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Molecular Links Between Autophagy and Apoptosis

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Summary

Macroautophagy (herein referred to as autophagy) contributes to the control of life and death throughout the animal and plant kingdoms. Bilateral links have been found between apoptosis and autophagy where inducers of apoptosis also induce autophagy and vice versa. In some cases, autophagy delays the onset of apoptosis and thus prolongs life although it may also promote apoptosis and other forms of cell death. It is thus of great biological and medical interest to understand the molecular connections between these two pathways, and try to utilize—or block—them selectively to aid induction of cell death (e.g., cancer cells) or inhibit death (e.g., in degenerative disorders). This chapter describes methods for studying apoptotic induction of autophagy and its effects on cell function. We also discuss potential pitfalls. Although cell lines are used as model systems, the substances and methods described here can be applied to primary cells and tissues.

Key Words: Apoptosis; autophagy; Bax; caspase; LC3; PARP cleavage.

1. Introduction

Although macroautophagy (henceforth referred to as autophagy) is activated primarily as a response to nutritional deprivation ***(1,2)***, especially through lack of nitrogen/amino acids, autophagy is also induced by many—if not all—apoptotic stimuli ***(3–6)***. The mechanisms by which apoptotic stimuli activate autophagosome formation are not well defined, but our studies on neurons have established that amino acid starvation—a classical method of inducing autophagy especially in liver, heart, and kidney ***(7,8)***—is not necessary ***(5)***. In fact, apoptotic stimuli that cause DNA damage ***(5)***, ER stress ***(9)***, or the ones that originate as extrinsic signals (Fas, tumor necrosis factor [TNF]-α) ***(10)***

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also promote autophagosome formation. Moreover, at least for induction of autophagy by TNF-α, no new protein synthesis is required as autophagy is activated in the presence of protein synthesis inhibitors (I. A. Ciechomska et al., unpublished data). Importantly, Beclin1, a key intermediate in the transduction of proautophagic signals to autophagosome formation, binds and alters the properties of some of the antiapoptotic Bcl2 family members and vice versa ***(11)***. Moreover, ER stress, which is a key mechanism of apoptosis induction, is also a mechanism for inducing autophagy ***(12–15)***. In this chapter, methods of apoptosis induction that produce autophagy and measurement of both in cell culture systems are described. Although the focus is on adherent cells, where detailed microscopic studies can be obtained, induction of apoptosis can be carried out on many types of nonadherent cells, as well as in isolated tissues and whole organisms (plants and animals). It is important to note that some cancer cell lines are highly resistant to apoptosis (e.g., some MCF7 lines lack expression of caspase 3). Hence, it is crucial to verify that apoptosis has been induced by the treatment being used before connections to autophagy can be considered.

2. Materials

1. Cell lines for studying apoptotic induction of autophagy (HeLa, 293, Cos7, MCF7) can be obtained from American Type Culture Collection (ATCC, www.atcc.org) or European Collection of Cell Cultures (ECACC, www.hpa.org.uk/business/ecacc.htm). Recommended growth conditions are provided with each batch as well as the passage number. It is important to restrict experiments to a limited number of passages as the genetic status of cells drifts on prolonged propagation.
2. For the above cells, use Dulbecco's modified Eagle's medium (DMEM, 5–10 m*M* D-glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (all from Sigma-Aldrich, www.sigmaaldrich.com; or Invitrogen, www.invitrogen.com).
3. Staurosporine (Sts, www.sigmaaldrich.com) is a potent kinase inhibitor that induces the intrinsic mitochondrial pathway of apoptosis in most cell types. Prepare a 1 m*M* stock in dimethyl sulfoxide (DMSO); store in aliquots at –20ºC.
4. Etoposide (www.sigmaaldrich.com) is representative of proapoptotic DNA-damaging agents (e.g., cisplatin and cytosine arabinoside). Prepare a 50–100 m*M* stock in DMSO; store in aliquots at –20ºC.
5. Thapsigargin (Tg, a sarco/endoplasmic reticulum Ca^{2+} ATPase [SERCA] inhibitor that causes release of Ca^{2+} from the ER) and tunicamycin (Tm, which disrupts N-glycosylation of newly synthesized proteins in the ER) promote ER stress-induced apoptosis. Prepare stocks of 2 m*M* Tg and 10 mg/mL Tm in DMSO and store aliquots at –20ºC.
6. TNF-α not only activates the extrinsic pathway of apoptosis through the death-inducing signaling complex (DISC) and caspase 8 but also activates a

protective mechanism via induction of nuclear factor (NF)-κB. Therefore, it is commonly applied together with the protein synthesis inhibitor cycloheximide to suppress NF-κB expression. Prepare stocks of 100 ng/μL TNF-α (Peprotech, www.peprotech.com) as instructed. Store in aliquots at –80ºC. Cycloheximide (www.sigmaaldrich.com) is dissolved at 10 mg/mL in medium or water. Store at –20ºC.

