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Rapamycin pre-treatment protects against apoptosis

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Macroautophagy (generally referred to as autophagy) mediates the bulk degradation of cytoplasmic contents, including proteins and organelles, in lysosomes. Rapamycin, a lipophilic, macrolide antibiotic, induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR). We previously showed that rapamycin protects against mutant huntingtin-induced neurodegeneration in cell, fly and mouse models of Huntington's disease [Ravikumar, B., Duden, R. and Rubinsztein, D.C. (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.*, 11, 1107–1117, Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. *et al.* (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.*, 36, 585–595]. This protective effect of rapamycin was attributed to enhanced clearance of the mutant protein via autophagy [Ravikumar, B., Duden, R. and Rubinsztein, D.C. (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.*, 11, 1107–1117, Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. *et al.* (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.*, 36, 585–595]. Here, we show that rapamycin may have additional cytoprotective effects—it protects cells against a range of subsequent pro-apoptotic insults and reduces paraquat toxicity in *Drosophila*. This protection can be accounted for by enhanced clearance of mitochondria by autophagy, thereby reducing cytosolic cytochrome *c* release and downstream caspase activation after pro-apoptotic insults. Thus, rapamycin (pro-autophagic) treatment may be useful in certain disease conditions (including various neurodegenerative diseases) where a slow but increased rate of apoptosis is evident, even if they are not associated with overt aggregate formation.

INTRODUCTION

The bulk degradation of cytoplasmic proteins or organelles by lysosomes is largely mediated by macroautophagy, generally referred to as autophagy. Autophagy involves the formation of a double membrane structure around a portion of cytoplasm to form an autophagosome/autophagic vacuole, which then fuses with a lysosome where its contents are degraded (1). Several genes/proteins that regulate mammalian autophagy have recently been identified. Mammalian target of rapamycin (mTOR) is a phosphatidyl inositol kinase-related kinase that negatively regulates autophagy (2). Rapamycin, a lipophilic, macrolide antibiotic, induces autophagy by inactivating mTOR (3). We have previously shown that rapamycin can protect against mutant huntingtin-induced degeneration in cell, fly and mouse models of Huntington's disease (4,5). This protection was attributed to enhanced autophagic clearance of mutant huntingtin fragments by rapamycin. Subsequently, we showed that induction of autophagy by rapamycin enhances the clearance of a wide range of aggregate-prone proteins and reduces their toxicity (4,6,7).

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Increased numbers of autophagic vacuoles are also seen in a variety of physiological and pathological states in the nervous system. However, in many cases, it is controversial if this phenomenon is the result of increased autophagic activity or decreased autophagosome–lysosome fusion. Although autophagy has been associated with both apoptotic and non-apoptotic cell death, the functional significance of autophagy and its exact relationship to cell-death pathways are poorly understood (reviewed in 8). Many recent studies have claimed that autophagy enhances cell death, whereas many others argued the converse. For instance, apoptotic signalling is activated in *Lurcher* mouse Purkinje cells, and recent studies show upregulated autophagy in these dying cells (9). In contrast, inhibition of autophagy using 3-methyl adenine (3MA) seems to increase sensitivity to pro-apoptotic insults (10). Others have subsequently shown that inhibition of autophagy enhances susceptibility to cell death induced by a variety of means (11). Although such experiments with autophagy inhibitors have led some to conclude that autophagy is protective, this interpretation is not conclusive because basal levels of mammalian autophagy may have important normal functions such as nutrient recycling. Indeed, constitutive and conditional knockouts (12) of autophagy genes are deleterious (12,13). To be able to claim that autophagy is protective against cell death, one needs to show reduced susceptibility to toxic agents in the presence of upregulated autophagy. This has not been reported previously either in cell lines or *in vivo*. We believe this is a key experimental approach that can help clarify certain aspects of the controversial and poorly understood relationships of autophagy and cell death (8).

RESULTS

Pre-treatment with rapamycin protects cells from pro-apoptotic insults

We previously showed that rapamycin protects against toxicity caused by a wide range of aggregate-prone proteins, including those causing Huntington's disease and taupathies (4,6,7). This protective effect was mediated by enhanced clearance of the mutant protein via the autophagic pathway. To test whether rapamycin had other protective effects independent of the clearance of aggregate-prone proteins, we began by pre-treating COS-7 cells with rapamycin or 3MA for 48 h, followed by induction of apoptosis with staurosporine (STA) for 4 h. Cells pre-treated with rapamycin showed reduced susceptibility to STA treatment when compared with control cells (Fig. 1A and B). We tested the above mentioned paradigm in four different cell lines (PC12, NRK, COS-7 and CSM-14) with three different apoptotic insults [STA, GFP-Bax and 3-NP (which has been used in toxin models that have some pathological abnormalities shared with Huntington's disease)] (14–16) and obtained similar results (data not shown; Supplementary Material, Fig. S1A–C). The protective effect of rapamycin appears to be mTOR-dependent, as we observed no effect with FK-506, which binds the same immunophilin (FKBP12) as rapamycin, but does not inhibit mTOR (Supplementary Material, Fig. S1D). However, this protection by rapamycin was reversed when the cells were simultaneously pre-treated with 3MA + rapamycin (Fig. 1B), suggesting that the anti-apoptotic effects of rapamycin pre-treatment are blocked when autophagy is inhibited.

