Autophagy prevents autophagic cell death in *Tetrahymena* in response to oxidative stress

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ABSTRACT

Autophagy is a major cellular pathway used to degrade long-lived proteins or organelles that may be damaged due to increased reactive oxygen species (ROS) generated by cellular stress. Autophagy typically enhances cell survival, but it may also act to promote cell death under certain conditions. The mechanism underlying this paradox, however, remains unclear. We showed that *Tetrahymena* cells exerted increased membrane-bound vacuoles characteristic of autophagy followed by autophagic cell death (referred to as cell death with autophagy) after exposure to hydrogen peroxide. Inhibition of autophagy by chloroquine or 3-methyladenine significantly augmented autophagic cell death induced by hydrogen peroxide. Blockage of the mitochondrial electron transport chain or starvation triggered activation of autophagy followed by cell death by inducing the production of ROS due to the loss of mitochondrial membrane potential. This indicated a regulatory role of mitochondrial ROS in programming autophagy and autophagic cell death in *Tetrahymena*. Importantly, suppression of autophagy enhanced autophagic cell death in *Tetrahymena* in response to elevated ROS production from starvation, and this was reversed by antioxidants. Therefore, our results suggest that autophagy was activated upon oxidative stress to prevent the initiation of autophagic cell death in *Tetrahymena* until the accumulation of ROS passed the point of no return, leading to delayed cell death in *Tetrahymena*.

Keywords: Autophagy; Autophagic cell death; Lysosome; Mitochondria; Reactive oxygen species; *Tetrahymena*

Abbreviations used: ROS: reactive oxygen species; ATG: autophagy-related genes; PND: programmed nuclear degradation; CQ: chloroquine; 3MA: 3-methyladenine; PI3K: phosphatidylinositol-3 kinase; MOMP: mitochondrial membrane potential.

INTRODUCTION

Autophagy is a well-conserved catabolic process used to degrade long-lived proteins and cytoplasmic organelles damaged by cellular stresses, such as reactive oxygen species (ROS), and involves the formation of double-membrane vesicles called autophagosomes by sequestering cytoplasmic materials and subsequently fusing with lysosomes for degradation (Levine & Klionsky, 2004). It is well accepted that autophagy plays dual roles in controlling the fate of cells (Maiuri et al, 2007). The pro-survival characteristic of autophagy is to maintain tissue homeostasis and sustain cell viability under conditions of nutrient deprivation, growth factor withdrawal or pathogen infection by recycling damaged proteins or organelles to generate anti-apoptotic ATP. Successful removal of damaged proteins or organelles followed by repair and adaption increases cell survival. Paradoxically, when failing to restore homeostasis, autophagy executes its death-promoting characteristic and facilitates cell death through the autophagic cell death (ACD) pathway (Kroemer et al, 2009; Levine & Kroemer, 2008). Apoptosis involves the activation of catabolic enzymes, especially caspases, in the signaling cascades, which leads to the rapid demolition of apoptotic cells. ACD is morphologically defined by the presence of autophagosomes and autolysosomes in dying cells and is referred to as cell death with autophagy (Kroemer & Levine, 2008). However, in contrast to autophagy, which is well characterized and requires nearly 30 autophagy-related genes (ATG) (Levine & Klionsky, 2004), little is known about the mechanisms that regulate autophagic cell death.

ROS play an important part in regulating autophagy and cell death in yeast, nematodes and higher eukaryotes. ROS can effectively serve as signaling molecules that induce adaptive response autophagy by degrading impaired cellular components to promote cell survival. In other cellular settings, however, autophagy induction has been shown to enhance cell death in yeast and mammalian cells in response to oxidative stress (Kang et al, 2007; Scherz-Shouval & Elazar, 2007). Our