7. UV-C irradiation is obtained using a Stratalinker® UV Crosslinker (www.stratagene.com).
8. Vinblastine activates autophagy so is a useful positive control. Dissolve at 10–50 m*M* in DMSO; store in aliquots at –20ºC.
9. Earle's or Hanks basal salt solution (EBSS, HBSS; www.sigmaaldrich.com) is used to activate autophagy by amino acid starvation.
10. Hoechst 33342 and propidium iodide (PI, www.sigmaaldrich.com). Prepare stocks at 100 μg/mL in water and store at 4ºC. More concentrated stocks can be prepared and stored at –20ºC.
11. Boc-aspartyl(*O*-methyl)fluoromethylketone (BAF, www.mpbio.com) is one of a family of cell-permeable caspase inhibitors that can be used to demonstrate that apoptosis is indeed occurring. Prepare 50 m*M* stocks in DMSO, aliquote, and store and –20ºC. Use at 50–100 μ*M*, but beware that these inhibitors may target other cysteine proteases (e.g., cathepsins) differentially depending on concentration.
12. Bafilomycin A1 (Baf A1, www.biomol.com) alkalinizes the lysosomal lumen by inhibiting the vacuolar-type (V-type) H^+ ATPase. Stock at 1 μ*M* in DMSO; store at –20ºC.
13. Cell lysis buffer (recipe is also good for collecting phosphoproteins): 50 m*M* Tris-HCl, pH 7.4, 1% NP-40; 120 m*M* NaCl, 1 m*M* EDTA, 25 m*M* sodium fluoride, 40 m*M* sodium glycerophosphate, 1 m*M* sodium orthovandate, 1 m*M* benzamidine, 5 m*M* sodium pyrophosphate, protease inhibitor cocktail (e.g., Complete™, Roche, www.roche.com).
14. 4X loading buffer: 50 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 m*M* dithiothreitol (DTT), and 0.01% bromophenol blue.
15. Caspase dilution buffer: 10 m*M* HEPES, pH 7.4, 42 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 5 μg/mL aprotinin, and 0.1% Triton X-100.
16. Caspase assay buffer: 20 m*M* HEPES, pH 7.4, 1 m*M* EDTA, 10% sucrose, 5 m*M* DTT, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 15 μ*M* DEVD-AMC (Asp-Glu-Val-Asp-aminomethylcoumarin).

3. Methods

Apoptosis can be induced by a variety of compounds or treatments depending on cell type and receptor/metabolic repertoire. For this reason, to demonstrate generalized (universal) mechanisms, it is important to use several

compounds or treatments and different cell types. Each of the treatments described here initiates apoptosis through a different mechanism although all of these ultimately converge on the mitochondrial pathway that comprises induction/activation of BH3 only proteins; activation of Bax and/or Bak; mitochondrial outer membrane permeabilization; release of cytochrome-*c* from the intermembrane space; activation of Apaf-1, caspase 9, and downstream caspases; and cleavage of multiple substrates. Key caspase substrates are acinus and caspase-activated DNase (CAD), which cause DNA clumping and fragmentation ***(16,17)***, and poly-ADP-ribose polymerase (PARP), which reports on caspase 3/7 activities within the cell ***(18–20)***. Evidence that apoptosis is occurring in intact cells is obtained by monitoring translocation of Bax to mitochondria using, for example, conformation-specific antibodies ***(21)*** or GFP-tagged Bax (not described here), release of cytochrome-*c* from mitochondria ***(5,22–24)***, nuclear morphological changes using DNA-binding dyes (e.g., *see* **ref. 5**), and phosphatidylserine (PS) exposure using fluorescent Annexin V staining ***(25)***. Biochemical events monitored by immunoblotting include PARP cleavage by immunoblotting and caspase activity using fluorogenic substrates. Concomitant autophagosome formation in cells can be demonstrated by recording the formation of LC3-II-dependent puncta (using fluorescent fusion protein tags or antibodies) and by immunoblotting for LC3-II. A parallel assay using an inhibitor of autolysosomal-mediated degradation of LC3-II ***(26,27)*** (e.g., by inhibition of the V-type H^+ ATPase with Baf A1) is crucial to demonstrate that productive autophagy, namely, LC3 turnover, is occurring. Otherwise, LC3-II puncta might be accumulating because there is a block in the formation of autolysosomes or in lysosomal proteolytic activity.

3.1. Induction of Apoptosis

1. Seed cells into 24-wells containing glass cover slips (13 mm, #0–1 thickness, or glass-bottom dishes, www.glass-bottom-dishes.com) at a density that will give 70–80% confluence the next day (*see* **Notes 1** and **2**). For biochemistry, seed proportionately in six-well plates (*see* **Note 3**).
2. Replace growth medium with medium containing the apoptotic inducer. Dose efficacy should be tested for each cell type at different times after addition as described below.
    a. Sts: efficacy range 0.1–2 μ*M*. Note that high concentrations can cause necrosis and that Sts can alter cell shape independently of apoptosis due to cytoskeletal deformation.
    b. Etoposide: efficacy range 20–200 μ*M*.
    c. Tg and Tm: efficacy range 1–4 μ*M* Tg and 5–20 μg/mL Tm. In some cells, these can be used separately to induce apoptosis, but we find them especially efficacious when they are added together.

d. TNF-α and cycloheximide: efficacy range 5–100 ng/mL TNF-α, 5–20 μg/mL cycloheximide.
e. UV: efficacy range 100–600 J/m^2. Replace medium with sterile PBS. Place plate/dish on bottom center of Stratalinker chamber, remove lid from wells to be irradiated (UV does not penetrate tissue culture plastic), and activate irradiation protocol as instructed after carefully calculating the time required to give the energy required. Replace the lid immediately. We usually activate the protocol once or twice before submitting the cells to irradiation as this also sterilizes the chamber.