To further investigate whether the anti-apoptotic effects of rapamycin were autophagy-dependent, we used genetic enhancers and suppressors of autophagy. Beclin-1 is the mammalian homolog of yeast Atg6p/Vps30p, and overexpression of beclin-1 induces autophagy in yeast and mammalian cells (17). A highly conserved region spanning amino acids 244–337 of human beclin-1 is important for its binding to the class-III PI-3-kinase Vps34 and for the induction of autophagy. Induction of autophagy in cells by overexpression of wild-type beclin-1 (18) also protected against STA-induced cell death (Fig. 1C). No protective effect was seen when we transfected cells with a construct encoding beclin-1 with residues 244–337 deleted (Δ244–337 beclin-1), which does not induce autophagy (18) (Fig. 1C). The protective effect of wild-type beclin-1 was abrogated when autophagy was blocked with 3MA (Fig. 1C). Furthermore, knockdown of the essential autophagy gene *Atg7* by RNAi enhanced cellular susceptibility to STA (Fig. 1D). We obtained identical results with *Atg12* (another essential autophagy gene) RNAi (data not shown). Thus, rapamycin pre-treatment protects against pro-apoptotic insults, and this effect is probably mediated via induction of autophagy.

Induction of autophagy protects against a subsequent toxic insult in *Drosophila*

We then used *Drosophila* to test whether rapamycin pre-treatment protected against cell death *in vivo*. Consistent with data obtained with cell models, wild-type flies pre-treated with rapamycin showed an increased survival rate after paraquat exposure (Fig. 2A). Paraquat is an MPTP-like chemical that causes apoptosis by production of free oxygen radicals via the mitochondrial pathway (19) and is frequently used for modelling Parkinson's disease (20). However, this protective effect of rapamycin was attenuated in flies with heterozygous loss-of-function of the essential autophagy gene *Atg1* ($Atg1^{\Delta 3D}$) (21) (Fig. 2B), confirming *in vivo*, as we had shown in cells, that the protective effects of rapamycin require autophagy and are accounted for largely or entirely by autophagy. In the absence of rapamycin pre-treatment, we did not see any difference in the survival after paraquat treatment between wild-type flies and flies with heterozygous loss-of-function of *Atg1* (Fig. 2B). These data are consistent with other findings (7) that suggest that hemizygous loss of Atg1 is not obviously deleterious under basal conditions but becomes limiting when autophagy is induced. Note that wild-type genotype survival is set to 1 in both +RAP and −RAP conditions in Figure 2B to clarify the effects of ATG1 loss-of-function. The effect of rapamycin on survival of wild-type flies after paraquat treatment is shown in Figure 2A.

Enhanced autophagy reduces mitochondrial load

A possible mechanism for these protective effects of rapamycin may be related to the fact that autophagy is the only known route for clearance of intact mitochondria (22,23). In general, the major form of apoptosis seen in vertebrate cells proceeds

