Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species

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Summary

Autophagy is a self-digestion process important for cell survival during starvation. It has also been described as a form of programmed cell death. Mitochondria are important regulators of autophagy-induced cell death and damaged mitochondria are often degraded by autophagosomes. Inhibition of the mitochondrial electron transport chain (mETC) induces cell death through generating reactive oxygen species (ROS). The role of mETC inhibitors in autophagy-induced cell death is unknown. Herein, we determined that inhibitors of complex I (rotenone) and complex II (TTFA) induce cell death and autophagy in the transformed cell line HEK 293, and in cancer cell lines U87 and HeLa. Blocking the expression of autophagic genes (beclin 1 and *ATG5*) by siRNAs or using the autophagy inhibitor 3-methyladenine (3-MA) decreased cell death that was induced by rotenone or TTFA. Rotenone and TTFA induce ROS production, and the ROS scavenger tiron decreased autophagy and cell death induced by rotenone and TTFA. Overexpression of manganese-superoxide dismutase (SOD2) in HeLa cells decreased autophagy and cell death induced by rotenone and TTFA. Furthermore, blocking *SOD2* expression by siRNA in HeLa cells increased ROS generation, autophagy and cell death induced by rotenone and TTFA. Rotenone- and TTFA-induced ROS generation was not affected by 3-MA, or by beclin 1 and *ATG5* siRNAs. By contrast, treatment of non-transformed primary mouse astrocytes with rotenone or TTFA failed to significantly increase levels of ROS or autophagy. These results indicate that targeting mETC complex I and II selectively induces autophagic cell death through a ROS-mediated mechanism.

Supplementary material available online at
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Key words: Electron transport chain, Autophagic cell death, Apoptosis, Reactive oxygen species

Introduction

Autophagy is a regulated lysosomal pathway involved in the degradation and recycling of long-lived proteins and organelles within cells (Baehrecke, 2005; Codogno and Meijer, 2005; Gozuacik and Kimchi, 2004; Guimarães and Linder, 2004; Levine and Yuan, 2005; Mariño and López-Otín, 2004). During autophagy, cytoplasmic constituents are sequestered into double-membraned 'autophagosomes', which then fuse with lysosomes to form autolysosomes, in which degradation occurs. Under conditions such as nutrient starvation, this process frees components that are essential for cell survival. Under certain conditions, autophagy can also promote cell death. Treatment with chemical agents such as arsenic trioxide (As_2O_3) (Kanzawa et al., 2003; Kanzawa et al., 2005), or overexpression of tumor-suppressor proteins such as the short mitochondrial form of $p19^{ARF}$ (Reef et al., 2006), initiate autophagy, leading to cell death. In addition, in systems in which apoptosis has been blocked by, for example, caspase inhibitors, autophagy-induced cell death has been achieved (Xu et al., 2006; Xue et al., 2001).

The mechanism of autophagy-induced cell death remains unclear, but it appears that mitochondria play a central role (Gozuacik and Kimchi, 2004). Autophagy often occurs when the mitochondria fail to maintain ATP levels, during starvation, for example (Levine and Yuan, 2005), or when the mitochondria are damaged (Elmore et al., 2001; Gozuacik and Kimchi, 2004). Furthermore, blocking mitochondria-mediated apoptosis through the elimination of mouse *Bax* and *Bak* (*BAK1*) expression promotes autophagy-induced cell death (Shimizu et al., 2004). The short mitochondrial form of $p19^{ARF}$ is required to be localized to the mitochondria to induce autophagy and cell death (Reef et al., 2006). Arsenic trioxide (As_2O_3) induces the mitochondrial localization of pro-cell-death BCL2 family member BNIP3, which contributes to As_2O_3-induced autophagic cell death (Kanzawa et al., 2005). Inhibition of the various mitochondrial electron-transport chain (mETC) complexes induces cell death (Albayrak et al., 2003; Wolvetang et al., 1994), but the role of autophagy in mETC-inhibitors-induced cell death is unknown.

Reactive oxygen species (ROS) are often generated following inhibition of the mETC (Li et al., 2003; Muller et al., 2004). ROS include free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2) (Pelicano et al., 2004). Excess ROS might detrimentally affect cellular functions and induce cell death. Cells produce antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase to prevent themselves from the damage by excess ROS (Pelicano et al., 2004). Blockage of caspase activation leads to degradation of catalase; this degradation is mediated by autophagy, indicating a role of autophagy in caspase-independent cell death (Yu et al., 2006). The role of ROS that is generated from the mETC in autophagy-induced cell death is unknown.

In this study, we demonstrated for the first time that complex-I inhibitor rotenone and complex-II inhibitor thenoyl trifluoroacetone (TTFA) can induce autophagy, contributing to cell death in transformed and cancer cell lines. This is mediated by ROS production. By contrast, in primary mouse astrocytes, rotenone and TTFA failed to induce autophagy. This suggests that the inhibition of mitochondrial complex I or complex II selectively induces autophagic cell death, mediated by ROS, in transformed and cancer cells.

Results

Rotenone and TTFA induce cell death in transformed and cancer cells

Human embryonic kidney cell line HEK 293 and glioma cell line U87 were incubated with the mETC-complex-I inhibitor rotenone (50 μM) or mETC-complex-II inhibitor TTFA (0.5 mM) for 0, 24, 48 and 72 hours, and cell death was measured by membrane permeabilization. Cell death was induced by rotenone and TTFA over a 72-hour time course in HEK 293 and U87 cells (Fig. 1). In HEK 293 cells, rotenone and TTFA induced 30% and 40% cell death, respectively, after 72 hours (Fig. 1A), whereas, in U87 cells, they induced 40% and 90% cell death, respectively (Fig. 1B). Rotenone and TTFA also induced a dose-dependent cell death in HEK 293 and U87 cells (data not shown).

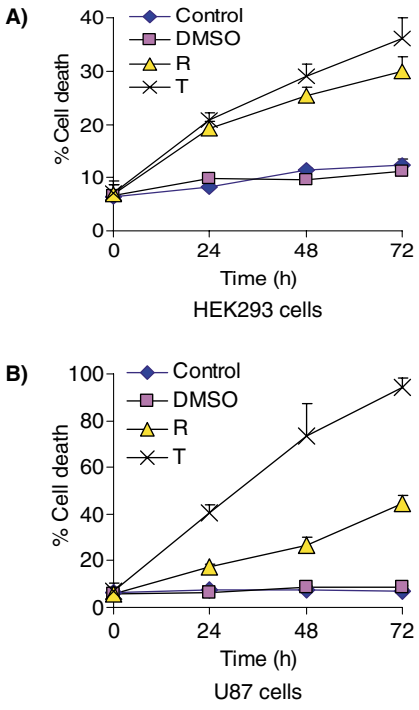
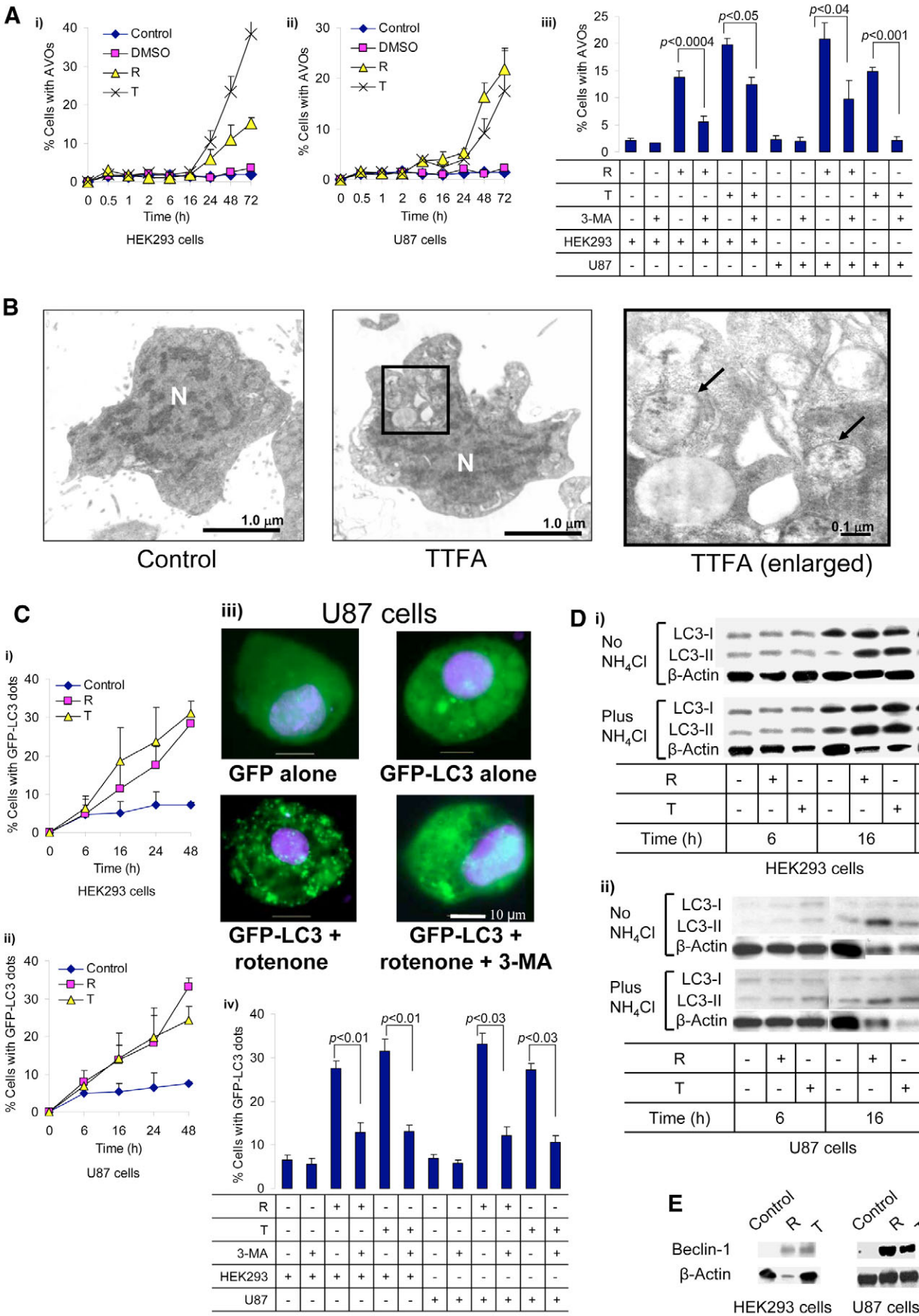