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previous studies demonstrated that the unicellular eukaryotic protozoan *Tetrahymena* is a good model to study the signal transduction pathway under cellular stresses such as cold, osmotic or oxidative stress (Li et al, 2009; Nakashima et al, 1999; Wang et al, 1998; Wang et al, 1999). Although a set of ATG genes and their homologues were discovered in yeast and mammalian cells (Klionsky, 2007), their counterparts in *Tetrahymena* are largely unknown. *Tetrahymena* is an organism with two distinct types of nuclei within the same cytoplasm. *Tetrahymena* enters a unique programmed nuclear degradation (PND) during conjugation, in which the parental macronucleus is eliminated from the progeny cytoplasm while other nuclei, such as new micro- and macro-nuclei, remain unaffected. PND triggers the destruction of macronuclei through an apoptosis-like autophagic degradation (Ejercito & Wolfe, 2003; Endoh & Kobayashi, 2006; Kobayashi & Endoh, 2003; Lu & Wolf, 2001). However, the mechanisms that regulate autophagy and the cellular self-decomposition of *Tetrahymena* under normal conditions or cellular stress, such as oxidative stress, have not yet been studied.

Here, we determined the effect of oxidative stress on cell death in *Tetrahymena* and the modulation of autophagy in the self-destruction of *Tetrahymena*. We found that *Tetrahymena* cells displayed increased double-membrane vesicles characteristic of autophagosomes and underwent autophagic cell death after exposure to hydrogen peroxide. Moreover, our data showed that starvation or blockage of the mitochondrial respiratory chain induced accumulation of ROS and activation of autophagy, followed by autophagic cell death, which was reversed by antioxidant treatment. Thus, our results indicated that mitochondrial ROS might play a pivotal role in regulating autophagy and autophagic cell death of *Tetrahymena*. Importantly, we found that treatment of cells with autophagy inhibitor resulted in a parallel suppression of autophagy associated with augmented autophagic cell death in response to oxidative stress. Taken together, our results suggest that autophagy was activated under oxidative stress to inhibit the initiation of autophagic cell death in *Tetrahymena* until increased ROS production surfeited the threshold to delayed cell death.

MATERIALS AND METHODS

Cell culture

Tetrahymena thermophila cells were kindly provided by Dr. Osamu Numata at the University of Tsukuba, Japan. Cells were grown in stock medium (2% polypeptone, autoclaved) at 28 °C, and were subcultured every week. Cells for experiments were grown in PYG medium (1% polypeptone, 0.5% yeast extract and 0.87% glucose, autoclaved) at 34 °C, as described previously (Nakashima et al, 1999; Wang et al, 1998; Wang et al, 1999).

Induction of ROS and antioxidant treatment

Tetrahymena cells were treated with 1 mmol/L, 10 mmol/L or 20 mmol/L hydrogen peroxide, respectively, by direct addition to 1 mL cell cultures. To induce the accumulation of ROS by starvation, *Tetrahymena* cells were concentrated and resuspended in sterilized phosphate buffer solution (PBS, pH 7.0) and cultured for the indicated durations. We used 40 μg/mL oligomycin (Sigma, USA), a pharmaceutical inhibitor for mitochondrial electron transport chain, or 50 μmol/L menadione/vitamin K3 (Sigma, USA) to induce oxidative stress, respectively. To determine the effect of antioxidants on ROS production, cells were pretreated with 2 mmol/L N-acetyl-L-cysteine (NAC) (Sigma, USA) for 2 h or 0.5 mmol/L catalase (Sigma, USA) for 10 min, respectively.

Inhibitors for autophagy

To suppress autophagy, cells were treated with 10 mmol/L 3-methyladenine (Sigma, USA), 100 μmol/L LY294002 (Cell Signaling, USA) or 250 nmol/L wortmannin (Cell Signaling), which are inhibitors for phosphatidylinositol-3 kinase (PI3K) required for autophagosome formation. Alternatively, *Tetrahymena* cells were treated with 500 nmol/L chloroquine (Sigma, USA), a lysosomotropic alkaline, to suppress the function of autophagy.