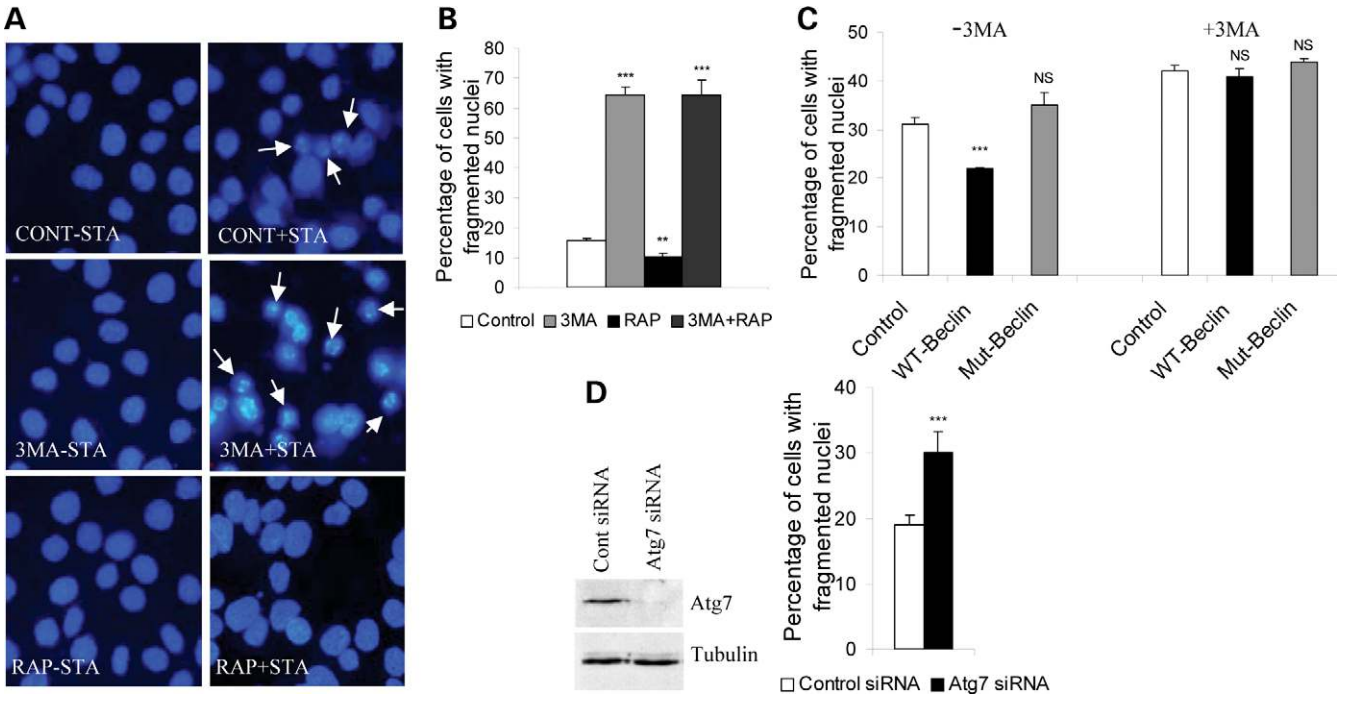

Figure 1. Pre-treatment with rapamycin protects against subsequent pro-apoptotic insults. (**A** and **B**) COS-7 pre-treated with rapamycin (R) or 3MA for 48 h or left untreated (Cont) were subsequently treated with (+) or without (−) 1 μM STA for 4 h. The cells were then fixed and stained with DAPI. Representative images of nuclei of cells (A) and percentages of cells with fragmented nuclei upon STA treatment (B) are shown (one representative data from three independent experiments are shown in the graph). (**C**) COS-7 cells were cotransfected for 48 h with either pcDNA3, wild-type beclin-1 or Δ244–337 beclin-1 and pEGFP in 3:1 ratio in the presence (+) or absence (−) of 3MA. The cells were then treated with STA as mentioned earlier and scored for cell death. Amino acids 244–337 of beclin-1 are required for autophagy and its binding to Vps34 but are not essential for vacuolar protein sorting and cathepsin D maturation. (**D**) HeLa cells were transfected for 72 h with siRNA against *Atg7* or a control siRNA, after which they were treated with STA as mentioned earlier and scored for nuclear fragmentation. The level of Atg7 after transfection with *Atg7* siRNA is also shown.

through the mitochondrial pathway (24). Mitochondrial outer membrane permeabilization leads to the release of pro-apoptotic molecules like cytochrome *c* and apoptosis-inducing factor (AIF), which are normally found in the mitochondrial intermembrane space. Cytochrome *c* release into the cytosol leads to caspase-9 cleavage and activation, which in turn cleaves/activates the executor caspase, caspase-3 (24). The cell death paradigms we have used earlier have a high dependency on this pathway (14–16,19). Autophagy is the only known route for clearance of intact mitochondria. Mitochondria are frequently seen in autophagosomes, and mitochondrial mass increases when autophagy is inhibited (22,23). Thus, we hypothesized that pre-treatment with autophagy-inducing agents like rapamycin would decrease mitochondrial load. This would, in turn, reduce the levels of cytochrome *c* released into the cytosol after mitochondrial outer membrane permeabilization stimulated by pro-apoptotic agents. Such effects would be predicted to be protective because other approaches that reduce cytochrome *c* release or cytochrome *c* knockouts reduce caspase activation and apoptosis in response to such agents (24–26). To test whether enhanced autophagy can reduce mitochondrial load, we pre-treated PC12 cells with rapamycin or 3MA for 72 and 48 h, respectively. Rapamycin decreased the levels of the mitochondrial proteins cytochrome *c*, AIF and complex-IV (Fig. 3A–C) in total cell lysates, consistent with a reduction in mitochondrial number. The converse was observed in cells treated with 3MA (Fig. 3A–C) or upon transfection with siRNA for Atg7 (Fig. 3D). Similarly, we found decreased levels of other mitochondrial proteins like porin and complex-I in cells treated with rapamycin (Supplementary Material, Fig. S1E). (We have measured levels of specific mitochondrial proteins to assess mitochondrial load. This is preferable to measures of mitochondrial number, which are confounded by fission and fusion events that, in the absence of changes in load, would not necessarily affect the amounts of proteins, such as cytochrome *c*, that are potentially releasable after pro-apoptotic insults.)