Fig. 1. Rotenone and TTFA induce cell death in HEK 293 and U87 cells over a 72-hour time course. Cell death was quantified as stated in the Materials and Methods section. HEK 293 (A) and U87 (B) cells were treated with 50.0 μM rotenone (R; complex-I inhibitor) or 0.5 mM TTFA (T; complex-II inhibitor) over a 72-hour time course. Error bars represent s.e. from three independent experiments.

Rotenone and TTFA induce autophagy in transformed and cancer cells

Autophagy is characterized by the formation of acidic vesicular organelles (AVOs) (autophagosomes and autolysosomes) and, during autophagy, autophagy proteins such as beclin 1 and ATG5 are expressed, and microtubule-associated protein 1 light chain 3 (LC3, MAP1LC3) locates on autophagosomes (Baehrecke, 2005; Codogno and Meijer, 2005; Gozuacik and Kimchi, 2004; Guimarães and Linder, 2004; Levine and Yuan, 2005; Mariño and López-Otín, 2004). In this study, the detection of autophagy was accomplished by measuring: (1) formation of AVOs by flow cytometry (FACS); (2) electron microscopy (EM) of AVOs; (3) formation of GFP-LC3-labeled vacuoles (from AVOs) by transfection and fluorescent microscopy; and (4) conversion of the cytoplasmic form of LC3 (LC3-I, 18 kDa) to the pre-autophagosomal and autophagosomal membrane-bound form of LC3 (LC3-II, 16 kDa) by western blot, and expression of beclin 1 by western

Fig. 2. Rotenone and TTFA induce autophagy. HEK 293 and U87 cells were treated as described in Fig. 1. (A) The percentage of cells with AVOs (autophagosomes and autolysosomes) was determined by flow cytometry. Rates of AVO formation induced by rotenone (R) and TTFA (T) are indicated in (i) HEK 293 and (ii) U87 cells over a 72-hour time course. (iii) Effect of 3-MA (2.0 mM) on the formation of AVOs that were induced by rotenone or TTFA after a treatment of 48 hours in U87 and HEK 293 cells. *P* values less than 0.05 represent significant difference between conditions, as indicated. (B) Electron-microscopy pictures were taken of HEK 293 cells that were untreated (control) or treated with TTFA (0.5 mM) for 48 hours. Arrows represent double-membrane vacuoles in TTFA-treated cells (enlarged image). N represents the nucleus. (C) Formation of GFP-LC3-labeled vacuoles (dots). The percentage of cells with GFP-LC3-labeled vacuoles (dots) in (i) HEK 293 and (ii) U87 cells following rotenone or TFFA treatment over a 48-hour time course was determined. Error bars represent s.e. of three independent experiments. (iii) Representative pictures from three independent experiments of U87 cells treated with GFP alone; GFP-LC3 alone; GFP-LC3 and rotenone; and GFP-LC3, rotenone and 3-MA (2.0 mM) were captured by an Olympus microscope and coolsnap camera. The nucleolus was stained with DAPI (blue). (iv) HEK 293 and U87 cells were treated with rotenone or TTFA in the presence or absence of 3-MA (2.0 mM). (D) The conversion of LC3-I to LC3-II was determined in (i) HEK 293 and (ii) U87 cells treated with rotenone or TTFA for 6, 16 or 24 hours in the presence or absence of the lysosomal inhibitor NH_4Cl (30 mM). Cells were lysed and western blotted for expression of LC3. Blots were stripped and re-probed with anti-actin antibody for equal loading. (E) Beclin 1 expression was determined after rotenone and TTFA treatment in HEK 293 and U87 cells after 48 hours of treatment. The cells were lysed and western blotted for beclin 1 and actin was used as a loading control.



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blot. Complex-I inhibitor rotenone and complex-II inhibitor TTFA significantly induced AVO formation in HEK 293 and U87 cells over a 72-hour time course (Fig. 2Ai-ii, supplementary material Fig. S1). The formation of AVOs induced by rotenone and TTFA in HEK 293 cells and U87 cells was suppressed by the autophagy inhibitor 3-methyladenine (3-MA) by approximately 40% and 50%, respectively, after cells were treated for 48 hours (Fig. 2Aiii). As a positive control, HEK 293 cells were placed under starvation conditions to increase AVO formation; this increase was blocked by 3-MA (supplementary material Fig. S2A). Using electron microscopy, we identified double-membraned autophagosomes (Fig. 2B, black arrows) containing cytosolic content in HEK 293 cells after treatment with TTFA for 48 hours; by contrast, the nuclei (N) were clearly visible in untreated cells (Fig. 2B). Because LC3 is a specific marker for autophagosome formation (Mizushima, 2004), *GFP-LC3* cDNA was transfected into cells and cells with GFP-LC3-labeled vacuoles were counted using a fluorescent microscope over a 48-hour time course. In agreement with the results of AVO formation, rotenone and TTFA induced significant formation of GFP-LC3-labeled vacuoles (25-30%) in HEK 293 and U87 cells after 48 hours of treatment, whereas 6 hours of treatment showed little GFP-LC3-labeled vacuoles (Fig. 2Ci,ii). Fig. 2Ciii shows the formation of GFP-LC3-labeled vacuoles after U87 cells were treated with rotenone for 48 hours; formation of these vacuoles was inhibited by 3-MA treatment. GFP-LC3-labeled vacuole formation was also inhibited by 3-MA after TTFA treatment in U87 cells and after rotenone or TTFA treatment in HEK 293 cells (Fig. 2Civ). As a positive control, HEK 293 cells were placed under starvation conditions to increase the amount of GFP-LC3-labeled vacuoles; this increase was blocked by 3-MA (supplementary material Fig. S2B). As a negative control, cells were transfected by GFP alone or treated with DMSO; as expected, no vacuole formation was observed following rotenone or TTFA treatment (data not shown).

Conversion of LC3-I to LC3-II is another specific marker for autophagy. In HEK 293 and U87 cells, both rotenone and TTFA induced a much higher amount of LC3-II expression compared to controls following 16 and 24 hours of treatment, whereas LC3-II expression failed to increase following 6 hours of treatment (Fig. 2D). In the presence of a lysosomal inhibitor, ammonium chloride (NH_4Cl), which prevents the degradation of LC3 in autophagosomes, the amount of LC3-II increased following treatment with rotenone or TTFA in both HEK 293 and U87 cells (Fig. 2D). However, NH_4Cl treatment failed to significantly increase the formation of GFP-LC3-labeled vacuoles following rotenone or TTFA treatment (data not shown). Similar to LC3-II accumulation, beclin 1 expression was also significantly increased by rotenone and TTFA in HEK 293 and U87 cells following 6 hours of treatment (Fig. 2E). Taken together, rotenone and TTFA can induce autophagy in transformed and cancer cells.