3.2. Quantification of Apoptosis

3.2.1. Analysis of Nuclear Morphology

Nuclear condensation and fragmentation during apoptosis can be quantified by co-staining with two nuclear dyes: Hoechst 33342 and PI. Because PI is a membrane-impermeable dye, it only stains the nucleus of dead cells that have lost plasma membrane integrity, that is, cells that have undergone necrosis or secondary necrosis (following on from apoptosis). Hoechst 33342 is membrane permeable so it stains live cells. Nuclei of healthy cells are relatively dimly stained by Hoechst and are not stained by PI. Nuclei of apoptotic cells are fragmented or highly condensed and display bright blue fluorescence due to Hoechst staining. Occasionally, nuclei will appear pink, indicating that the plasma membrane is beginning to be permeable to PI. Necrotic cells show a bright red fluorescence staining with PI, with normal or slightly condensed size. If in doubt, use phase microscopy to examine whether the plasma membrane is shattered as these cells are phase dark. Note that this method cannot be used for fixed cells as PI will stain live as well as dead cells.

1. At the indicated time after treatment, add Hoechst 33342 and PI (1–5 μg/mL final concentration of each) directly to the growth medium.
2. Incubate cells for 10 min at 37ºC.
3. Examine cells by fluorescence microscopy using a UV filter (340–380 nm excitation, 425 nm emission) and count the number healthy and apoptotic blue-stained cells and PI-positive red dead cells in at least four randomly selected fields, at least 200 cells in total.
4. Calculate the percentage of apoptotic and necrotic cells out of the total (apoptotic + necrotic + healthy) cells counted.

3.2.2. PARP Cleavage

PARP, a 113-kDa protein in humans, binds specifically to DNA strand breaks and is a substrate for caspase 3, which is activated during apoptosis in most cell types. However, in MCF7 cells lacking caspase 3, caspase 7 cleaves

PARP albeit inefficiently. Caspase 3 cleaves PARP into two fragments of approximately 89 and 24 kDa. Thus, detection of the 89-kDa PARP fragment serves as a biochemical marker of apoptosis. To detect PARP in untreated cells and after exposure to drugs:

1. Collect healthy and dead cells from a six-well plate by scraping them off the dish in the culture medium using the plunger of a 1-mL syringe.
2. Centrifuge at 380×g for 5 min; resuspend cells in 1 mL ice-cold PBS (to remove serum remnants), transfer cell suspension to a microfuge tube, and pellet cells again.
3. After removing PBS, add 100–150 µL cell lysis buffer. Keep on ice for 20–30 min with occasional vortexing to swell the cells and facilitate protein extraction.
4. Centrifuge the lysate at 13,000×g at 4ºC for 10 min. Transfer the supernatant to a fresh microcentrifuge tube and discard the pellet (containing nuclei; transcription factors will be extracted but histones remain with the pellet).
5. Estimate the amount of total protein in 2–5 µL of each sample using a bicinchoninic acid assay (BCA). BCA protein assay kit (www.sigmaaldrich.com) is used according to the manufacturer's instructions.
6. Add the appropriate amount of 4X gel loading buffer to each sample and heat at 98ºC for 5–10 min.
7. Separate proteins (at least 20 µg per lane) on a 10% denaturing polyacrylamide gel (SDS–PAGE).
8. Transfer the separated proteins to a nitrocellulose/PVDF filter by electroblotting.
9. Stain the filter with Ponceau Red solution (1% Ponceau S [www.sigmaaldrich.com] in 5% acetic acid/water) for 5 min to visualize protein bands.
10. Rinse the membrane in water until protein bands are distinct and mark the position of the molecular weight markers with a ballpoint pen or pencil. Scan, photograph, or photocopy the blot. Image may be used to demonstrate equality of protein loading.
11. Cut membrane across into two pieces at the approximately 70 kDa marker. PARP (113 and 89 kDa) is on the upper piece and α-tubulin (~50 kDa) is on the lower piece of membrane. Detection of α-tubulin is a useful loading control for immunoblotting. If you are using an anti-PARP monoclonal antibody, ensure that it recognizes the 89-kDa fragment, namely that the epitope is not within the 24-kDa fragment.
12. After blocking with 5% nonfat milk in TBST (Tris-buffered saline/Tween-20: 10 m*M* Tris-HCl, pH 7.4; 150 m*M* NaCl; 0.1% Tween-20), incubate the membranes overnight at 4ºC with primary antibodies diluted in blocking buffer or TBST. Antibodies can be reused several times if stored in the presence of 0.02% sodium azide.
13. Wash with TBST four times for 5 min each and incubate for 1 h at room temperature with appropriate horseradish peroxidase (HRP)-conjugated anti-species-specific IgG. For best results, choose antibodies that have been subtracted against

all species other than the species in which the first antibody was raised (e.g., *see* www.jacksonimmuno.com). Note that HRP is potently inhibited by sodium azide.