Reduced cytochrome *c* release and decreased activated caspase 3 and 9 after pre-treatment with rapamycin

We next examined whether the reduced mitochondrial load attenuated mitochondrial-dependent activation of the apoptotic cascade after a pro-apoptotic stimulus. Rapamycin pre-treatment resulted in lower levels of cytochrome *c* release into the cytosol after subsequent treatment of cells with the pro-apoptotic agent STA (Fig. 4A). Rapamycin pre-treatment also reduced the levels of cleaved (activated) caspase-9 and caspase-3 (Fig. 4B and C) in cells treated with rapamycin +STA. Conversely, when autophagy was inhibited by pre-treating cells with 3MA, we observed increased levels of cytochrome *c* release and activated caspase-9 and caspase-3 when they were exposed to STA (Fig. 4A–C). If rapamycin is mediating its effects via autophagy clearing mitochondria,

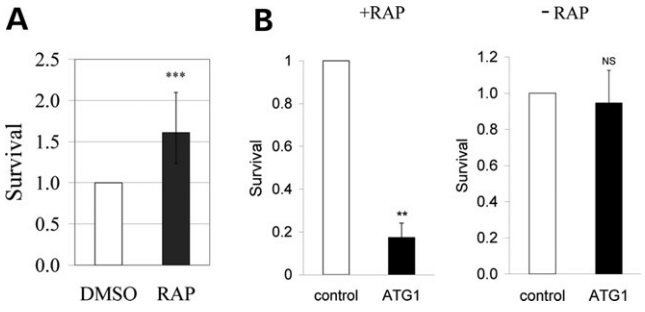

Figure 2. Induction of autophagy protects against cell death in *Drosophila.* (**A**) Flies reared on rapamycin or carrier vehicle (DMSO) as control were treated after eclosion with paraquat (15 mM for 3 days) and the number of flies that were alive was scored as shown in the graph. Typically, paraquat kills around 40% of wild-type flies, whereas no lethality is observed without paraquat treatment. We tested separate vials of 100 wild-type flies each for rapamycin or DMSO for each sex (five vials for each sex for each condition; total 1000 flies). Data are shown for females; similar significant effects were seen with males. The proportion of flies that survived in the control conditions was set to 1. (**B**) Similar experiments as in (A) were performed in wild-type or heterozygous $Atg1^{\Delta 3D}$ mutant flies reared either with (+RAP) or without (−RAP) rapamycin and treated with paraquat as in (A). The number of flies that survived was scored as depicted in the graph (+RAP: four vials for each condition, with a total of 700 flies; −RAP: 10 vials for each condition, with a total of 2000 flies). Note that it was not feasible to do all of these pairwise comparisons at the same time because of the large numbers of flies involved, co-ordination of the timing of eclosion of all flies and the time needed for scoring. As experiments in (A) and (B) were performed at different times and similar but not identical conditions, the fold changes observed cannot be expected to be identical. Note that wild-type genotype survival is set to 1 in both +RAP and −RAP conditions in (B) to clarify the effects of ATG1 loss-of-function. The effect of rapamycin on survival of wild-type flies after paraquat treatment are shown in (A). $^{***}P < 0.0001$, $^{**}P < 0.001$, $^{*}P < 0.01$ and NS indicates not significant ($P > 0.05$).