Fluorescent microscopy

The lysosomal bodies and nuclear degradation were visualized in *Tetrahymena* by staining with apofluor, a dye mixed with two parts 100 μg/mL acridine orange (Sigma, USA) and one part 1 mg/mL Hoechst 33342 (Sigma, USA) (Lu & Wolf, 2001). Cells were concentrated by centrifugation at 5 000 r/min for 5 min and then fixed with 2% formalin (37% formaldehyde). Cells were observed with an Olympus IX-71 fluorescence microscope (Japan) using the filter for blue light with an exposure time of 1/1.5 or 1/2.0 s. Photos were taken automatically and analyzed with DP controller software (Japan)

Transmission electron microscopy

Nuclear structures, lysosome aggregation and autophagosome formation were observed by transmission electron microscopy (TEM). Treated cells were concentrated and fixed with 2.5% glutaraldehyde at 4 °C for 2 h. TEM was performed with a Philips CM120 electron microscope (German) following the procedure at the electron microscope laboratory of Fudan University School of Medicine in Shanghai, China.

Flow cytometric analysis

Variations in *Tetrahymena* cells with degraded DNA were determined by flow cytometry combined with propidium iodide labeling (Sigma, USA). *Tetrahymena* cells were permeated with 0.03% Triton 100× (Sigma, USA) in PBS. After washing with PBS, cells were resuspended in PBS with PI, and incubated for 30 min at room temperature. Cells were sorted and analyzed using a FACS Calibur (Becton Dickinson, USA) from collections of 2×10^4 cells.

The percentage of cells with degraded DNA and ROS accumulation were determined by flow cytometry. Cells were stained with propidium iodide (Sigma, USA) and 2',7'-dichlorofluorescescin diacetate (DCF-DA) (Sigma, USA) at 37 °C for 30 min. Cells were sorted and quadrant diagrams were analyzed using a FACS Calibur (Becton Dickinson, USA) from

collections of 2×10^4 cells.

Mitochondrial membrane potential assay

A JC-1 Detection Kit (KeyGEN, China) was used to determine changes in mitochondrial membrane potential. *Tetrahymena* cells were incubated with 5 μg/mL JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine) at 34 °C for 30 min, and then washed and resuspended with the kit buffer. Cells were sorted under wavelengths of 488 nm and 530 nm simultaneously by a FACS Calibur (Becton Dickinson, USA) from collections of 2×10^4 cells.

RESULTS AND DISCUSSION

Induction of autophagy in *Tetrahymena* cells after exposure to hydrogen peroxide.

Autophagy occurs at low basal levels in all cells and involves the delivery of damaged organelles or proteins sequestered inside double-membrane vesicles to lysosomes for degradation in order to maintain tissue homeostasis. Autophagy is rapidly activated as an adaptive catabolic process when cells need to generate energy in response to different forms of metabolic stress, including growth factor deprivation and nutrient shortages (Shintani & Klionsky, 2004). In mammalian cells and plants, ROS act as signaling molecules in various intracellular processes and play a regulatory role in autophagy leading to, under certain circumstances, cell survival or cell death (Baehrecke, 2005; Klionsky, 2007).

To study the effect of oxidative stress on autophagy in *Tetrahymena* cells, we used apofluor staining to specifically stain the acid vesicles incorporated into lysosomes acridine orange so as to visualize the lysosome-containing vesicles characteristic of autophagy (Lu & Wolf, 2001). Our results indicated that lysosome-containing vesicles located at the posterior end of the cells under normal conditions tended to be aggregated and clustered near the macronucleus 10 min after exposure to 1 mmol/L H_2O_2, and reached maximal accumulation 30 min after treatment (Figure 1A). Apofluor staining showed that the nucleus color gradually turned into a combination of blue and orange, indicating that it became acidified and gradually underwent degradation after treatment with 1 mmol/L H_2O_2 (Figure 1A). To substantiate this observation,