14. Visualize immunocomplexes using an enhanced chemiluminescence detection system (ECL, www.amershambiosciences.com).
15. After exposure of blots to X-ray film, bands can be digitized using a scanner. To obtain meaningful data, blots must not be overexposed, and band intensities should be in the linear range of the densitometric sensitivity of the film. This can be tested by running increasing amounts of lysates on parallel lanes and scanning the X-ray film. The range of linearity of the scanner also has to be determined. For this purpose, special calibration strips are commercially available (www.kodak.com). Density can be quantified using ImageJ (NIH Image) analysis system (open source software at http://rsb.info.nih.gov/ij/). *See* **Fig. 1** for image of blot probed for PARP cleavage.

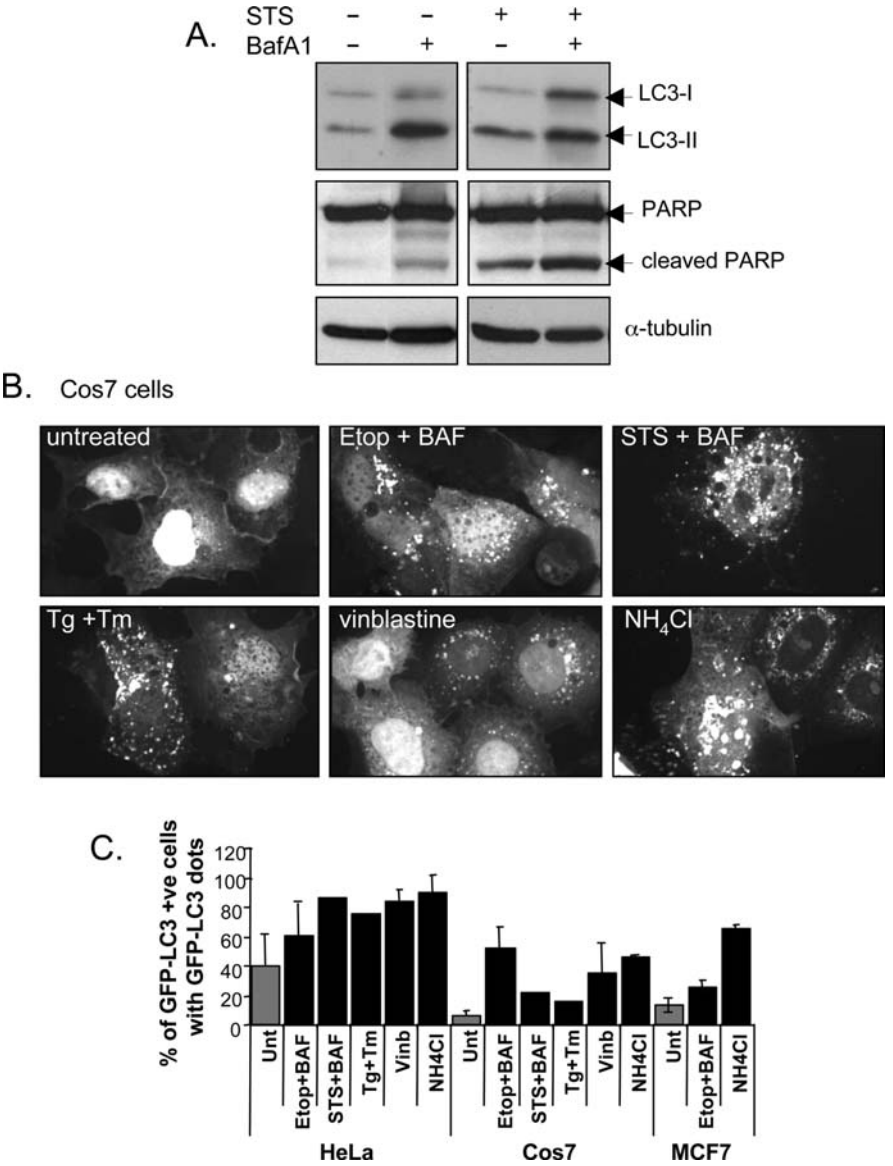
3.2.3. Caspase 3 Activity

Caspase 3 is one of the key executioners of apoptosis, being responsible for the proteolytic cleavage of many key proteins, including PARP. Caspase 3 activity can be sensitively assessed by measuring the cleavage of the fluorogenic substrate Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin); the signal is emitted when AMC is released on cleavage. Specificity can be determined by adding excess DEVD aldehyde (DEVD-CHO), a high-affinity reversible inhibitor, to a parallel cell extract. Indeed, the DEVD amino acid sequence is derived from the caspase 3 cleavage site in PARP. The same protein extracts used for immunoblotting can be used for caspase activity measurement as the protease inhibitor mix Complete in the extraction buffer does not seem to interfere with the caspase activity assay. A key requirement is the presence of DTT as caspases are cysteine proteases that require a reduced thiol group for activity. Measurement of caspase 3 activity with this substrate is best performed in a 96-well microplate format using a fluorometric microplate reader.

1. Add 20 µg protein extract to ice-cold caspase dilution buffer to produce a final volume of 50 µL. Alternatively, cells can be lysed directly in the caspase dilution buffer by sonication on ice.
2. Transfer each sample to a 96-well microplate.
3. Prepare blank without protein extract (only 50 µL hypotonic buffer) and an AMC calibration standard (0.5–10 µ*M* AMC) to determine the linear range of detection.
4. To each well, add 150 µL of ice-cold caspase assay buffer.
5. Cleavage of DEVD-AMC (intensity of fluorescence) is measured at 340 nm (excitation) and 460 nm (emission) in the microplate reader. Record fluorescence at 5- to 10-min intervals for up to 120 min (for low protein concentrations, one

can measure up to 24 h as long as the readings are linear). *See* **Fig. 2** for caspase assay results.