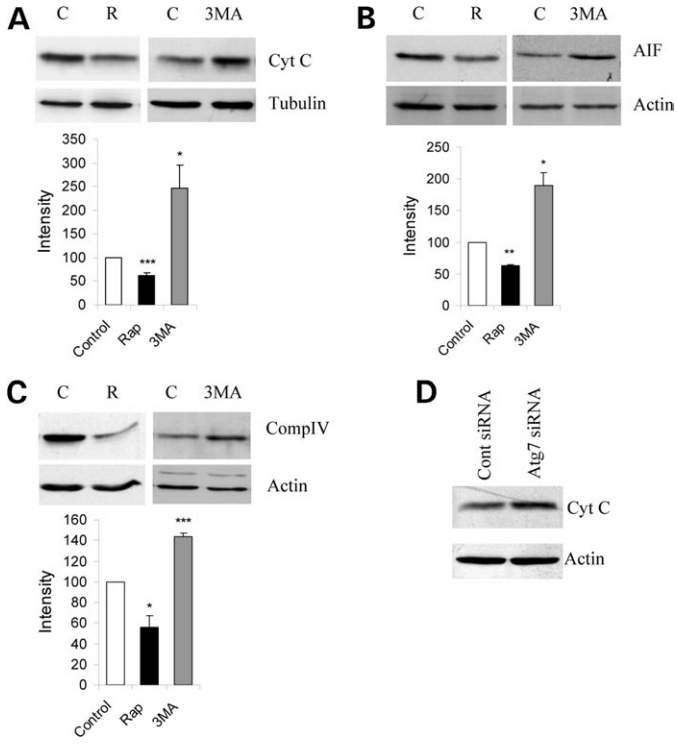

Figure 3. Enhanced autophagy can reduce mitochondrial load. (**A–C**) PC12 cells pre-treated with rapamycin (R) or 3MA for 72 and 48 h, respectively, or left untreated (C) were harvested and total lysates were subjected to western blot analysis using antibodies against cytochrome *c* (Cyt *C*) (A), anti-AIF (B) or anti-complex-IV (CompIV) (C). The blots were probed for tubulin/actin as a control. Quantification of band intensities from at least three independent experiments is shown. (**D**) Lysates from HeLa cells treated without STA were blotted for Cyt *C* and actin as control. Similar results were obtained with complex IV (data not shown).

we should only see protection in cells pre-treated for a relatively long period (at least 48 h) sufficient to allow some mitochondrial clearance. Cells treated with rapamycin for a short period (6 h) have unaltered cytochrome *c* levels, although there is a dramatic inhibition of mTOR signalling (Supplementary Material, Fig. S1F). Consistent with an autophagy mechanism, short-term rapamycin treatment (that impaired mTOR signalling) afforded no protection against a subsequent pro-apoptotic insult (Supplementary Material, Fig. S1F). Thus, the rapamycin pre-treatment effect on apoptosis is likely to be mediated via autophagy (consistent with the beclin-1 data) and not due to other signalling consequences of mTOR inhibition. If this protective effect of rapamycin is mediated predominantly by reducing mitochondrial load and the potential for cytochrome *c* release, then rapamycin should not further protect against pro-apoptotic insults in cells expressing E1B19K, a viral Bcl-2 homologue that blocks cytochrome *c* release. Consistent with this prediction, rapamycin pre-treatment did not further suppress apoptotic insults in cells expressing E1B-19K (Fig. 4D).

Effect of rapamycin against mitochondrial-independent apoptotic inducers

The apoptotic insults that we used in the above experiments induce cell death via the mitochondrial pathway. We wanted to test whether rapamycin could protect against apoptotic stimuli that are initiated independently or upstream of mitochondria, such as extrinsic death-receptor-mediated apoptosis (that involves activation of the initiator caspase, caspase-8) (27) and ER stress-induced apoptosis (28) (that may involve activation of caspase-12). We first pre-treated HeLa cells with rapamycin and subsequently treated them with either tunicamycin (to induce ER stress) (28) or TNFα (to induce death-receptor-mediated apoptosis) (29) for 48 and 5 h, respectively. Cells pre-treated with rapamycin showed decreased susceptibility to tunicamycin (Fig. 5A). However, recent studies have shown that activation of ER stress induces apoptosis in an apoptosome-dependent (and thus mitochondrial-dependent) manner and is independent of caspase-12 (30). Thus, apoptosis induced by ER stress seems to have a major dependence on mitochondria and thus can explain our data. HeLa cells pre-treated with rapamycin also showed reduced susceptibility to TNFα (Fig. 5B). There are two different categories of cells, namely type I and II, classified on whether or not they require mitochondria to die after the activation of the death-receptor-mediated apoptotic pathway (31). HeLa cells are type II cells that require mitochondria to die through the death-receptor-mediated pathway (31). Thus, our rapamycin data are consistent with a role for