Rotenone and TTFA induce autophagic cell death in transformed and cancer cells

Autophagy is often treated as a survival mechanism induced by starvation, and its role as a cell-death mechanism is controversial (Mariño and López-Otín, 2004). We determined whether autophagy induced by rotenone or TTFA contributes to cell death. Cell death induced by rotenone or TTFA in both HEK 293 and U87 cells was inhibited by 3-MA by 40%, whereas the caspase inhibitor zVAD-fmk (zVAD) failed to inhibit rotenone- and TTFA-induced cell death in HEK 293 cells (Fig. 3A). Furthermore, zVAD alone failed to induce autophagy in HEK 293 and U87 cells. In U87 cells, zVAD was able to reduce both rotenone- and TTFA-induced cell death, suggesting that both autophagy and apoptosis are occurring in U87 cells (Fig. 3A). To confirm these results, we determined the amount of DNA fragmentation (a hallmark of apoptosis) by sub-G1 peak and TUNEL assay. HEK 293 cells treated with rotenone or TTFA failed to induce apoptosis, whereas etoposide (DNA-damaging agent) induced apoptosis in HEK 293 cells (Fig. 3B). In U87 cells, both rotenone and TTFA induced apoptosis but to a lesser extent than etoposide treatment (Fig. 3B). The amount of apoptosis in HEK 293 or U87 cells following rotenone or TTFA treatment failed to change in the presence of 3-MA (supplementary material Fig. S3). Because the expression of beclin 1 and ATG5 proteins contributes to the induction of autophagy, we reduced their expression by the transfection of siRNAs against beclin 1 and *ATG5* into U87 cells (Fig. 3Ci) and HEK 293 cells (supplementary material Fig. S4). The effects of rotenone and TTFA treatment on autophagy, cell death and apoptosis were determined. Transfection of beclin 1 and *ATG5* siRNAs into cells decreased rotenone- and TTFA-induced AVO formation and GFP-LC3-labeled vacuole formation (Fig. 3Cii,iii), and inhibited the level of cell death induced by rotenone and TTFA (Fig. 3Di). Rotenone- and TTFA-induced apoptosis (formation of sub-G1 peaks) was not affected by beclin 1 and *ATG5* siRNAs (Fig. 3Dii). Similar results were found for HEK 293 cells (supplementary material Fig. S4). These results indicate that autophagy induced by rotenone and TTFA contributes to cell death.

ROS are a mediator for autophagic cell death induced by rotenone and TTFA

Because ROS have been implicated in autophagy (Xu et al., 2006; Yu et al., 2006) and apoptosis (Pelicano et al., 2004), we determined whether ROS mediate autophagy and autophagic cell death induced by rotenone and TTFA. Both rotenone and TTFA induced elevated ROS generation in a 72-hour time course in HEK 293 and U87 cells (Fig. 4A, supplementary material Fig. S5). ROS generation was detected following 1 hour of treatment and remained elevated throughout the 72-hour time course. Presence of the ROS scavenger tiron (1.0 mM) reduced ROS generation following rotenone and TTFA treatment in both HEK 293 and U87 cells (Fig. 4Bi). A reduction in AVO formation and in the formation of GFP-LC3-labeled vacuoles was also detected in these tiron-treated cells (Fig. 4Bii,iii). Similar results were found with the ROS scavengers glutathione and L-cysteine (supplementary material Fig. S6A-C). Expression of beclin 1 and conversion of LC3-I to LC3-II induced by rotenone and TTFA were significantly reduced by the presence of tiron (Fig. 4C). Total cell death was also reduced by tiron in HEK 293 and U87 cells (Fig. 4Di). Similar findings were found with other ROS scavengers in HEK 293 cells (supplementary material Fig. S6D). By contrast, tiron failed to affect apoptosis (formation of sub-G1 peaks) following rotenone or TTFA treatment in HEK 293 and U87 cells (Fig. 4Dii). This indicates that ROS

scavengers decreased autophagy and autophagic cell death induced by rotenone and TTFA in transformed and cancer cells.

Manganese-superoxide dismutase (SOD2) is one of the mitochondrial antioxidant enzymes that reduce superoxide levels within cells (Pelicano et al., 2004). The effects of rotenone and TTFA on ROS generation, autophagy, cell death and apoptosis were investigated in wild-type and SOD2-overexpressing HeLa cells (western blot of SOD2, see supplementary material Fig. S7). After a treatment of 24 hours, rotenone and TTFA induced 40% and 60% ROS generation, respectively, in the wild-type cells; these figures were reduced to 14% and 24%, respectively, in the SOD2-

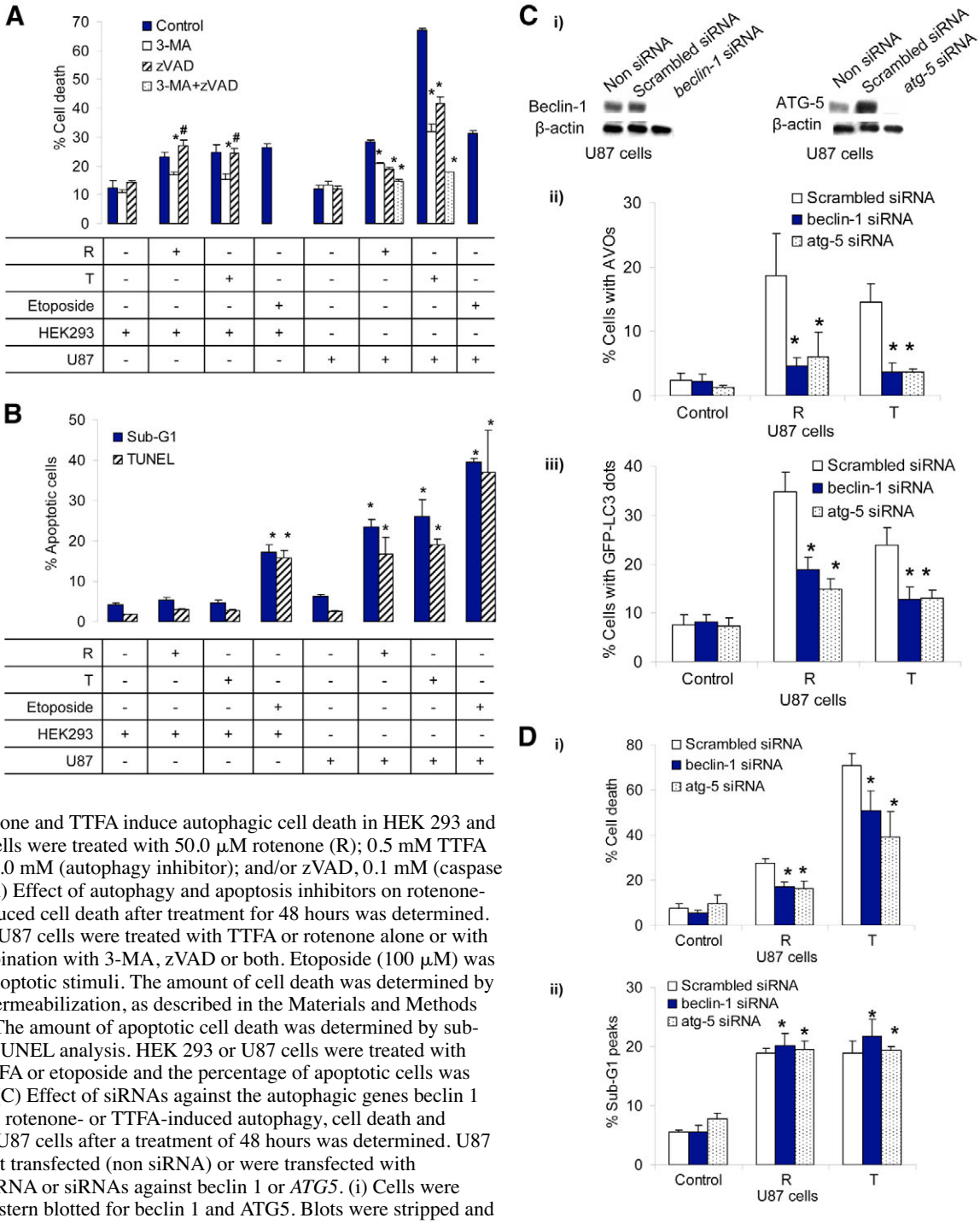

Fig. 3. Rotenone and TTFA induce autophagic cell death in HEK 293 and U87 cells. Cells were treated with 50.0 μM rotenone (R); 0.5 mM TTFA (T); 3-MA, 2.0 mM (autophagy inhibitor); and/or zVAD, 0.1 mM (caspase inhibitor). (A) Effect of autophagy and apoptosis inhibitors on rotenone- or TTFA-induced cell death after treatment for 48 hours was determined. HEK 293 or U87 cells were treated with TTFA or rotenone alone or with each in combination with 3-MA, zVAD or both. Etoposide (100 μM) was used as an apoptotic stimuli. The amount of cell death was determined by membrane permeabilization, as described in the Materials and Methods section. (B) The amount of apoptotic cell death was determined by sub-G1 peak or TUNEL analysis. HEK 293 or U87 cells were treated with rotenone, TTFA or etoposide and the percentage of apoptotic cells was determined. (C) Effect of siRNAs against the autophagic genes beclin 1 and *ATG5* on rotenone- or TTFA-induced autophagy, cell death and apoptosis in U87 cells after a treatment of 48 hours was determined. U87 cells were not transfected (non siRNA) or were transfected with scrambled siRNA or siRNAs against beclin 1 or *ATG5*. (i) Cells were lysed and western blotted for beclin 1 and ATG5. Blots were stripped and re-probed with anti-actin antibody for equal loading. (Cii,Ciii,Di,Dii) The effects of siRNA against beclin 1 and *ATG5*, and of scrambled siRNA, on AVO formation (Cii), GFP-LC3-labeled vacuoles (Ciii), cell death (Di) and apoptosis (formation of sub-G1 peaks) (Dii) following rotenone or TTFA treatment were determined. Error bars represent s.e. from three independent experiments. * Represents significant difference from control conditions ($P<0.05$). # (A) Represents a lack of significant difference from control conditions ($P>0.05$).