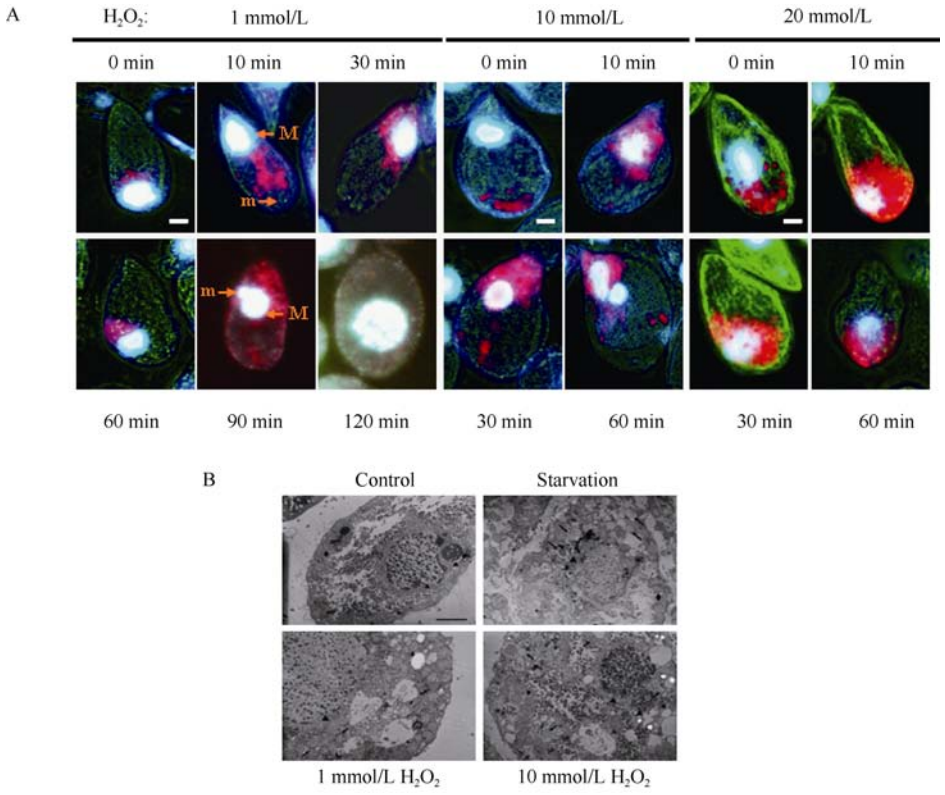

Figure 1 Autophagy activated in *Tetrahymena* upon exposure to hydrogen peroxide

A: *Tetrahymena* cells were treated with 1 mmol/L or 20 mmol/L H_2O_2, respectively, and harvested at the indicated time points for apofluor staining. The micronucleus (m) and macronucleus (M) are blue, and lysosome-containing vesicles are stained orange red. B: *Tetrahymena* cells were treated as indicated and subjected to transmission electron microscopic analysis. Arrows indicate double-membrane autophagosome-like vesicles. M represents macronucleus of *Tetrahymena*. Scale bars (1 μm) are shown.

we determined the induction of autophagy by TEM. The results revealed that *Tetrahymena* cells displayed intact mitochondria and endoplasmic reticula, condensed cytoplasm, and importantly increased large cytoplasmic inclusions, which were the membrane-bound vacuoles characteristic of autophagy (Figure 1B). The consistent results obtained by apofluor staining and TEM analysis suggest that autophagy was activated in *Tetrahymena* cells in response to hydrogen peroxide treatment.

Induction of autophagy preceded autophagic cell death in *Tetrahymena* after exposure to hydrogen peroxide

The crosstalk between autophagy and cell death is complicated in the sense that, in several scenarios, autophagy constitutes a stress adaptation to promote cell survival, whereas under other cellular settings, autophagy follows an alternative pathway leading to cell death (Baehrecke, 2005; Klionsky, 2007). In higher eukaryotes, autophagy serves as a double-edged sword in the cellular response to oxidative stress. High levels of ROS oxidize cell components, such as lipids, proteins and DNA, and thus cause cell death. Various defense mechanisms have been developed to protect cells from oxidative stress through the removal of damaged proteins or organelles by autophagy. When survival mechanisms fail, however, death programs are activated in response to oxidative stress and thus contribute to autophagic cell death (Scherz-Shouval & Elazar, 2007). To study the effect of hydrogen peroxide treatment on *Tetrahymena* cell death and its association with autophagy, we used flow cytometry combined with PI staining to determine variations in cells with degraded DNA after exposure to H_2O_2. Our results indicated that *Tetrahymena* cells undergo cell death accompanied by an accumulation of double-membrane vacuoles in the cytoplasm, and that cell death increased in a time-dependent fashion after H_2O_2 treatment (Figure 1, 2). Using the unified criteria on the definition of cell death proposed by the Nomenclature Committee on Cell Death, that is, autophagic cell death occurs together with the appearance of autophagy (Kroemer et al, 2009; Kroemer & Levine, 2008), we defined cell death with autophagic vacuolization in *Tetrahymena* as autophagic cell death induced by oxidative stress. Importantly, a time course analysis comparing the results in Figure 1 revealed that the increased autophagic vacuolization in the cytoplasm occurred before *Tetrahymena* underwent cellular self-destruction, suggesting that activation of autophagy preceded the initiation of autophagic cell death in *Tetrahymena* after exposure to H_2O_2.