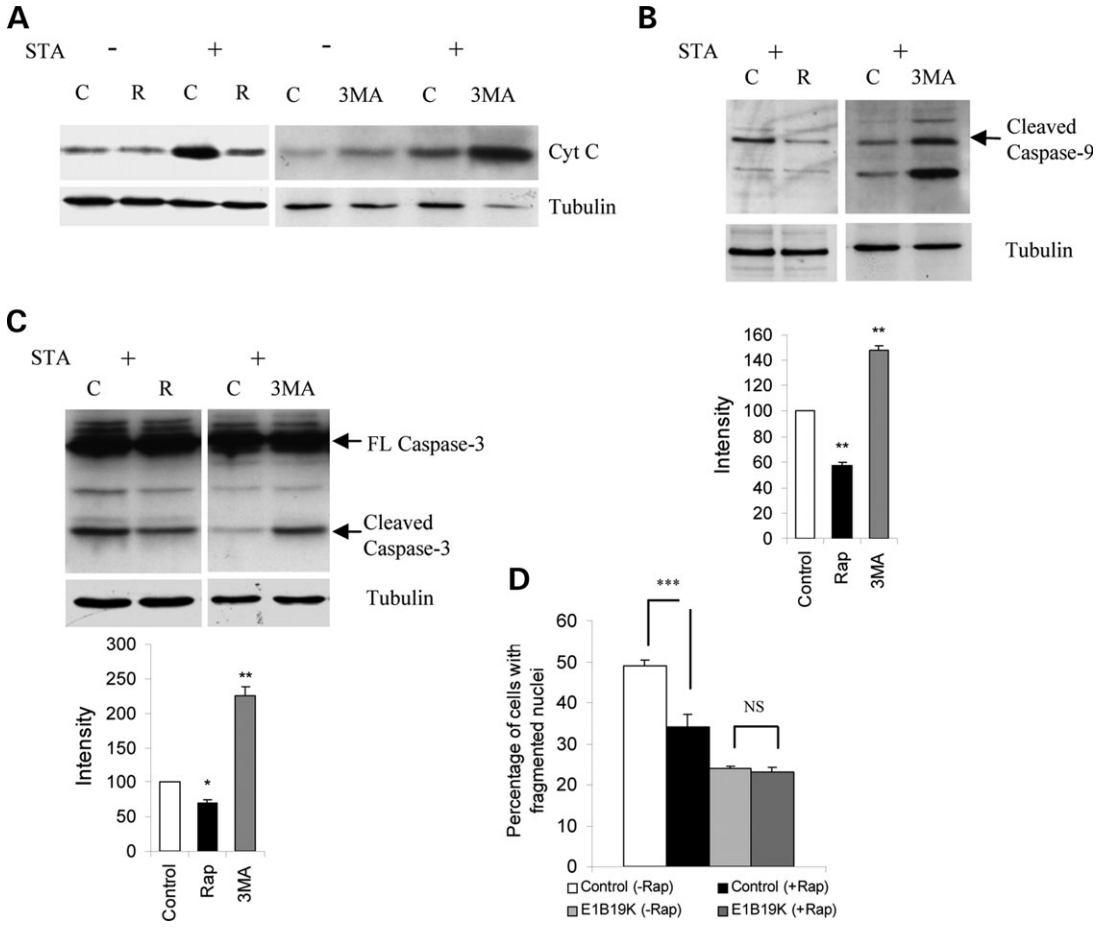

Figure 4. Induction of autophagy decreases mitochondrial-dependent activation of apoptotic cascade. (**A**–**C**) PC12 cells pre-treated with rapamycin (R) or 3MA as mentioned earlier or left untreated (**C**) were subsequently treated with (+) or without (−) 1 μM STA for 4 h. Cells were collected at the end of the 4 h period, and the cytosolic fractions (A) or total lysates (B and C) were isolated for western blot analysis using anti-cytochrome *c* antibody (Cyt *C*) (A), anti-cleaved caspase-9 (Asp353) antibody (B) or anti-caspase-3 antibody (C). Tubulin was used as a loading control. Quantification of the band intensities from three independent experiments is shown. Note that Figure 3A shows total cell lysate, whereas Figure 4A measures only the amounts of cytochrome *c* released into the cytosol of untreated cells. (**D**) COS-7 cells were pre-treated with (+Rap) or without (−Rap) rapamycin for 24 h, and subsequently they were transfected with either a control DNA or E1B19K and GFP (in 3:1 ratio) for 48 h with or without Rap treatment. The cells were then treated with STA for 4 h, and quantification of cells with fragmented nuclei is shown in the graph. $^{***}P < 0.0001$, $^{**}P < 0.001$, $^{*}P < 0.01$.

mitochondria for this apoptotic pathway in HeLa cells. In contrast, type I cells (like the T47D human breast carcinoma cell line) are completely independent of mitochondria for their death through the extrinsic pathway (31). T47D cells pre-treated with rapamycin were stimulated with anti-Fas antibody to induce Fas-ligand-mediated cell death through the death-receptor pathway (32). Rapamycin showed no protection in these cells (Fig. 5C), suggesting that the protective effects of rapamycin pre-treatment may be restricted to apoptotic pathways with a major mitochondrial dependency.

