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Original Article

The heme oxygenase-1 inhibitor ZnPPIX induces non-canonical, Beclin 1-independent, autophagy through p38 MAPK pathway

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Zinc protoporphyrin IX (ZnPPIX), a heme oxygenase-1 enzyme inhibitor, has been reported to induce apoptosis and to have antitumor properties. Here, we report that ZnPPIX triggers autophagy and causes defective autophagy flux in HeLa cells. Autophagosome formation was independent of Beclin 1, indicating non-canonical autophagy activity in ZnPPIX-treated cells. Furthermore, western blot results indicated that p38 MAPK (mitogen-activated protein kinase) was phosphorylated in treated cells. Consistently, SB203580 (a p38 inhibitor) obviously inhibited the accumulation of autophagosomes. Our results indicated that p38 MAPK may be a key regulator for non-canonical Beclin1-independent autophagy.

Keywords ZnPPIX; autophagy; Beclin1-independent autophagy; p38

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Introduction

Autophagy is a catabolic process through which cytosol and organelles are sequestered into double-membraned autophagosomes and delivered into lysosomes for degradation [1]. A set of autophagy-related (ATG) proteins are recruited to the isolation membrane, known as a phagophore, for the construction of the autophagosomes [2]. Among these proteins, Beclin 1 acts pivotally by transporting phosphatidylinositol 3-kinase class III (PtdIns3KC3) to defined membrane templates that serve as a phagophore assembly site [2]. However, recent findings suggest that there is also non-canonical autophagy where autophagosome biogenesis occurs in the absence of Beclin 1 [3–6]. Currently, the signaling pathways that regulate Beclin 1-independent autophagy are poorly elucidated. What is known, however, is that arsenic trioxide-induced reactive oxygen species (ROS) can increase the expression of SnoN/SkiL and elicit Beclin 1-independent autophagy [7].

Zinc protoporphyrin IX (ZnPPIX), a potent heme oxygenase-1 (HO-1) inhibitor, exhibits significant antitumor properties both *in vitro* and *in vivo* [8,9]. The antitumor mechanism of ZnPPIX related to HO-1 is complex. It may be that ZnPPIX induces oxidative stress, consequently triggering apoptotic death *via* the inhibition of HO-1 activity [8,10]. However, in an *in vitro* model, ZnPPIX-induced cell death was accompanied by high HO-1 expression [11]. In addition, ZnPPIX itself can serve as a signaling molecule in many physiological processes, such as iron insufficiency and heme catabolism.

Here, we demonstrate that ZnPPIX induces autophagy in HeLa cells. Interestingly, autophagosome formation was independent of Beclin 1. Furthermore, we observed that the phosphorylation of p38 increased with the induction of autophagy. The addition of SB203580 (a p38 inhibitor) diminished the autophagic response. Our results suggest that p38 participates in the regulation of non-canonical Beclin1-independent autophagy.

Materials and Methods

Chemicals and antibodies

Zn(II)-protoporphyrin IX (ZnPPIX), staurosporine (STS), SB203580, benzyloxy-carbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD-fmk), rapamycin, 3-methyladenine (3-MA), and amphotericin were purchased from Sigma (St Louis, USA). Lipofectamine™ 2000 and tetramethylrhodamine methyl ester (TMRM) were from Invitrogen (Carlsbad, USA). Anti-p38 antibody, anti-phosphorylated p38 antibody, and rabbit anti-Beclin 1 antibody were from Cell Signaling Technology (Beverly, USA). LC3B antibody (Sigma), mouse anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA), goat anti-mouse/rabbit horseradish peroxidase-linked antibody (ZSGB-BIO, Beijing, China) were used. Z18 was synthesized in our lab [4].

Cell culture and plasmid transfection

Wild-type HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; HyClone). Beclin 1 knock-down (KD) HeLa cells and wild-type Beclin 1 HeLa cells were generous gifts from Dr Quan Chen (Institute of Zoology, Chinese Academy of Science, Beijing, China) and cultured in DMEM supplemented with 10% FBS and 200 μg/ml amphotericin in a humidified incubator at 37°C and 5% CO_2. For transfection, cells were seeded in 24-well plates at a density of 1×10^5 cells per well. Twenty-four hours later, GFP-LC3 plasmid (Addgene, Cambridge, USA) transfection at 600 ng/well was performed using Lipofectamine^{TM} 2000 according to the manufacturer's protocol.

Cell viability and treatments

About 5×10^5 cells were seeded into a 24-well plate for each experiment. After 24 h, ZnPPIX dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium and cell viability was detected at different time points using Trypan blue exclusion assay.

For the combined treatments, HeLa cells were co-incubated with ZnPPIX and 10 mM 3-MA, 100 μM z-VAD-fmk, or 30 μM SB203580, respectively. After co-incubation for 24 h, the extent of cell death was also analyzed using the Trypan blue exclusion assay.

For cell death assay after co-incubation with z-VAD-fmk, HeLa cells treated with 50 nM STS for 12 h were used as the positive control. For autophagosome formation assay, HeLa cells treated with 10 μM Z18 and 500 nM rapamycin for 12 h were used as positive and negative control, respectively.