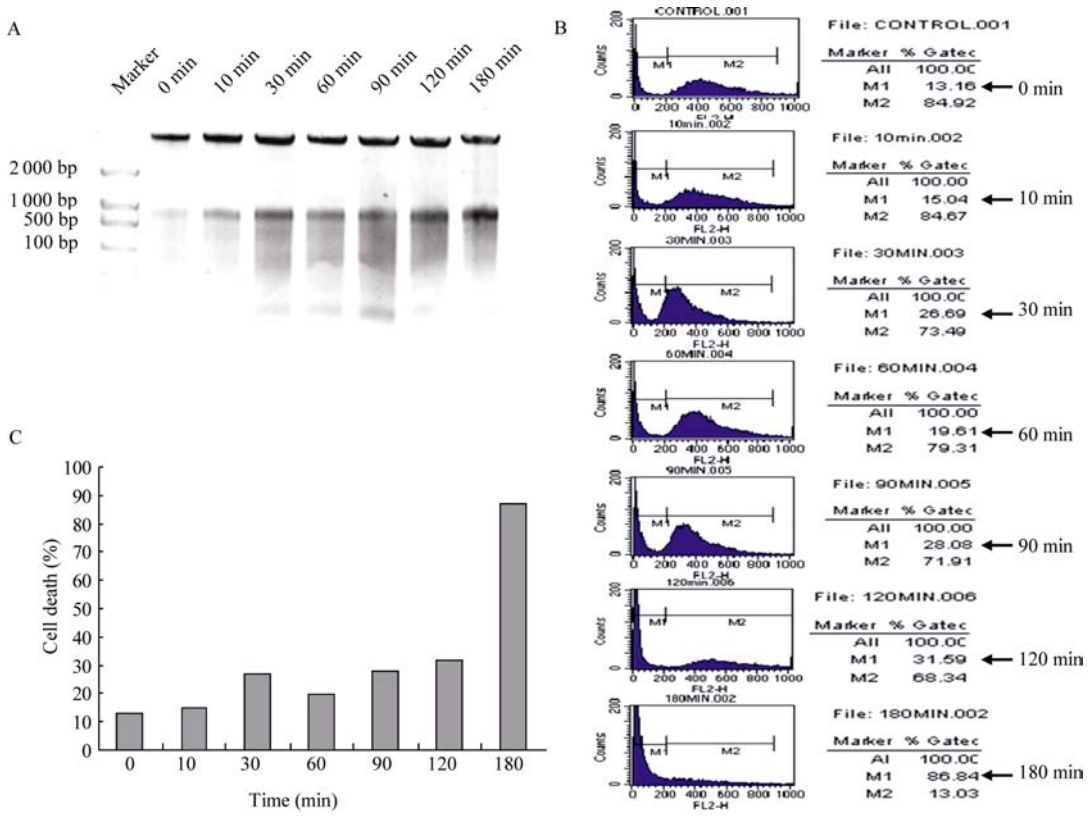

Figure 2 Autophagy occurred prior to autophagic cell death of *Tetrahymena* after hydrogen peroxide treatment

Tetrahymena cells were treated with 1 mmol/L H_2O_2 for the indicated time periods. DNA fragmentation (A) and percentages of cells with degraded DNA determined by flow cytometry combined with PI staining are shown (B and C).