DISCUSSION

The current understanding of the relationship between autophagy and cell death is unclear and controversial. Although previous studies have shown that inhibition of autophagy enhances susceptibility to cell death, the enhanced cell death in response to toxic insults in cells with downregulated autophagy may be due to additive and even unrelated effects of pro-apoptotic agents and blockade of constitutive autophagy. For example, Lum *et al*. (33) postulated that basal levels of autophagy allow Bax/Bak-deficient cells to survive after growth factor withdrawal by maintaining ATP production from catabolism of intracellular nutrients. Also, the entire literature linking autophagy to apoptotic insults is based on data in transformed or genetically modified (e.g. Bax/Bak-deficient) cells (33).

We believe that our data with a diversity of chemical and genetic autophagy inducers and suppressors allow us to show for the first time that enhanced autophagy, under certain conditions, may have a beneficial role by protecting against subsequent pro-apoptotic insults, and we provide a plausible mechanism for this phenomenon. We cannot exclude the possibility that there may be additional non-mitochondrial pro-apoptotic proteins that are cleared by autophagy—unfortunately, we are not aware of an experimental approach that would allow us to differentiate between these

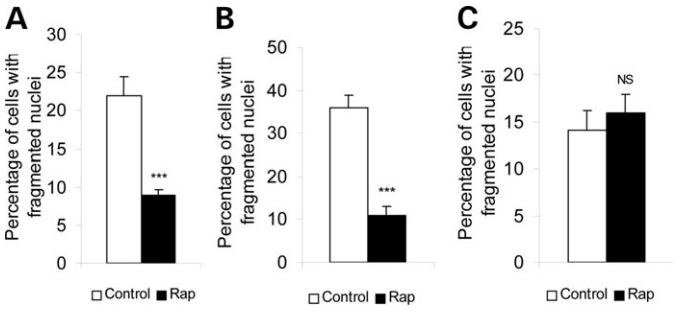

Figure 5. Effect of rapamycin on ER stress-mediated and death-receptor-mediated apoptosis. (**A**) HeLa cells were first pre-treated either with or without rapamycin for 48 h, followed by treatment with 1 μg/ml tunicamycin (with or without rapamycin) for the next 48 h. The cells were then fixed and the nuclei stained with DAPI. Percentages of cells with apoptotic nuclear morphology are shown in the graph. (**B**) HeLa cells treated with rapamycin as in (A) were subsequently treated with 20 ng/ml TNFα and 10 μg/ml cycloheximide for 4 h, after which they were fixed and scored for cells with apoptotic nuclei. (**C**) T47D cells were treated with rapamycin as in (A). For Fas stimulation, the cells were suspended in 1 ml media with serum to which 1 μg/ml of activated anti-Fas antibody was added and incubated at room temperature for 10 min. This was followed by the addition of 2 ml of media with serum, and the cells were plated back onto tissue culture plates and incubated at 37°C for a further 24 h (32). Rapamycin was included throughout this treatment period, where relevant. The cells were then fixed and apoptotic nuclei scored as mentioned earlier.

possibilities. However, we believe that the anti-apoptotic effects caused by preventing cytochrome *c* release by other strategies strongly argue that the reduction in mitochondrial load after autophagic induction is sufficient to explain the protective responses we have observed (24–26). Furthermore, the inability of rapamycin to further protect against apoptosis in cells overexpressing E1B19K that blocks cytochrome *c* release and the failure of rapamycin to protect against Fas-ligand-mediated apoptosis in a type I cell line both suggest that the protective effect of rapamycin is mitochondrial-dependent.

Our data suggest that rapamycin is reducing mitochondrial load to ∼50% (Fig. 3) while having a protective effect against subsequent pro-apoptotic insults. Steady-state levels of mitochondria are set when the absolute degradation rate equals the absolute synthetic rate. If absolute synthetic rates are fixed, and one induces autophagy, then one increases the fractional degradation rate (analogous to decreasing half-life). The cells will then adjust to a new steady state where the absolute degradation rate is maintained as it was previously. This can be explained as the mitochondrial pool decreases and the absolute degradation rate is a function of concentration as well as fractional degradation rate. In other words, the absolute degradation rate can stay the same in the presence of enhanced autophagy (fractional degradation rate) when the mitochondrial load (concentration) drops. So, increasing autophagy decreases the mitochondrial load to a lower steady-state level but does not result in eventual total depletion of mitochondria (unless it is much more dramatic than the conditions we used).