6. Analyze the initial rate (slope) of fluorescence signal along the linear portion of the curve depicting fluorescence accumulation.
7. To ensure that the fluorescence is due to caspase activity, add 5- to 10-fold excess DEVD-CHO over DEVD-AMC to parallel samples.



3.2.4. Bax Activation

As discussed above, Bax is a key mediator of apoptosis induced through the mitochondrial pathway on which both extrinsic and intrinsic signals converge. On apoptotic stimulation, Bax forms oligomers and translocates from the cytosol to the mitochondrial outer membrane and increases the membrane's permeability, which leads to the release of apoptogenic factors, such as cytochrome-*c*. Active Bax can be distinguished from the inactive cytosolic form using conformation-specific antibodies that bind to the N-terminal domain of the protein. The monoclonal antibodies described here were originally raised in Dr. R. J. Youle's laboratory ***(28)*** and are available from commercial sources (e.g., www.labvision.com). Polyclonal antibodies that recognize the exposed N-terminus are also available (www.scbt.com). Note that the same immunostaining protocol described here for Bax can be used to examine cytochrome-*c* release from mitochondria, where cytochrome-*c* displays a punctate staining pattern when confined to mitochondria, but a diffuse cytoplasmic staining pattern when it is released into the cytoplasm ***(5)***. *See* **Fig. 3** for example of cytochrome-*c* and active Bax staining.

1. Treat cells with proapoptotic drugs in the presence of 50–75 μ*M* BAF to prevent caspase-dependent execution of cell death as this may cause the cells to dislodge from the plate. BAF does not prevent Bax translocation (but *see* **Note 4** for an important exception with regard to TNF-α). Fix cells in 3% paraformaldehyde in PBS for 15 min at room temperature.

Fig. 1. **(A)** Immunoblot for LC3-II and poly-ADP-ribose polymerase (PARP) from HeLa cells treated with 1 μ*M* staurosporine (Sts) for 15 h in the absence or presence of 20 n*M* bafilomycin A1 (Baf A1). Postnuclear extracts were separated on a 10% (PARP, tubulin) or a 15% (LC3) gel. Immunoblots were probed for PARP (here we used a monoclonal antibody from Drs. Said Aoufouchi and Sidney Shall but have also successfully used PARP Antibody #9542; www.cellsignaling.com). LC3 was probed with an antibody raised by Dr. E. Kominami, but we have also successfully used the monoclonal antibody 5F10 from www.nanotools.de. α-Tubulin was probed with T5168 from www.sigmaaldrich.com. Note that STS induces an increase in LC3-II whose intensity increases further in the presence of Baf A1. The intensity of LC3-I also increases possibly because some LC3-II that cannot be consumed by the lysosomes is regenerated into LC3-I (note that Baf A1 is slightly toxic to the cells when incubated for long times). **(B)** Images of Cos7 cells transfected with GFP-LC3, treated as indicated in the figure. were captured using a PerkinElmer™ UltraView system ***(6)***. **(C)** Autophagy induced by apoptotic stimuli compared with proautophagic stimuli in HeLa, Cos7, and MCF7 cells after transfection with GFP-LC3.

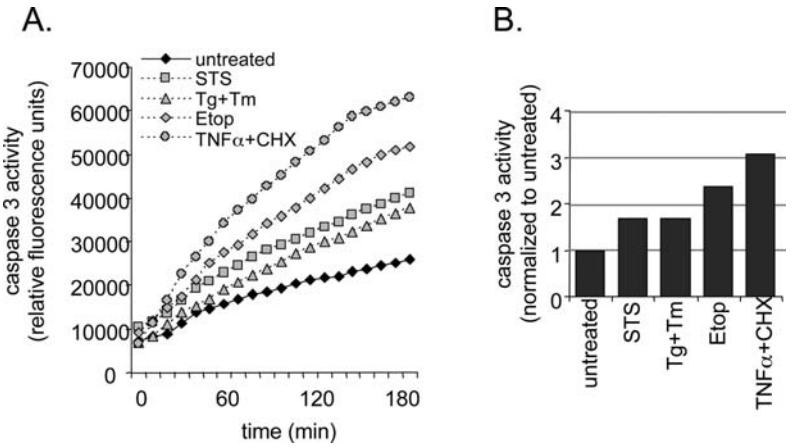


Fig. 2. Caspase 3 activity measured in HeLa cells treated with the indicated proapoptotic factors using DEVD-AMC and a fluorescent microplate reader. **(A)** Raw traces. **(B)** Rates calculated from the early portion of the slopes in (A).