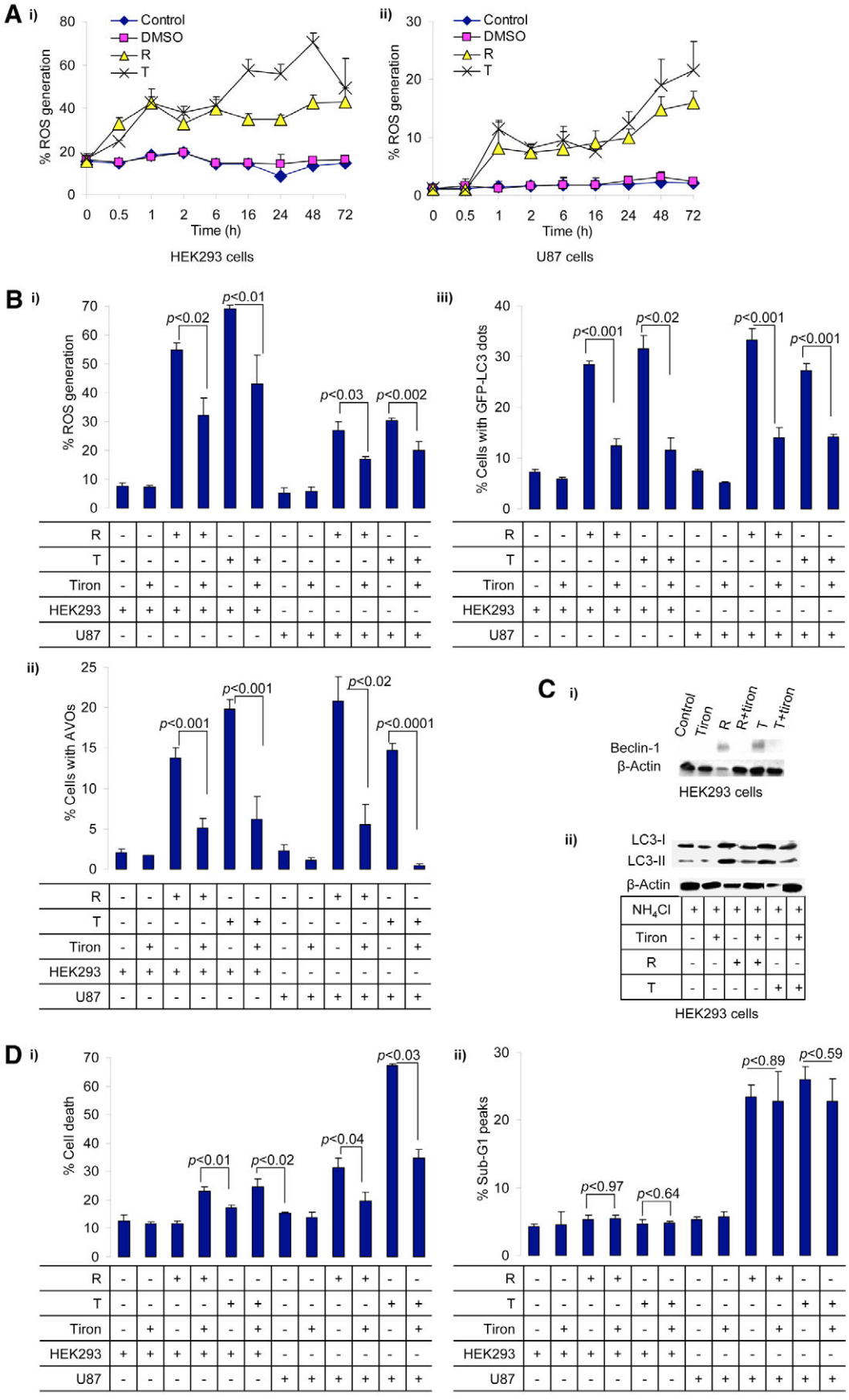

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Fig. 4. ROS scavenger tiron decreases autophagy and autophagic cell death induced by rotenone and TTFA in HEK 293 and U87 cells. Cells were treated with 50.0 μM rotenone (R), 0.5 mM TTFA (T), and/or tiron (1.0 mM). (A) ROS generation after (i) HEK 293 and (ii) U87 cells were treated with rotenone or TTFA over a 72-hour time course. (B) HEK 293 and U87 cells were treated with tiron alone or in combination with rotenone or TTTF. The percentages of (i) ROS generation, (ii) AVO formation and (iii) GFP-LC3-labeled vacuoles (dots) were determined after 48 hours. (C) Expression of beclin 1 (i) and conversion of LC3-I to LC3-II (ii) were determined by western blotting after 48 hours in the presence or absence of tiron. Actin was used as a loading control. NH_4Cl was used as a lysosomal inhibitor. (Di) Cell death was determined by membrane permeabilization following rotenone or TTFA treatment in the presence or absence of tiron in HEK 293 and U87 cells after 48 hours. (Dii) Apoptosis (formation of sub-G1 peaks) was determined in HEK 293 and U87 cells treated as above. Error bars represent s.e. from three independent experiments. *P* values less than 0.05 represent significant difference between conditions, as indicated.

overexpressing cells (Fig. 5A). Importantly, the overexpression of SOD2 in HeLa cells also reduced rotenone- or TTFA-induced autophagy. Rotenone and TTFA induced 20% and 30% formation of AVOs, respectively, in HeLa cells, and this was reduced to 6% and 4%, respectively, in SOD2-overexpressing cells (Fig. 5Bi). In agreement with the results of AVO formation, rotenone and TTFA induced the formation of GFP-LC3-labeled vacuoles in wild-type cells but not in SOD2-overexpressing cells (Fig. 5Bii, supplementary material Fig. S8). Again, compared with controls, rotenone and TTFA significantly induced beclin 1 expression and conversion of LC3-I to LC3-II in wild-type cells, whereas these processes were blocked in SOD2-overexpressing cells (Fig. 5C). Treatment with NH_4Cl increased LC3-II expression in wild-type cells following rotenone or TTFA treatment but failed to increase LC3-II expression in SOD2-overexpressing cells (Fig. 5Cii). Overexpression of SOD2 also decreased the levels of cell death induced by rotenone and TTFA by 34% and 45%, respectively (Fig. 5D). The autophagy inhibitor 3-MA inhibited rotenone- and TTFA-induced cell death by 30% and 34%, respectively, in the wild-type cells, but it had no effect on the rotenone- and TTFA-induced cell death when SOD2 was overexpressed (Fig. 5D). As a control, 3-MA was able to reduce the formation of GFP-LC3-labeled vacuoles induced by rotenone and TTFA in these cells (supplementary material Fig. S8). When zVAD was added, cell death was reduced in wild-type cells and in SOD2-overexpressing cells, and, when 3-MA and zVAD were combined, cell death was further reduced in wild-type cells. This indicates that both autophagy and apoptosis that occur in wild-type cells contribute to overall cell death induced by rotenone or TTFA, whereas only apoptosis was induced in SOD2-overexpressing cells. To confirm that apoptosis is occurring, wild-type and SOD2-overexpressing cells were treated with rotenone or TTFA and sub-G1 peak analysis or TUNEL assay was preformed. Rotenone and TTFA induced apoptosis both in wild-type and SOD2-overexpressing cells (Fig. 5E). As a positive control, HeLa cells were treated with etoposide, which induced apoptosis to a greater extent than rotenone or TTFA (Fig. 5E). Etoposide induced total cell death to a similar extent as rotenone in wild-type cells (data not shown). Taken together, these results indicate that the rotenone and TTFA-induced cell death in SOD2-overexpressing cells might be mainly apoptotic because it was not inhibited by 3-MA (Fig. 5D). This is in agreement with the fact that rotenone and TTFA did not induce autophagy when SOD2 was overexpressed (Fig. 5B,C).