From our experiments, it is difficult to know exactly what reduction of mitochondrial load will be sufficient to translate into protection against subsequent pro-apoptotic insults. Effects may be seen even with fairly modest reductions in mitochondrial load, particularly in cell-death pathways where there is a major positive-feedback component involving mitochondria. Very large decreases in mitochondrial load may be associated with deleterious effects due to losses of oxidative phosphorylation. However, it should be noted that although such relationships are complex and tissue-dependent, the activities of some respiratory complexes can be reduced by 25–80% before affecting respiration or ATP synthesis in brain mitochondria, and the activity of rat liver complex III can be decreased by 45% before respiration is affected (34). Thus, it is likely that one will be able to reduce mitochondrial load to levels that have substantial protective effects against pro-apoptotic insults without affecting respiration. Indeed, it is worth noting that rapamycin (which acts in the brain and peripherally) is used chronically in humans. Pro-autophagic treatments may thus be useful in certain disease conditions, such as certain neurodegenerative diseases, where a slow but increased rate of apoptosis is evident. Our current data suggest that inducing autophagy may have two distinct beneficial effects in protein conformational diseases such as Huntington's disease. First, it can be beneficial by clearing the toxic mutant proteins (5). Secondly, enhanced autophagy attenuates apoptotic responses to various insults. In Huntington's disease and various other neurodegenerative conditions, such insults may include excitotoxicity and elevated levels of reactive oxygen species.

In conclusion, we show for the first time that enhancing autophagy can protect against subsequent pro-apoptotic insults. This is likely to occur, as induction of autophagy results in reduced levels of mitochondrial proteins (consistent with decreased mitochondrial load).

MATERIALS AND METHODS

Mammalian cell culture and transfection

PC12, NRK, COS-7, CSM-14, HeLa and T47D cells used for the experiments were cultured using standard protocols. The cells were pre-treated with 0.2 μg/ml rapamycin (LC Laboratories) or 10 mM 3MA (Sigma) for 72 and 48 h, respectively. Pro-apoptotic insults used were as follows: 1 μM STA (Sigma) for 4 h, 5 mM (low) 3-nitro-propionic acid (3-NP; Sigma) for 15 h or 50 mM (high) 3-NP for 6 h, transient transfection of GFP-Bax for 48 h, 1 μg/ml tunicamycin (Sigma) for 48 h, 20 ng/ml THFα and 10 μg/ml cycloheximide (Sigma) for 5 h and 1 μg/ml activated anti-Fas antibody (Upstate) for 24 h. Transfection was performed using lipofectamine reagent (Invitrogen). Nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI, 3 μg/ml, Sigma). Cells were considered dead if the DAPI-stained nuclei showed apoptotic morphology (fragmentation or pyknosis). Pyknotic nuclei are typically <50% diameter of normal nuclei and show obvious increased DAPI intensity. We have demonstrated that these criteria are specific for cell death, as they show a very high correlation with propidium iodide staining in live cells (35). Furthermore, these nuclear abnormalities are reversed with caspase inhibitors (36). Analysis was performed with the observer blinded to the identity of the slides, and all experiments reported in the figures were done in triplicate at least

twice. A total of 20 nmol of control or Apg7 siRNA was transfected using siPORT NeoFX transfection reagent (Ambion) for 72 h, according to manufacturer's instructions.

Antibodies

We used the following antibodies in the study: anti-cytochrome *c*, anti-cleaved caspase-9 (Asp353) and anti-caspase-3 (8G10) (Cell Signalling technology), anti-AIF (Chemicon), anti-complex-IV subunit IV 20E8 (Invitrogen), anti-complex-I 39 kDa subunit 20C11 (Invitrogen), anti-porin (Calbiochem), activated anti-Fas (Upstate) anti-tubulin (Sigma), anti-actin (Sigma) and anti-Apg7 (Rockland Immunochemicals). Western blotting was performed using standard protocols, and densitometry analysis was performed using Scion Image Beta 4.02 software.

Drosophila crosses and treatment

We used [w1118] flies isogenized for X chromosome and two major autosomes (37). Female flies [w1118] were crossed either to males of the same genotype or to males of genotype *+;ATG1[delta 3D]* (21). The flies were allowed to mate on the normal fly food for 2–3 days and then transferred onto food with 1 μM rapamycin (LC laboratories) or DMSO (carrier vehicle). Flies were allowed to eclose for up to 3 days (flies of similar age were used for the experiment), females and males were separated into vials containing 50 or 100 flies and multiple vials were used for each experiment (as detailed in text). Flies were starved for 6 h and then fed with 5% sucrose solution containing 15 mM paraquat for 3 days. The number of flies that were dead or alive was counted. All experiments were done at 25°C and 60–80% humidity.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. D.R. and B.R. are inventors and Z.B., C.V. and C.O'K. are contributors on a patent describing the use of rapamycin for the treatment of diseases caused by aggregate-prone proteins.

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