2. Wash cells twice with PBS and permeabilize the cells in PBS containing 0.1% CHAPS (*see* **Note 5**).
3. Dilute the anti-Bax antibody (0.4 µg/mL) in PBS-CHAPS, add to the cells, and incubate for 1 h at room temperature. Note that the monoclonal antibodies described in **ref. *28*** are species specific.
4. Wash in PBS four times (we dip the cover slips sequentially in four pots of PBS) and incubate with the appropriate secondary antibody conjugated to a fluorescent reporter (e.g., anti-mouse FITC).
5. Wash the cells and stain nuclei with Hoechst 33342 (1–5 µg/mL) added for 5 min at room temperature.
6. Dip cover slips in water and mount face down onto slides using an appropriate mounting medium such as Fluoromount-G (a water-soluble permanent mounting medium that suppresses photobleaching, www.southernbiotech.com) and analyze by fluorescence (or even better, by confocal) microscopy.
7. Count a minimum of 100 cells that display Bax translocation from a few separate visual fields, typically revealed by intense punctate staining (*see* **Note 6** for method that confirms co-localization of Bax and mitochondria). Determine the percentage of cells displaying active Bax.

3.2.5. Detection of a Subdiploid Population by Fluorescence-Activated Cell Sorting

Although detection of apoptotic cells by method in **Subheading 3.2.1** is fast after some practice, counting cells under the microscope is painstaking, which can be avoided using fluorescence-activated cell sorting (FACS). One

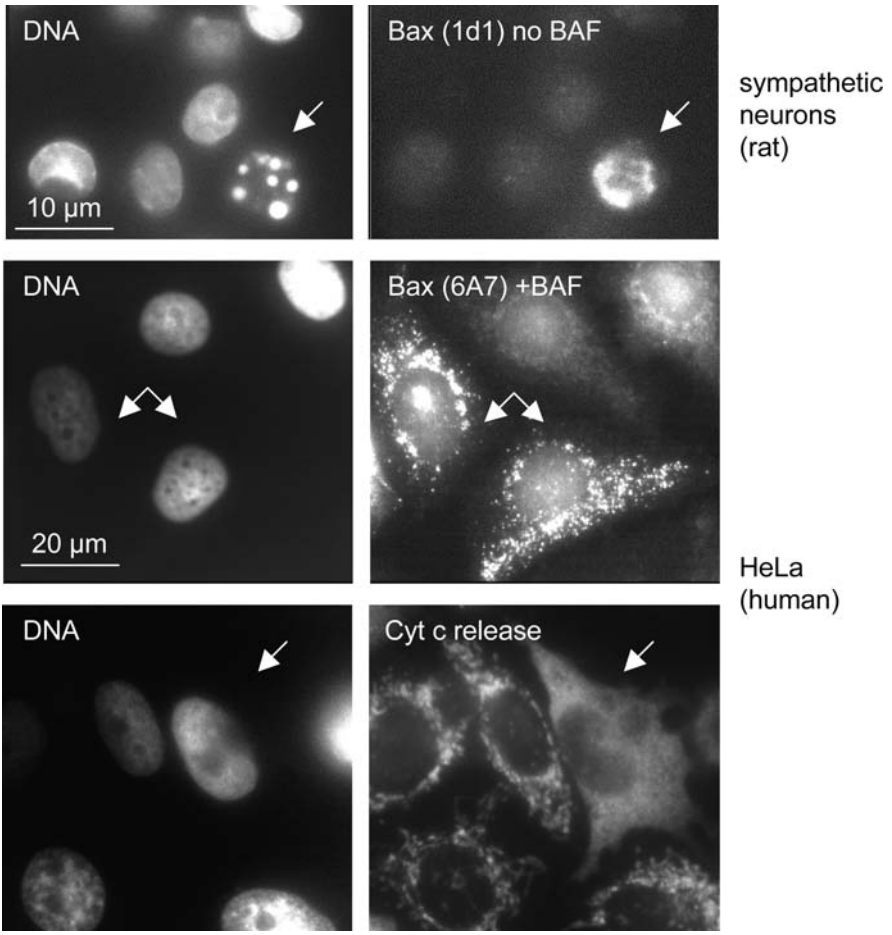


Fig. 3. Cells stained for active Bax and cytochrome-*c* release. Top row shows cultured rat sympathetic neurons from which nerve growth factor (NGF) has been withdrawn for 15 h. Cells were stained with rat-specific anti-Bax monoclonal antibody 1D1 followed by anti-mouse Alexa488. DNA was stained with Hoechst 33342. Note that the cell undergoing DNA fragmentation (arrow) is the only Bax-positive cell. Middle row shows HeLa cells treated with etoposide in the presence of BAF for 21 h. Antiactive Bax was probed with monoclonal antibody 6A7 followed by anti-mouse Cy3. Note that the cells indicated express active Bax although the nuclei have not fragmented due to the presence of BAF. Bottom row shows the same set of HeLa cells probed with anti-cytochrome-*c* (cyt-*c*). The cell indicated shows diffuse cytoplasmic staining for cytochrome-*c*, whereas the adjacent cells show cyt-*c* localized to mitochondria.

of the characteristics of apoptosis is the degradation of DNA due to the activation of several endonucleases, some of which are activated by caspases. Fixation of cells with precipitating fixatives (such as ethanol) causes the leakage of the cleaved low-molecular-weight DNA fragments that are produced during apoptosis. As a consequence, apoptotic cells can be identified as a hypodiploid peak (sub-G1 peak), whereas healthy cells generate a typical cell cycle histogram (without sub-G1 peak). *See* **ref.** ***(30)*** for detailed information on using FACS to analyze apoptosis.