The above results indicate that overexpression of SOD2 prevents ROS accumulation, autophagy and autophagy-induced cell death. Inversely, the suppression of SOD2 expression might increase ROS generation, autophagy and autophagy-induced cell death. The expression of SOD2 was suppressed by transfection of HeLa cells (wild type) with siRNA against *SOD2* (Fig. 6A). Fig. 6B demonstrates that rotenone or TTFA-induced ROS generation was increased from approximately 30% to 50% following transfection with siRNA against *SOD2*. Similarly, rotenone or TTFA-induced autophagy (formation of AVOs and GFP-LC3-labeled vacuoles) was increased by silencing SOD2 expression with siRNA (Fig. 6C,D). Finally, rotenone and TTFA-induced cell death was increased from 27% to 37% and from 33% to 44%, respectively, by silencing SOD2 expression, and treatment with 3-MA decreased rotenone- and TTFA-induced total cell death to 23% and 27%, respectively, in cells lacking SOD2 (Fig. 6E). However, apoptosis (formation of sub-G1 peaks) induced by rotenone or TTFA was not affected by *SOD2* siRNA (Fig. 6F). When *SOD2* siRNA was transfected into HEK 293 cells, similar results were obtained to those of HeLa cells, with TTFA-induced ROS generation, autophagy (formation of AVOs) and cell death (supplementary material Fig. S9).

Because it was reported that ROS generation could be a downstream effect of autophagy (Yu et al., 2006), we investigated whether the inhibition of autophagy could affect ROS generation induced by mETC inhibitors. Fig. 7 shows that rotenone- and TTFA-induced ROS generation was not affected by the autophagy inhibitor 3-MA or by siRNAs against the autophagy genes beclin 1 or *ATG5* in HEK 293 cells. A similar result was obtained in U87 cells: TTFA-induced ROS generation was not affected by siRNAs against the autophagy genes beclin 1 or *ATG5* (supplementary material Fig. S10).

Rotenone and TTFA induce apoptosis but not autophagy in non-transformed primary mouse astrocytes

Rotenone and TTFA can induce autophagy and autophagic cell death in transformed cells (HEK 293 cells) and cancer cells (U87 and HeLa cells). The effect of rotenone and TTFA in normal, non-transformed cells is unknown. We isolated normal primary astrocytes from mice and treated the cells with rotenone and TTFA. As shown in Fig. 8A, in mouse astrocytes, rotenone and TTFA failed to significantly induce ROS generation compared with controls over a 48-hour time course. Rotenone and TTFA also failed to significantly increase the amount of AVO formation or GFP-LC3-labeled vacuoles compared with controls, even in the presence of lysosomal inhibitor NH_4Cl, over a 48-hour time course (Fig. 8B). In addition, rotenone and TTFA failed to induce a higher amount of beclin 1 expression (Fig. 8Ci). LC3-I expression was undetectable in mouse primary astrocytes compared with the cancer cell lines (Fig. 8Cii). The conversion of LC3-I to LC3-II was unchanged compared to control and NH_4Cl increased LC3-II expression, but rotenone or TTFA treatment failed to further increase LC3-II expression (Fig. 8Cii). Mouse astrocytes were still capable of inducing autophagy. Under

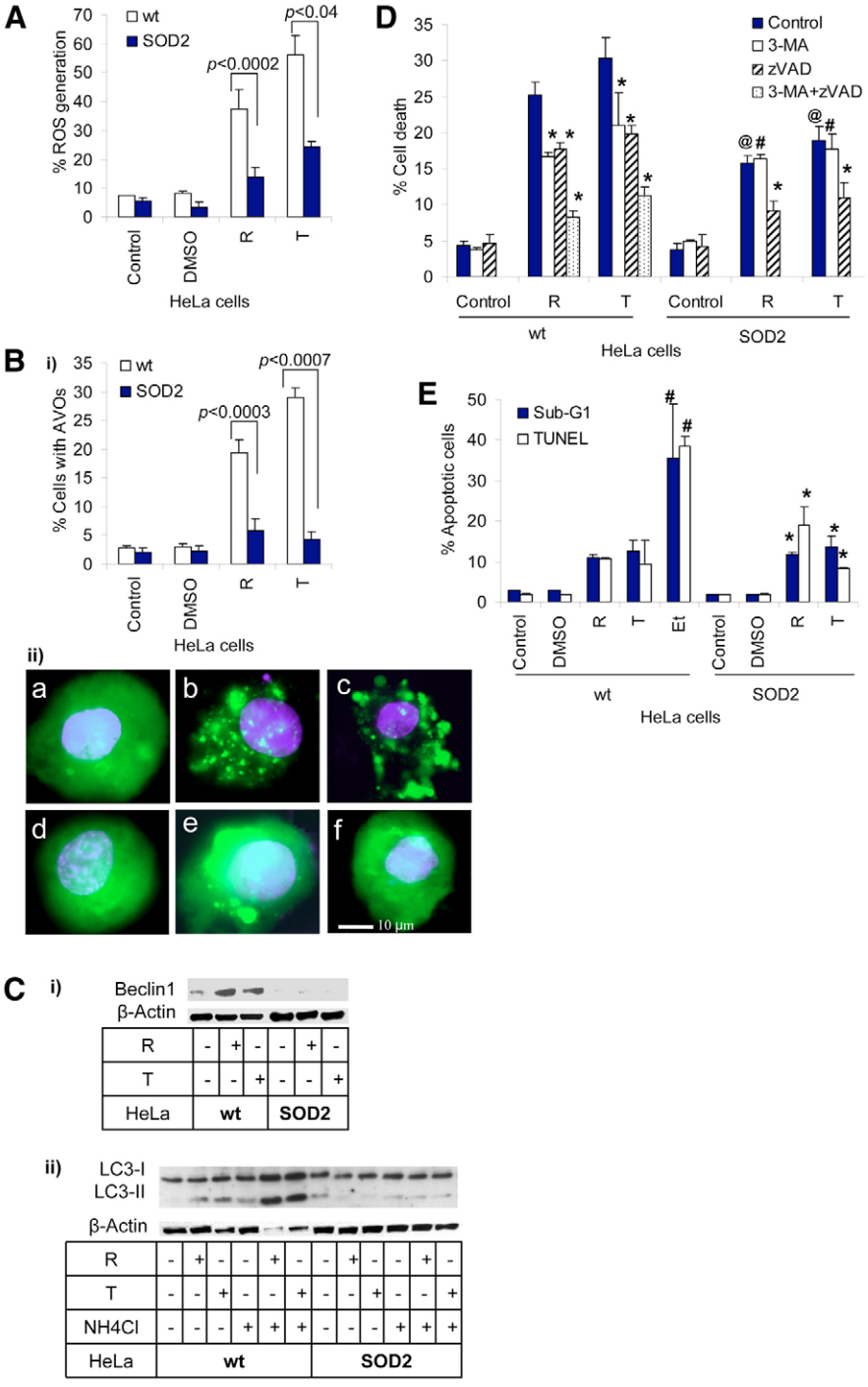

Fig. 5. Overexpression of SOD2 in HeLa cells decreases autophagy and autophagic cell death induced by rotenone and TTFA. Wild-type (wt) and SOD2-overexpressing (SOD2) HeLa cells were treated with rotenone (R, 50.0 μM) or TTFA (T, 0.5 mM) as indicated. (A) ROS generation was determined following 48 hours of rotenone or TTFA treatment. DMSO is a solvent control. (B) AVO formation (i) was determined after 48 hours of treatment with rotenone or TTFA and representative pictures of HeLa (wt and SOD2) cells with GFP-LC3-labeled vacuoles (green dots, ii) were obtained by a fluorescent microscope. In HeLa (wt) cells: a, control; b, rotenone; c, TTFA. In HeLa (SOD2) cells: d, control; e, rotenone; f, TTFA. The nucleolus was stained with DAPI (blue). (C) Beclin 1 expression (i) and conversion of LC3-I to LC3-II (ii) in the presence or absence of NH_4Cl (30 mM) was determined by western blot. Actin was used as a loading control. (D) Cell death was determined after cells were treated with rotenone or TTFA in the presence or absence of 3-MA (2.0 mM) and/or zVAD (0.1 mM). * Represents significant difference between rotenone or TTFA treatment alone and combined treatment with 3-MA and/or zVAD (P<0.05). @ Represents significant differences between wt and SOD2 cells treated with rotenone or TTFA (P<0.05). # Represents a lack of significant difference between rotenone or TTFA treatment alone and the combined treatment with 3-MA in SOD2 cells (P>0.05). (E) Apoptosis (formation of sub-G1 peak and TUNEL assay) was determined after treatment with rotenone, TTFA or etoposide (Et, apoptotic stimuli). # Represents significant differences between etoposide treatment and control wt cells. * Represents a lack of significant differences between wt and SOD2 cells treated with rotenone or TTFA alone (P>0.05). Error bars represent s.e. from three independent experiments. P values less than 0.05 represent significant difference between conditions, as indicated.

starvation conditions, LC3-II expression was increased (Fig. 8Cii). In addition, both AVO formation and the amount of GFP-LC3-labeled vacuoles were increased following starvation of mouse astrocytes and were reduced in the presence of 3-MA (supplementary material Fig. S11). Rotenone and TTFA, however, induced cell death in mouse astrocytes (Fig. 8D). This was mainly caused by apoptosis (formation of sub-G1 peaks), as shown in Fig. 8Dii: rotenone and TTFA increased sub-G1 peaks. Therefore, rotenone and TTFA preferentially induced apoptosis in normal non-transformed astrocytes, unlike in the transformed cells.

