control (a, e), A23187 (2.5 µM, b, f), TM (5 µg/ml, c, g), or TG (0.5µM, d, h) for another 24 hours. Percentage of GFP-LC3B positive cells showing puncta were determined (mean±SD). (B) Wild type and atg5-deficient MEFs were treated with A23187 (A, 1 µM), TM (5 µg/ml), TG (0.5 µM), BA (5 µM) or vehicle control (Ctr) for 24 hours. Immunoblot analysis was performed on total lysates. Atg5 was detected as the complex with Atg12. (C) Wild type MEF cells were treated as in panel A for 24 hours and subjected to electron microscopy. Number of autophagic vacuole were quantified and presented as mean±SD per 100 µm² area from different cells.

Figure 7. Suppression of ER stress-induced autophagy reduces cell death in MEFs. Wild type (a-d) and atg5-deficient (e-h) MEFs were treated for 24 hours with A23187 (2.5 µM, a, e), TM (5 µg/ml, b, f), TG (0.5 µM, c, g), BA (2.5 µM, d, h) for 24 hours. The morphology of cells was examined by phase microscopy (A). Note the lack of cellular vacuolization in treated cells and the reduced cell death in atg5-deficient cells. The percentages of PI positive cells (B) and apoptotic cells (C) were then determined by PI or Hoechst 33342 staining, respectively. Caspase-3 activities (D) were measured using Ac-DEVD-AFC as the substrate. (E) atg5-deficient MEFs were transfected with murine Atg5 or the vector control for 24 hours and then treated with A23187 or TG for another 24 hours before being analyzed for apoptosis as described above. The insert shows an immunoblot assay on Atg5 and β-actin expression in these cells. Atg5 was detected as the complex with Atg12.
Figure 8. Suppression of ER stress-induced autophagy reduces cell death in CCD-18Co cells. (A). CCD-18Co cells were first infected with Adeno-GFP-LC3 (50 m.o.i) for 24 hours, and then treated with vehicle control (a), A23187 (2.5 µM, b-c) or TM (5 µg/mL, d-e) in the presence (c, e) or absence (a, b, d) of 3-MA (10 mM) for another 24 hours. Percentages of GFP-LC3B positive cells showing the punctated signals were determined (mean±SD). (B-D). CCD-18Co cells were treated with A23187 (2.5 µM), TG (0.5 µM) or TM (5 µg/mL) in the absence or presence of 3-MA for 24 hours. Cell death was determined by PI staining (B) and apoptotic cells were quantified by nuclear staining with Hoechst 33342 (C). Phase contrast microscopy was conducted to examine cellular vacuolization, which was not observed (D).
Figure 1

A. Diagram showing the effects of A23187 on HCT 116 cells with or without Bax expression. The table lists the concentrations of A23187 (1, 2.5, 5 µM) and the corresponding Western blot images of LC3B-II and GAPDH. The images show a decrease in LC3B-II levels with increasing A23187 concentration.

B. Table showing the effects of A23187, TM, and TG with or without 3-MA on HCT 116 cells. The bar graph represents the percentage of punctated GFP-LC3+ cells in different conditions. Control, A23187, TM, and TG are compared with or without 3-MA.

C. Images showing punctated GFP-LC3+ cells under different conditions. Images a and b show control conditions, while images c and d show treatments with A23187, TM, and TG. Images e and f show the effect of Atg6 siRNA.

D. Bar graph showing the percentage of punctated GFP-LC3+ cells under different conditions. Control, A23187, TM, and TG are compared with or without 3-MA. The graph includes error bars for each condition.

E. Bar graph showing the effect of Atg6 siRNA on HCT 116 cells with or without Bax expression. The graph compares control, A23187, and TM conditions with or without Atg6 siRNA.
Figure 2

A

[Images of electron micrographs of autophagic vacuoles]

B

[Bar graph showing the number of autophagic vacuoles per 100 μm² for different conditions: Control, A23187, TG, TM, and BA. The bars are divided into two groups: HCT 116 Bax (-) and DU145.]
Figure 3

A

|       | Ctr | A23187 | TM | TG |
|-------|-----|--------|----|----|
| CHX   | -   | -      | +  | -  |
|       | -   | -      | +  | -  |
| β-actin | - | -      | +  | -  |

B

![Graph](http://example.com/graph.png)

Punctated GFP-LC3 B+ Cells (%)
Figure 4

A

B

C

Vacuolated Cells (%)

3-MA: - - +             - +

Vacuolated Cells (%)

Neg Atg6

Control A23187 BA

Control A23187 BA

HCT116 Bax (+) HCT116 Bax (-)

HCT116 Bax (+) HCT116 Bax (-)
Figure 4

**D**

| siRNA: | Neg | Atg6 |
|--------|-----|------|
| Ctr    | A   | TM   |

| Ubiquitin | β-actin |
|-----------|---------|
|           |         |

**E**

| 3-MA: | -  | -  | +  | +  |
|-------|----|----|----|----|
| A23187 | TM | A23187 | TM |

| Ubiquitin | β-actin |
|-----------|---------|
|           |         |

**F**

- Ctr
- A23187
- TG

**G**

| Ub-aggregate+Cells (%) |
|------------------------|
| Control                |
| A23187                 |
| TG                     |
| TM                     |
Figure 5

A

![Graph showing Apoptotic Cells (%)]

B

![Graph showing Apoptotic Cells (%)]

C

![Graph showing PI Positive Cells (%)]
Figure 5

D

Caspase-3 Activities (Fold of control)

|        | A23187 | TM   | TG   | A23187 | TM   | TG   |
|--------|--------|------|------|--------|------|------|
| Neg    |        |      |      |        |      |      |
| Atg6   |        |      |      |        |      |      |
| Atg8   |        |      |      |        |      |      |
| HCT116 Bax (+) |     |      |      | HCT116 Bax (-) |     |      |

E

Caspase-4 Activities (Fold of control)

|        | A23187 | TM   | A23187 | TM   |
|--------|--------|------|--------|------|
| Neg    |        |      |        |      |
| Atg6   |        |      |        |      |
| Atg8   |        |      |        |      |
| HCT116 Bax (+) |     |      | HCT116 Bax (-) |     |
Figure 6

A

**Figure 6A**

(a) Control; (b) A23187; (c) TG; (d) TM

9.2±3.5, 77.2±3.0, 77.7±5.5, 78.3±5.2

9.4±0.5, 22.3±2.4, 24.7±3.6, 13.1±3.7

B

|         | atg5 +/+ | atg5 -/- |
|---------|----------|----------|
| Ctrl    | A        | TM       |
| A       |          |          |
| TM      |          |          |
| TG      |          |          |
| BA      |          |          |

C

**Figure 6C**

Bar chart showing the number of autophagic vacuoles per 100 µm².

- Control
- A23187
- TG
- TM

- atg5 +/+ (open bars)
- atg5 -/- (filled bars)
Figure 7

A
Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival
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