Inhibition of autophagy potentiated the hydrogen peroxide-induced autophagic cell death of *Tetrahymena*

We found that the accumulation and clustering of double-membrane vacuoles around the macronucleus occurred prior to the self-decomposition of *Tetrahymena* after H_2O_2 treatment (Figure 1). This interesting observation drove us to determine the role of autophagy in H_2O_2-induced autophagic cell death. Chloroquine (CQ), a specific inhibitor for autophagy, effectively impairs lysosomal acidification and autophagic protein degradation (Poole & Ohkuma, 1981). By blocking the final step of the autophagy pathway, CQ treatment can lead to the accumulation of ineffective autophagosomes and increased death in cells reliant on autophagy for survival (Amaravadi et al, 2007; Maclean et al, 2008; Lum et al, 2005). To study the effect of autophagy inhibition and its modulation on *Tetrahymena* cell death, we pretreated cells with CQ or inhibitors for PI3K required for autophagy (Lu & Wolf, 2001), followed by H_2O_2 treatment. We found that CQ, 3-MA, LY294002 and wortmannin pretreatment, which block the function of autophagy, significantly increased autophagic cell death in *Tetrahymena* after H_2O_2 exposure (Figure 3A, B). Our results suggest, therefore, that autophagy inhibition augmented H_2O_2-induced autophagic cell death in *Tetrahymena* and that autophagy was activated to act as a "guardian" (colorful "clouds" around nucleus in Figure 1) for the *Tetrahymena* genome upon H_2O_2 treatment and protect the cells from further oxidative injury.

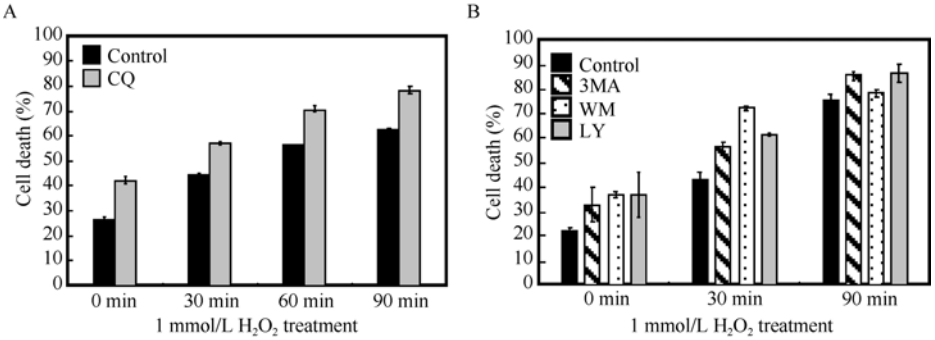

Figure 3 Autophagy inhibition led to augmented autophagic cell death in *Tetrahymena* in response to hydrogen peroxide treatment

A: *Tetrahymena* cells were pretreated with 500 nmol/L chloroquine for 18 h followed by 1 mmol/L H_2O_2 for the indicated time periods. B: *Tetrahymena* cells were pretreated with 10 mmol/L 3-methyladenine for 24 h, 100 μmol/L LY294002 for 1 h or 250 nmol/L wortmannin for 14 h, respectively, and then treated with 1 mmol/L H_2O_2 for the indicated time periods. Cell death was analyzed by flow cytometry combined with PI staining. Data are from three independent experiments and presented as mean±*SD*.

ROS formation in mitochondria was a regulatory event in autophagy and autophagic cell death in *Tetrahymena*