Discussion

Autophagy is normally considered to be a cell-survival mechanism induced by starvation and its role in cell death is controversial (Baehrecke, 2005; Codogno and Meijer, 2005; Gozuacik and Kimchi, 2004; Guimarães and Linder, 2004; Levine and Yuan, 2005; Mariño and López-Otín, 2004). Recently, increasing reports have provided evidence for the existence of autophagic cell death. When the apoptotic genes *Bax* and *Bak* are both knocked-out from mice embryonic fibroblasts, the apoptosis-inducing reagents etoposide and saurosporine induce autophagic cell death (Shimizu et al.,

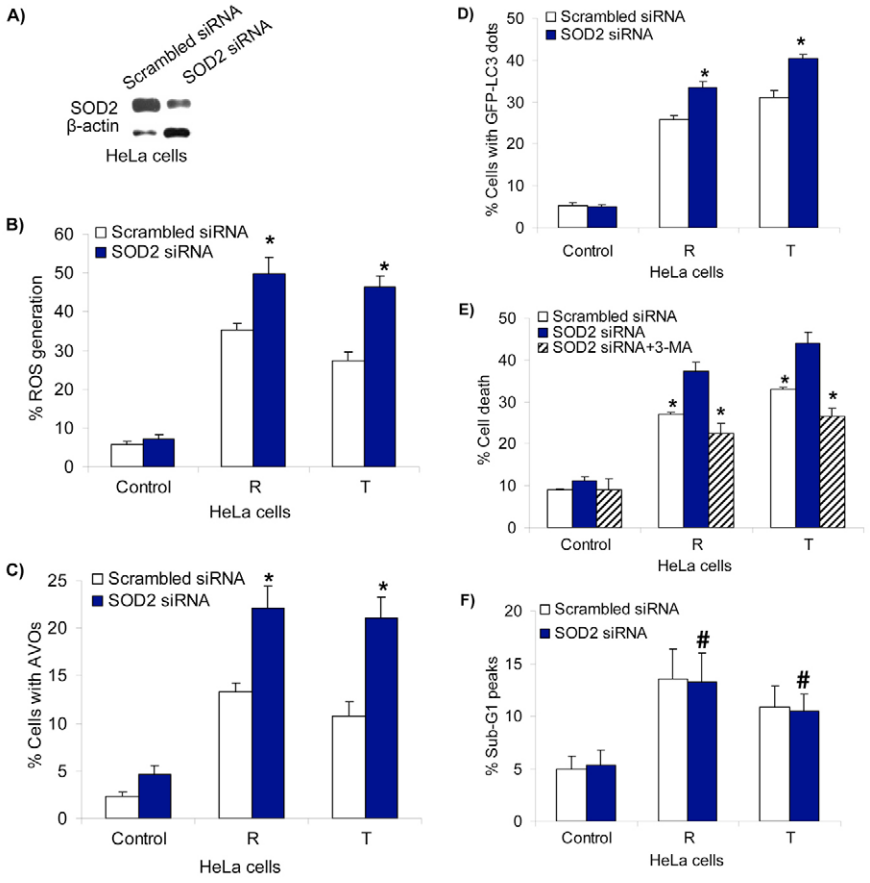

Fig. 6. Silencing SOD2 expression by siRNA increases autophagy and autophagic cell death induced by rotenone or TTFA in HeLa cells. (A) HeLa cells were transfected with scrambled or *SOD2* siRNAs. The cells were lysed and western blotted for SOD2. The blot was stripped and re-probed for actin. (B-F) After HeLa cells were treated with rotenone (R, 50.0 μM) or TTFA (T, 0.5 mM) for 24 hours, (B) ROS generation, (C) AVO formation, (D) formation of GFP-LC3-labeled vacuoles (dots), (E) cell death and (F) apoptosis (formation of sub-G1 peaks) were determined as described in the Materials and Methods section. Error bars represent s.e. from three independent experiments. * Represents significant difference from control conditions (P<0.05). # Represents a lack of significant difference from control conditions.

2004). A short mitochondrial form of the ARF tumor suppressor protein $p19^{ARF}$ induces autophagic cell death in HEK 293T cells (Reef et al., 2006). Autophagic cell death is also induced after macrophage cells are treated with lipopolysaccharides (LPS) and when apoptosis is inhibited by the caspase inhibitor zVAD (Xu et al., 2006). The metabolic toxin As_2O_3 induces an autophagic cell death mediated by the upregulation of the BCL2 family member BNIP3 (Kanzawa et al., 2005). In this study, we demonstrated for the first time that mitochondrial complex-I inhibitor rotenone and complex-II inhibitor TTFA induce autophagy leading to cell death in HEK 293, U87 and HeLa cells.

Targeting mitochondria for cancer therapy has been an area of intense investigation (Armstrong, 2006; Dias and Bailly, 2005). Mitochondria produce ROS as a byproduct of mETC (Pelicano et al., 2004). It is estimated that 2% of oxygen is converted to ROS by mETC (Pelicano et al., 2004). Compared with normal cells, cancer cells generally have a higher metabolic rate, leading to increased levels of ROS (Pelicano et

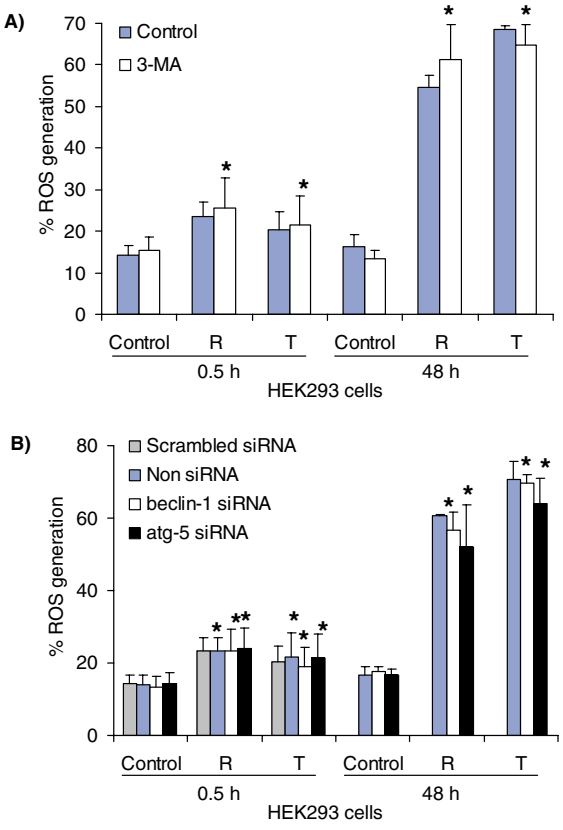

Fig. 7. Blockage of autophagy response failed to affect ROS generation induced by rotenone or TTFA. (A) HEK 293 cells were treated with rotenone (R, 50 μM) or TTFA (T, 0.5 mM) in the presence or absence of 3-MA (2.0 mM), and ROS generation was determined. (B) Expression of beclin 1 and ATG5 was silenced by siRNAs, and cells were treated with rotenone or TTFA as described in Fig. 1. ROS generation was determined as described above. * Represents a lack of significant difference from control conditions (P>0.05).