1. After induction of apoptosis, harvest cells in the appropriate manner (trypsinize adherent cells) and prepare a single-cell suspension in PBS.
2. Wash cells twice with PBS (centrifuge 380×g for 5 min) and resuspend at 1×10^6 cells in 200 µL.
3. Fix cells in cold 70% ethanol (2 mL) by adding it dropwise to the cell pellet while vortexing. This should ensure fixation of all cells and minimize clumping.
4. Fix cells for at least 30 min at 4°C. Specimens can be left at this stage at –20ºC for several weeks.
5. Wash cells twice with PBS. Spin at 380×g and be careful to avoid cell loss while discarding supernatant especially after spinning out of ethanol (it may be necessary to centrifuge cells at a slightly higher "*g*" to pellet after ethanol fixation as the cells become flocculent).
6. Resuspend pellet in 1 mL staining solution containing 50 µg/mL PI and 100 µg/mL RNase A. RNase A should be boiled for 10 min to inactivate any contaminating DNase. RNase A is used to eliminate all free RNA, so that PI will only stain cellular DNA (*see* **Note 7** for the often used complementary method of Annexin V staining).
7. Incubate for 30 min at room temperature.
8. If cells are clumped, pass them through a 25-gauge needle using a 1-mL syringe.
9. Store samples at 4ºC until analyzed by flow cytometry (within 24 h).
10. Collect a minimum of 20,000 events per datafile.

3.3. Analysis of Autophagy

The term macroautophagy comprises two processes: (1) formation of autophagosomes and the transport of vesicles containing cargo and (2) lysosomal degradation of the cargo after fusion of the autophagosomes with endo/lysosomes (also called amphisomes or autolysosomes; *see* **refs.** ***31*** and ***(32)***). As with apoptosis, which is regulated at several levels (second-messenger signaling, mitochondrial events, postmitochondrial events, etc.), one must distinguish between the different phases of autophagy-execution while assessing the interaction between apoptosis and autophagy. To this end, it is important to recognize the molecular principles of each assay. Here, we restrict ourselves to the analysis of microtubule-associated protein light-chain-3

(MAP-LC3) commonly named LC3. During the formation of autophagosomes, LC3 is proteolytically cleaved and lipidated. This LC3–phospholipid conjugate (LC3-II) is localized on autophagosomes and autolysosomes (prior to LC3-II digestion by lysosomal enzymes or removal from the membrane due to cleavage of the conjugated lipid; *see* **ref.** ***(33)***), forming numerous small puncta ***(34)***. We describe measurement of LC3-II formation by techniques similar to those we have described for apoptosis. It should be noted, however, that despite the advent of molecular markers for autophagy, demonstration of autophagosome formation and maturation by electron microscopy is still an indispensable technique, for example as practiced in **ref.** ***(36)***. There are also important pitfalls in the analysis of autophagy: in a system in which productive macroautophagy is running at a high pace, there may be even lower steady state levels of LC3-II than in unstimulated cells and this might lead to the false conclusion that cells are hardly undergoing autophagy. Hence, as pointed out by Tanida et al. ***(33)***, the true measurement of autophagic flux requires a control where lysosomal degradation of LC3-II is prevented. We use Baf A1 but cysteine and asparate protease inhibitors E64d and pepstatin are also efficacious ***(27)***. *See* also **Subheading 3.4.** for discussion regarding use of inhibitors of autophagy that impact on apoptosis and vice versa.

3.3.1. Quantification of Autophagosomes by Detection of GFP-LC3 Punctation

To determine whether apoptotic stimuli activate autophagosome formation, cells can be transfected with GFP-tagged LC3 plasmid and the change in the distribution of GFP-LC3 from a diffuse cytoplasmic pattern to a punctate pattern after induction of apoptosis can be documented. The mammalian expression plasmid for GFP-LC3 is available through the Addgene plasmid collection (www.addgene.org). As a positive control for autophagic stimulation, cells can be treated with vinblastine ***(31,35)***, starved of amino acids, or treated with ammonium chloride ***(6)***.

1. Transfect cells (grown on glass cover slips) with GFP-LC3 using appropriate reagents.
2. After 24 h, treat the cells with an apoptotic stimulus. Stain the nuclei with Hoechst 33342 and analyze GFP fluorescence by microscopy. To prevent the loss of cells due to execution of cell death, treat cells in the presence of a pan-caspase inhibitor, such as 50 μ*M* BAF.
3. Treat a cohort of cells with 20 n*M* Baf A1 to examine the net flux of LC3-II through the autophagic pathway.
4. Prepare a positive control for autophagy induction (GFP-LC3 punctation) by treating cells with 50 μ*M* vinblastine or 20 m*M* ammonium chloride (1–2 h), or

starve cells by washing them five times with EBSS or HBSS and incubating them in this medium for 1–2 h.
5. Determine the percentage of GFP-LC3-positive cells that contain GFP-LC3 puncta (*see* **Fig. 3**), counting at least 100 cells per sample.
6. Alternatively, stain nuclei with Hoechst 33342 and assess the number of autophagosomes per cell, counting GFP-LC3-positive puncta per nucleus. In cells that have high basal autophagy, this technique may be difficult.

3.3.2. Quantification of LC3-II by Immunoblotting

Lipidated LC3-II migrate more rapidly (~16 kDa) than LC3-I (~18 kDa) when proteins are separated by SDS–PAGE. Hence, the amount of LC3-II can be used to indicate autophagic activity with the proviso that a parallel experiment is conducted in the presence of lysosomal inhibitors that prevent LC3-II degradation to measure the net extent of autophagic induction ***(27)***.