The mitochondrial respiratory chain, which comprises four enzymes complexes, transfers electrons from NADH to molecular oxygen to generate ATP and H_2O (Bedard & Krause, 2007; Lambeth, 2004). However, partial one-electron reduction occurring primarily at complexes 1 and 3 results in the accumulation of ROS and oxidative stress, which can be detoxified by anti-oxidizing agents (Lambeth, 2004). To study the effect of mitochondrial ROS on autophagy induction and cell death in our system, we examined the effect of starvation on ROS production and cell viability in *Tetrahymena* by flow cytometry combined with PI and DCF-DA staining. We observed that *Tetrahymena* cells swam slower, experienced shrinkage and finally underwent decomposition during starvation. Furthermore, we found that ROS were accumulated in *Tetrahymena* cells during starvation in a time-dependent manner and the elevated ROS production resulted in a time-dependent increase in cell death (Figure 4A). Importantly, TEM analysis revealed an increased amount of vacuoles characteristic of autophagosomes in *Tetrahymena* after 24 h starvation (Figure 1B), supporting the notion that autophagy was activated before the initiation of autophagic cell death in starved *Tetrahymena* cells. We also used oligomycin treatment to disrupt the mitochondrial respiratory chain to induce oxidative stress, which resulted in the accumulation of ROS and loss of mitochondrial membrane potential in *Tetrahymena* cells (Figure 4B). Oligomycin treatment also significantly increased autophagic cell death in *Tetrahymena* (Figure 4C). In addition, analyses of cells treated with vitamin K3, a free radical generator, showed consistent results that autophagic cell death was significantly induced in *Tetrahymena* (Figure 4D). Our results indicated that CQ treatment enhanced autophagic cell death in *Tetrahymena* induced by oligomycin and vitamin K3 treatment (Figure 4C, D). To further examine the effect of mitochondrial ROS in regulating autophagy and cell death, we pretreated cells with antioxidants in the presence or absence of CQ during starvation. Our results showed that pretreatment with antioxidants, specifically N-acetyl-L-cysteine (NAC) or catalase, partially reversed the accumulation of ROS and loss of mitochondrial membrane potential during starvation (Figure 5A, B). Importantly, we found that autophagy inhibition increased autophagic cell death in *Tetrahymena* in response to the elevated ROS production from starvation, which was reversed by antioxidant treatment (Figure 5B). These results suggest, therefore, that ROS production in the mitochondria plays an important role in modulating the induction of autophagy and autophagic cell death in *Tetrahymena*.

Taken together, our current study revealed that autophagy was dramatically induced following oxidative stress. Interestingly,

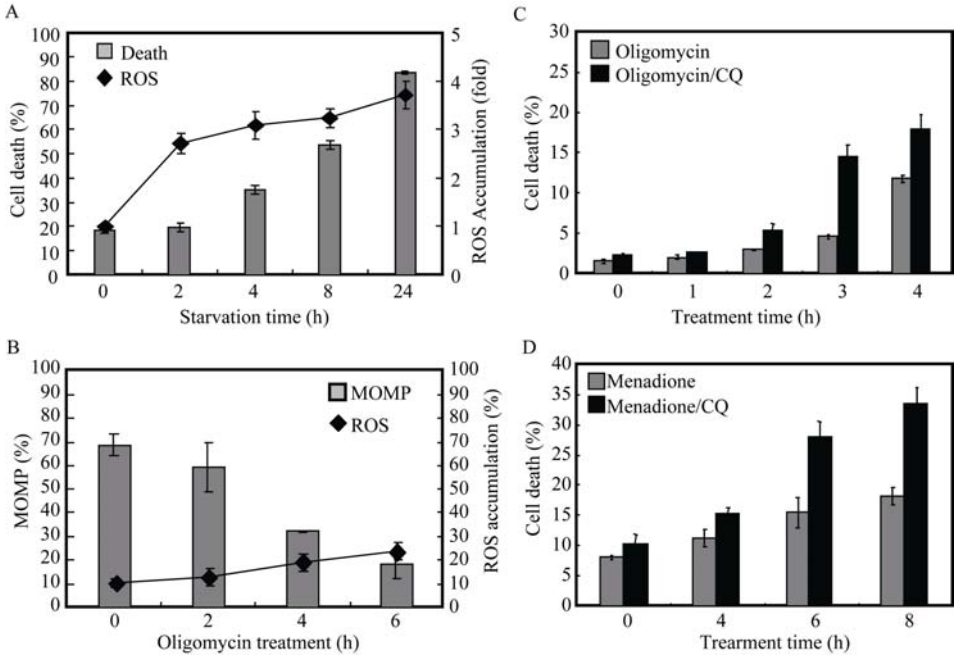

Figure 4 Mitochondrial ROS modulated autophagy and autophagic cell death in *Tetrahymena* during starvation or in response to oligomycin or vitamin K3 treatment