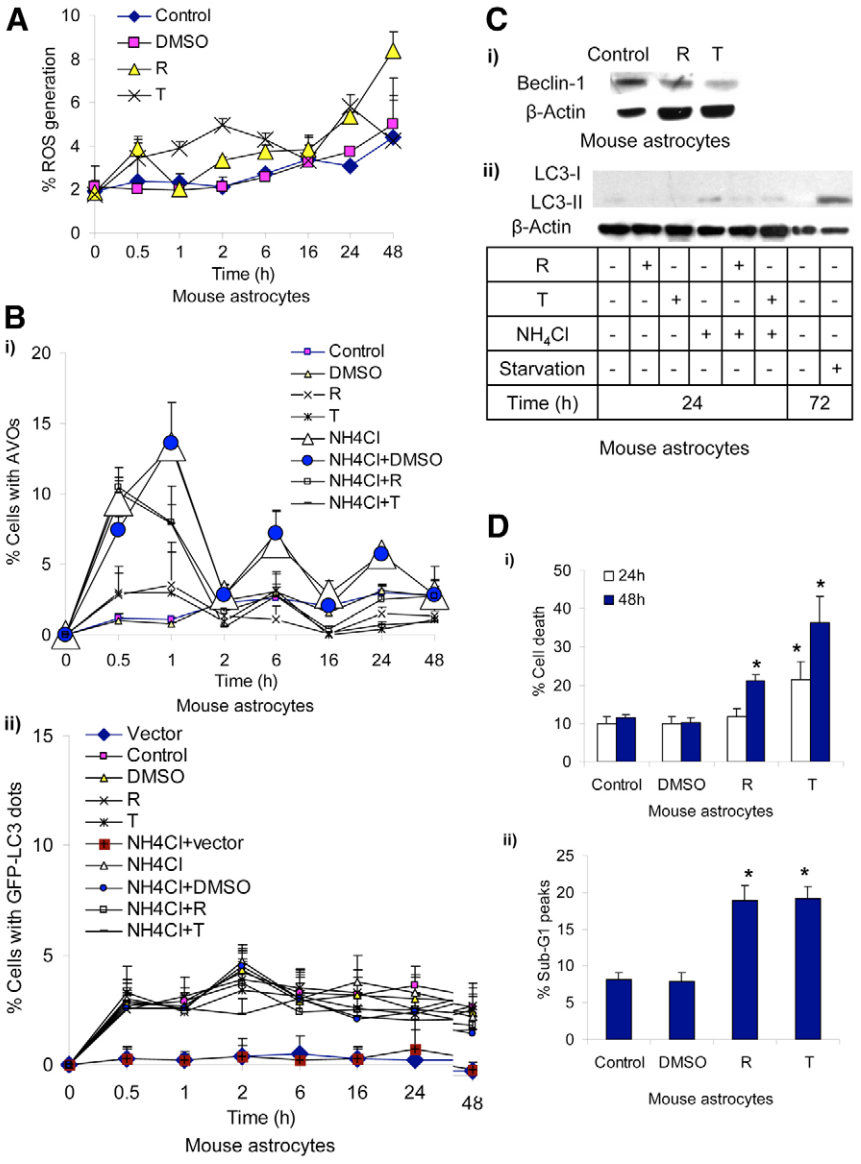

Fig. 8. Rotenone and TTFA do not induce autophagy in primary mouse astrocytes. Normal mouse astrocytes were treated with rotenone (R, 50.0 μM) or TTFA (T, 0.5 mM) over a 48-hour time course. (A) ROS generation and (B) autophagy [AVO formation and formation of GFP-LC3-labeled vacuoles (dots)] were determined as described in the Materials and Methods section. (C) Expression of beclin 1 (i) and conversion of LC3-I and LC3-II (ii) were determined by western blotting. As a positive control for conversion of LC3-I to LC3-II, astrocytes were starved of glucose and pyruvate for 3 days. Astrocytes were also incubated in the presence or absence of NH_4Cl (30 mM) and conversion of LC3-I to LC3-II was determined. (D) Cell death was determined by membrane permeabilization (i) and apoptosis was determined by the formation of sub-G1 peaks (ii). Error bars represent s.e. from three independent experiments. * Represents significant difference from control conditions (P<0.05).

al., 2004). To maintain ROS at tolerable levels so that they will not damage proteins, lipids or DNA, cells reduce ROS by antioxidant enzymes such as SOD (Pelicano et al., 2004). Blockage of caspase activation leads to degradation of catalase and increased ROS, leading to cell death (Yu et al., 2006). The degradation of catalase is mediated by autophagy. A recent report by Xu et al. shows that ROS could be involved in the induction of caspase-independent cell death in macrophage cells (Xu et al., 2006). Under nutrient-starvation conditions, ROS is increased, contributing to autophagy (Scherz-Shouval et al., 2007). It has been shown that mETC inhibitors of complexes I (Li et al., 2003; Wolvetang et al., 1994) and II (Albayrak et al., 2003) also induce ROS generation. Our study demonstrates that ROS produced from the mETC inhibition by rotenone and TTFA mediate autophagy and autophagic cell death in transformed cells and cancer cell lines.

Many brain cancers develop from astrocytes. U87 cells are glioma cells derived from astrocytes that undergo autophagy-induced cell death following treatment with mETC toxins. We demonstrated that, unlike U87 cells, normal mouse astrocytes fail to undergo autophagy following rotenone and TTFA treatment. This is correlated with a lack of ROS generation after rotenone or TTFA treatment. This could be due to reduced energy requirements for non-transformed cells compared with cancer cells, leading to lower levels of ROS generation (Pelicano et al., 2004). By inhibiting mETC, cancer cells have a higher capacity to induce ROS production and trigger autophagy. Another potential difference between primary mouse astrocytes and cancer cells is that astrocytes have low expression of LC3 compared to HEK 293, HeLa and U87 cells. This lowers the capacity of these cells to induce autophagy. Astrocytes are, however, capable of undergoing autophagy under starvation conditions. This suggests that mETC inhibitors could selectively target glioma cells to undergo autophagy-induced cell death compared with normal non-transformed astrocytes.

Rotenone and TTFA could induce apoptosis as well as autophagy in U87 and HeLa cells. TTFA was able to induce higher levels of cell death, especially in U87 cells, compared with rotenone. This corresponded to increased ROS generation induced by TTFA compared with rotenone. These differences in rotenone- and TTFA-induced cell death could be cell-type specific. Blocking both apoptosis and autophagy reduced TTFA-induced cell death to a greater extent than rotenone in U87 cells. By contrast, TTFA and rotenone failed to induce apoptosis in HEK 293 cells. These results are similar to the effect of As_2O_3 on cell death in human T-lymphocytic leukemia and myelodysplastic syndrome (MDS) cell lines (Qian et al., 2007). Furthermore, traditional apoptotic stimuli such as etoposide (Shimizu et al., 2004), rapamycin (Paglin et al., 2005), ionizing radiation (Ito et al., 2005) and temazolomide (Kanzawa et al., 2004) have been demonstrated to induce autophagy. However, inhibition of caspase activation

failed to significantly lower cell death following TTFA treatment in HEK 293 cells, indicating that TTFA-induced cell death could occur in a caspase-independent manner. Conversely, siRNAs against beclin 1 and *ATG5* significantly reduced TTFA-induced cell death but failed to reduce the TTFA-induced apoptotic response in U87 and HeLa cells, indicating that TTFA-induced autophagic cell death is independent of the apoptotic pathway. Rotenone-induced autophagic cell death also occurs separately from apoptosis. Because cancer cells usually develop resistance to apoptosis treatments (Dias and Bailly, 2005; Olie and Zangemeister-Wittke, 2001), selective prolonged activation of autophagy, such as treatment with mETC inhibitors of complex I and II in cancer cells, could be a viable strategy to treat cancers resistant to apoptosis.

Materials and Methods

Reagents

Acridine orange (AO), ethidium bromide, trypan blue, 3-MA, rotenone, TTFA, NH_4Cl, glutathione (reduced form, GSH), L-cysteine (Cys) and tiron (4,5-dihydroxy-1,3-benzene disulfonic acid-disodium salt) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Benzyloxycarbonyl-Val-Ala-Asp (zVAD-fmk, zVAD) was purchased from Calbiochem (Mississauga, Ontario), and dihydroethidium (HE) from Invitrogen (Burlington, Ontario). Rotenone, TTFA, zVAD and HE were dissolved in dimethyl sulphoxide (DMSO). GSH, Cys, tiron and 3-MA were dissolved in double distilled water. AO, ethidium bromide and trypan blue were dissolved in 1×PBS. The final concentration of DMSO in media was less than 0.1%. The concentrations of some reagents used in this study were: rotenone, 50 μM; TTFA, 0.5 mM; 3-MA, 2.0 mM; NH_4Cl, 30 mM; GSH, 10.0 mM; Cys, 10.0 mM; tiron, 1.0 mM; zVAD, 0.1 mM; and HE, 3.2 μM. GFP-expressing and GFP-LC3-expressing constructs were a kind gift from Michael Mowat (Manitoba Institute of Cell Biology, Winnipeg, Canada).