1. After treatment with apoptotic inducers, isolate cell proteins and normalize loading as described in **Subheading 3.2.2**.
2. Resolve proteins on a 15% denaturing gel (SDS–PAGE) and blot.
3. After blocking with 5 % nonfat milk, probe membranes with anti-LC3. A commercially available monoclonal anti-LC3, which we find very clean, is available from www.nanotools.de.
4. Follow the procedures outlined in **Subheading 3.2.2., steps 12–16**.

3.4. Use of Inhibitors to Examine the Relationship Between Apoptosis and Autophagy

A standard way to address whether autophagy contributes to apoptotic regulation or signaling (e.g., **refs.** ***(37–42)***) is to inhibit autophagy with chemical inhibitors. 3-Methyl adenine (3-MA) is widely used as a specific inhibitor of autophagy ***(43)***, although the authors used the word "specific" to indicate only that it did not inhibit other ATP-dependent processes. 3-MA is a low-affinity PI3-kinase inhibitor that inhibits the class III PI3-kinase Vsp34, which partners Beclin1 and initiates autophagy ***(44,45)***. In some reports, other PI3-kinase inhibitors such as wortmannin or LY294002 are used for the same reason. However, our laboratory has demonstrated that 3-MA exhibits—at concentrations used to suppress autophagy—profound inhibitory effects on stress-activated MAPK kinases JNK and p38 that are in many cases key regulators of apoptosis ***(5)***. Indeed, 3-MA alters many metabolic processes in cells ***(46)***. Moreover, LY294002 and wortmannin are also widely used and potent inhibitors of class I PI3-kinases, which activate an antiapoptotic signaling branch mediated by Akt in many cell types but also inhibit autophagy ***(47)***. Therefore, in studies addressing the interplay of apoptosis and autophagy,

one cannot pay enough attention to the molecular targets of chemical inhibitors whose use otherwise could undermine the purpose of the study. For this reason, it is highly advisable to back up experiments by using a variety of independent inhibitors, or, if possible, to knockdown key regulators in the pathways of autophagy or apoptosis using highly specific RNAi constructs.

4. Notes

1. Glass cover slips should be grease free. Using 100–200 cover slips at a time, we soak cover slips in nitric acid for a few hours in the fume cupboard while slowly rotating on a shaker, then wash them in water to remove all traces of the acid, followed by one wash in methanol (so that they do not stick to each other during drying) and oven-bake in a glass Petri dish at 250ºC overnight.
2. Some cells adhere better to poly-lysine-coated cover slips. Prepare stocks of 1 mg/mL poly-D-lysine in water (e.g., P-0899, Sigma-Aldrich). Keep frozen at −20ºC. For coating cover slips, prepare a 10 μg/mL solution in 0.1 *M* sodium borate, pH 8.4, immerse the cover slips, and coat from 1 h to overnight at room temperature. Wash three times with water. Air-dry in a sterile cabinet and store at 4ºC.
3. For electron microscopy, it is best to prepare a plastic cover slip made of polychlorotrifluoroethylene such as Aclar® which is consistent with embedding chemicals (www.proscitech.com.au/catalogue/g2.asp). Light microscopy on these cover slips is of low quality.
4. When using TNF-α, apoptotic signaling begins by activating caspase 8, and thus, BAF will inhibit the apoptotic signaling from reaching the stage of Bax activation. BAF can be omitted as long as cells are fixed early enough before execution of apoptosis, resulting in blebbing and/or detaching of the cells from the plate. As cell types vary remarkably in the rate of progression through the different stages of the apoptotic program, we recommend to include BAF when possible so that the cells keep beyond the mitochondrial stage of apoptosis.
5. CHAPS in used in lieu of Triton X-100 because Triton—but not CHAPS—can cause Bax to display the N-terminus associated with its activation independently of an apoptotic stimulus ***(29)***. Although the cells are fixed, it is best to be safe while performing this assay.
6. It is possible to counterstain mitochondria prior to initiation of apoptosis using a mitochondrial dye that resists paraformaldehyde fixation such as MitoTracker Orange (MTO) (CM-H2TMRos [M7511], www.invitrogen.com). Load cells by adding 0.3–1 μ*M* final concentration of MTO into the growth medium. Incubate for 15–30 min in the incubator. Wash the medium and commence apoptosis by using the appropriate treatment. Note that while using MTO, the secondary antibody used for detection of Bax should not be coupled to TRITC or Cy3. Alternatively, cells can be transfected with a plasmid expressing a mitochondrial-targeted fluorescent protein (available through www.clontech.com) to visualize mitochondria.

7. Another established marker for apoptosis is PS translocation from the inner leaflet of the plasma membrane to the outer leaflet, which can occur in both caspase-dependent and caspase-independent manners. Externalized PS can be visualized with fluorophore-conjugated Annexin V. Apoptotic cells are those that are Annexin V positive and PI negative ***(25)***. When Annexin V is applied to cells, cells must not be fixed or else Annexin V will enter the plasma membrane and stain PS inside the cell (PS is abundant in the inner leaflet of the plasma membrane). *See* **ref.** ***30*** for comprehensive description of analysis of apoptosis by FACS.

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