A: Cells grown at the mid-log phase were concentrated and resuspended in sterilized phosphate buffer solution (PBS, pH 7.0). Starved cells were harvested at the indicated time points. Cell death and ROS accumulation was determined by flow cytometry combined with PI and DCF-DA staining. B: *Tetrahymena* cells were treated with 40 μg/mL oligomycin at the indicated time periods. Treated cells were stained with JC-1 as described in the MATERIALS AND METHODS, and MOMP was determined by flow cytometry. C: *Tetrahymena* cells were pretreated with 500 nmol/L chloroquine for 18 h followed by 50 μmol/L menadione treatment at the indicated time periods. D: *Tetrahymena* cells were pretreated with 500 nmol/L chloroquine for 18 h followed by 40 μmol/L oligomycin treatment at the indicated time points. Cell death and ROS production were determined by flow cytometry combined with PI and DCF-DA staining. Data are from three independent experiments and presented as mean±*SD*.

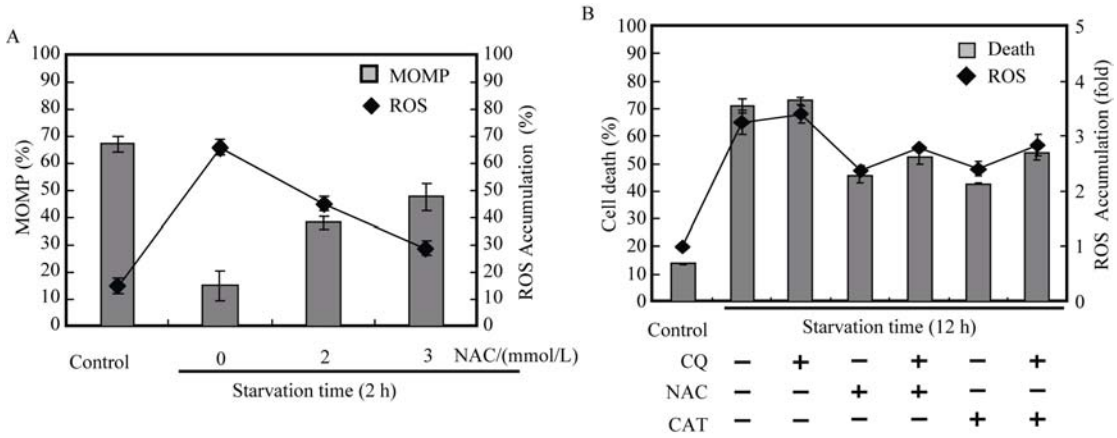

Figure5 Antioxidant reversed ROS accumulation and CQ-induced cell death in *Tetrahymena* during starvation.

A: Cells were grown in the presence or absence of 2 mmol/L or 3 mmol/L N-acetyl-L-cysteine for 2 h, respectively, and subjected to starvation for 2 h. Mitochondrial membrane potential and ROS production were measured as described in the MATERIALS AND METHODS. B: *Tetrahymena* cells were pretreated in the presence or absence of 500 nmol/L chloroquine for 18 h, 2 mmol/L N-acetyl-L-cysteine for 2 h or 0.5 mmol/L catalase for 10 min, respectively, and then subjected to starvation for 12 h. Cell death and ROS production were determined by flow cytometry combined with PI and DCF-DA staining. Data are from three independent experiments and presented as mean±*SD*.

suppression of autophagy enhanced autophagic cell death in *Tetrahymena* in response to ROS accumulation resulting from starvation or blockage of mitochondrial electron transport, suggesting that autophagy might prevent the initiation of

autophagic cell death in *Tetrahymena* until the accumulation of ROS switches autophagy into an alternative process, leading to delayed cell death. However, the molecular basis that regulates autophagic cell death, especially the genes responsible for autophagy in unicellular eukaryotic protozoans such as *Tetrahymena* has not been identified. Further studies are needed to investigate the mechanisms underlying the paradox that autophagy promotes cell survival in some instances but can lead to cell death under other cellular settings.

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