Antibodies and small interfering RNAs (siRNAs)

Beclin 1 (sc-10086) and ATG5 (sc-8667) primary antibodies and their secondary antibody donkey anti-goat HRP (sc-2020) were purchased from Santa Cruz Biotechnology (CA, USA). Rabbit anti-manganese superoxide dismutase (SOD2) polyclonal antibody (product #: SOD-110) was purchased from StressGen Biotechnologies (Victoria, Canada) and its secondary antibody goat anti-rabbit IgG (H+L) HRP from Bio-Rad laboratories (Hercules, CA). Rabbit anti-actin antibody was purchased from Sigma, rabbit anti-LC3 antibodies from Abgent or Nano-Tools (Teningen, Germany), and their secondary antibody goat anti-rabbit IgG (H+L) HRP and anti-mouse IgG (H+L) HRP from Bio-Rad Laboratories. The siRNA specific for human beclin 1 was purchased from Dharmacon (Lafayette, CO, USA) and the sequences used are the same as previously published (Degenhardt et al., 2006). The siRNA specific for *ATG5* was purchased from Sigma Proligo (The Woodlands, TX, USA) and the sequences are the same as previously published (Boya et al., 2005). The siRNA specific for *SOD2* was purchased from Ambion (Austin, TX, USA) and sequences used are same as previously published (Comhair et al., 2005).

Cell culture

HEK 293 cells, human glioma cancer cell line U87, human cervical cancer cell line HeLa and primary mouse astrocytes were maintained in a humidified 5% CO_2, 37°C incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen). Media for HEK 293 and HeLa were supplemented with 10% bovine calf serum (BCS) and 10% fetal bovine serum (FBS) (Invitrogen), respectively. Medium for the stabilized HeLa cells overexpressing SOD2 was also supplemented with 0.2 mg/ml G418 (Life Technologies). Medium for U87 was supplemented with 10% FBS, 1.0 mM sodium pyruvate and 2.0 mM glutamine without the addition of penicillin and streptomycin. Medium for normal mouse astrocytes was supplemented with 10% FBS and 1.6% glucose.

Analysis of cell death

Cell death was analyzed by measuring the permeability of the plasma membrane of the cell to acridine orange-ethidium bromide (AO/EB) (Gabai et al., 2000) or trypan blue. Cell suspension was centrifuged in an Eppendorf tube. Supernatant was removed by aspiration and cell pellet was resuspended in 100-300 μl PBS. Cells were stained with 5 μl cocktail of AO (100 μg/ml) and ethidium bromide (EB) (100 μg/ml) in PBS. A cell suspension of 10 μl was applied to a microscope slide, covered with a coverslide and cells were viewed under a fluorescent microscope. Live cells are permeable to AO but not to EB and stained green. Dead cells are permeable to both AO and EB and stained red. At least 200 cells were counted for each condition tested. Cell death can also be analyzed by staining cells with trypan blue and analyzing them by flow cytometry, similar to staining by AO/EB (Bohmer, 1985). Briefly, cells were harvested and suspended in 0.5 ml PBS in FACS tubes. Then cells were stained with Trypan blue with a final concentration of 0.04% for 5-10 minutes at room temperature. Stained cells were analyzed on a flow cytometer using CellQuest software (Becton Dickison, San Jose, CA). The red filter (675 nm, FL3-H) was used and histogram data on log scale were collected. Two peaks in the histograph were observed. The first peak represents the viable cells, which were dimly fluorescent and not permeable to Trypan blue. The second peak represents the dead cells, which were brightly fluorescent because of membrane permeabilization of Trypan blue. According to our experiments, these two methods (AO/EB-stain counting and Trypan-blue stain-flow cytometry) give similar results at least in HEK 293, U87, HeLa and mouse primary astrocyte cells. Cell death was determined by microscope counting (AO/EB staining) and the results were confirmed at least once by flow cytometry (Trypan-blue staining) unless otherwise stated.

Silencing of beclin 1/*ATG5*/*SOD2* genes by siRNAs

The same number of cells was seeded in each Petri plate (100×20 mm) on the first day and incubated at 37°C and 5% CO_2. On the second day, cells (with 30-50% confluency) were transfected with siRNA (scrambled, beclin 1, *ATG5* or *SOD2*). On the fourth day, cells from each Petri plate were split in six-well plates with the same amount of cells in each well. On the fifth day, old media were sucked off and fresh media, rotenone and TTFA were added. Cells in all plates were incubated at 37°C and 5% CO_2. On the sixth or seventh day, cells were harvested and analyzed. Cells were lysed to make protein lysates for western blot. Transfection of siRNA into cells followed the Invitrogen protocols with some modifications. For each Petri-plate transfection, 10 μl of Oligofectamine Reagent (Invitrogen) was diluted with 40 μl of plain DMEM medium (without serum) in an eppendorf tube and the diluted reagent was incubated at room temperature for 5-10 minutes. In another Eppendorf tube, 10 μl of 20 μM siRNA was added into 440 μl of plain DMEM medium. Diluted Oligofectamine Reagent was added to diluted siRNA solution, mixed gently and incubated at room temperature for 15-20 minutes. Cells were washed once with plain DMEM medium. Two ml of plain DMEM medium and 500 μl of the siRNA-Oligofectamine-Reagent complex was added to each plate containing cells and mixed. For the 'non-siRNA plate', 2.5 ml plain DMEM medium was added without Oligofectamine Reagent and siRNA. Cells were incubated for 4 hours at 37°C and 5% CO_2. Following incubation, 2.5 ml plain DMEM medium and 400 μl serum (FBS or BCS) was added to each plate. This was then mixed and incubate at 37°C and 5% CO_2. The final concentration of siRNA in medium was 40 nM.

Flow-cytometric quantification of AVOs with AO staining

Autophagy is characterized by the formation of AVOs (autophagosomes and autolysosomes) (Codogno and Meijer, 2005; Levine and Yuan, 2005). AVOs were quantified by flow cytometry after cells were stained by AO (Daido et al., 2004; Kanzawa et al., 2005; Traganos and Darzynkiewicz, 1994). AO is a fluorescent weak base that accumulates in acidic spaces and fluoresces bright red. In AO-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas AVOs fluoresce bright red (Traganos and Darzynkiewicz, 1994). The intensity of the red fluorescence is proportional to the degree of acidity. Thus, the volume of AVOs can be quantified. Cell pellet was collected in an Eppendorf tube and resuspended in 1 ml PBS. The cell suspension was stained with AO (100 μg/ml) for 15-20 minutes. Cells were washed twice with PBS, resuspended in 0.3 ml PBS and analyzed on a flow cytometer using CellQuest software.

Staining of autophagosomes with GFP-LC3

Cells were transfected with 1 μg of *GFP*/*GFP-LC3* cDNA in a mammalian expression vector. After 4 hours, cells were treated with rotenone or TTFA with or without 3-MA, anti-oxidant tiron or NH_4Cl, the fluorescence of *GFP*/*GFP-LC3* was viewed and the rate of GFP-LC3-labeled vacuoles (autophagosomes) was counted under a fluorescent microscope (Daido et al., 2004; Pattingre et al., 2005). GFP-LC3-labeled vacuoles are denoted as dots in all figures. When 3-MA or tiron was included in the treatment, it was pre-incubated in an incubator (37°C, 5% CO_2) for 1 hour. The nucleolus was stained with DAPI (4′, 6-diamidino-2-phenylindole, Sigma).

Flow-cytometric analysis of apoptosis

Apoptosis was analyzed by measuring sub-G1 peaks on a flow cytometer after cells were fixed with ethanol and stained with propidium iodide as previously described (Gibson et al., 2002). TUNEL assay was performed according to the manufacturer's instructions (Roche).

Flow-cytometric analysis of ROS

ROS generation was determined by flow cytometry after cells were stained with HE (Castedo et al., 2002). HE is oxidized by ROS into ethidium bromide (emission at 605 nm) and fluoresces red. The cell pellet was collected in an Eppendorf tube and resuspended in 0.5 ml PBS. HE with a final concentration of 3.2 μM was added

into the cell suspension. Then, the cell suspension was incubated in a water bath at 37°C for 15 minutes and analyzed on a flow cytometer using CellQuest software within 10 minutes.

Electron microscopy

Cells were collected and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium cacodylate for 2 hours, post-fixed with 1% OsO_4 for 1.5 hours, washed and finally stained for 1 hour in 3% aqueous uranyl acetate. The samples were then washed again, dehydrated with graded alcohol and embedded in Epon-Araldite resin (Canemco). Ultrathin sections were cut on a Reichert ultramicrotome, counterstained with 0.3% lead citrate and examined on a Philips EM420 electron microscope.

Western blot analysis

Western blot analysis was performed as stated previously (Kabore et al., 2006). Tris-glycine SDS-PAGE was used, except for the detection of conversion of LC3-I (18 kDa) to LC3-II (16 kDa), for which Tris-Tricine SDS-PAGE was used.

Statistical analysis

All experiments were repeated at least three times and each experiment was performed at least in duplicate. The data were expressed as means ± s.e. (standard error). Statistical analysis was performed using Student's *t*-test. The criterion for statistical significance was $P<0.05$. The software used was Excel and SigmaPlot.

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