Western blot analysis

To analyze protein expression, western blotting was performed as described previously [4]. Cells were harvested and lysed in buffer containing 100 mM Tris/HCl, 1 mM EDTA and 2% sodium dodecyl sulfate (SDS). Protein concentration was quantified using Bradford protein assay. After heat denaturation for 5 min, 50 μg proteins were separated by 12% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose filters and blocked with 5% low-fat milk in TBS (Tris-buffered saline) for 1 h. The filters were incubated with antibodies (anti-LC3, 1 : 1000 dilution; anti-beclin 1, 1 : 2000; anti-p38, 1 : 1000 dilution; anti- phosphorylated p38, 1 : 1000 dilution; and anti-actin, 1 : 1000 dilution) for 1 h or longer at room temperature with gentle shaking. Nitrocellulose filters were washed three times for 5 min each at room temperature in TBS, incubated with secondary antibody for 1 h, and then washed with TBS three times for 5 min each. The signals were detected with enhanced chemiluminescence kit (Thermo Fisher Scientific, Rockford, USA).

Cell staining and confocal microscopy

Both fixed and live cells were imaged using an A1R-si Laser Scanning Confocal Microscope (Nikon, Kanagawa, Japan). To determine the mitochondrial membrane potential, HeLa cells treated with ZnPPIX were stained with 150 nM TMRM for 15 min. Cells were then washed three times with 1× PBS and observed under the microscope. Fluorescence images were collected with a 543-nm excitation light.

To measure autophagosome formation, HeLa cells were transfected with GFP-LC3. The accumulation and distribution of GFP-LC3 puncta were recorded using the A1R-si Laser Scanning Confocal Microscope. To measure the effect of 3-MA or Beclin1 KD on the autophagosome formation, HeLa cells were transfected with GFP-LC3 and co-incubated with 10 μM ZnPPIX and 10 mM 3-MA for 12 h. To determine the effect of SB203580 (p38 inhibitor) on the autophagy activity, HeLa cells were transfected with GFP-LC3 and co-incubated with 10 μM ZnPPIX and 30 μM SB203580 for 12 h. HeLa cells with GFP-LC3 dots and the average number of GFP-LC3 dots per cell were quantified using ImageJ (National Institutes of Health, Bethesda, USA). Fifty fields of cells with GFP-LC3 puncta were counted. The number of GFP-LC3 puncta per cell was counted for 50 cells.

For detecting the autophagic flux, HeLa cells transfected with mTagRFP-mWasabi-LC3 [12] were treated with 10 μM ZnPPIX for 12 h and analyzed for the localization of mTagRFP and mWasabi of tandern LC3. Fluorescence images were collected with both 488 and 543 nm excitation light.

FACS analysis of caspase activation

For the analysis of caspase activation, HeLa cells were seeded into 12-well plates at a density of 3×10^5 cells per well and incubated with 10 μM ZnPPIX for 12 h. After treatment, cells were harvested using trypsin/EDTA and centrifuged to pellet at a low speed of 1500 rpm for 3–5 min. The pellet was resuspended in 0.5 ml of PBS and stained with 10 μM CaspACE^{TM} FITC-VAD-FMK (Promega Corporation, Madison, USA) in the dark for

30 min at 37°C. Cells incubated with 50 nM STS for 12 h were used as the positive control. The cytofluorometric analysis was performed using an FACSCalibur (Becton Dickinson, San Jose, USA).

Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out as previously described [4]. After 10 μM ZnPPIX treatment for 12 h, cells were harvested, rinsed with PBS, and then fixed by 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After three washes with 0.1 M phosphate buffer, cells were further fixed in 0.1 M sodium phosphate buffer containing 1% OsO_4 (pH 7.2) for 2 h at 4°C. After being dehydrated in a graded series of ethanol, cells were embedded into Ultracut (Leica, Wetzlar, Germany). The cell pellet was sliced into 60 nm sections and stained with uranyl acetate and lead citrate, and examined with a JEM-1230 TEM (JEOL, Akashima, Japan).

Statistical analysis

Data were presented as mean ± SD from at least three independent experiments. Statistical analysis was performed using Origin Pro 7.5 (Origin Lab Corporation, Northampton, USA). Data were analyzed using Student's t test. A threshold of $P < 0.05$ was set for statistical significance.

Results

ZnPPIX is toxic and induces cell death in HeLa cells

Several studies have reported that ZnPPIX exhibits antitumor properties [8,13]. We first determined the concentration of ZnPPIX that led to cell death. ZnPPIX completely suppressed the proliferation of HeLa cells at 15 μM [**Fig. 1(A)**]. Treatment of HeLa cells with 20 μM ZnPPIX for 48 h resulted in almost 100% cell death. The IC_{50} value of ZnPPIX was ∼7.5 μM [**Fig. 1(A)**]. Thus, treatment with 10 μM ZnPPIX for 12 h was used for further research. During cell death, mitochondrial membrane potential was completely lost and caspases became activated [**Fig. 1(B,C)**]. Meanwhile, treatment of caspase inhibitor z-VAD-fmk partially inhibited ZnPPIX-induced cell death [**Fig. 1(D)**].

ZnPPIX induces autophagy in HeLa cells

Since HO-1 deficiency leads to the activation of autophagy [14], we then investigated autophagy activation in ZnPPIX-treated cells. To characterize autophagosomes in ZnPPIX-treated cells, we first performed ultrastructural analyses by TEM. Double-membraned autophagosomes containing cytoplasmic content as well as membranous structures were found in HeLa cells treated with 10 μM ZnPPIX for 12 h [**Fig. 2(A)**]. In GFP-LC3-expressing cells

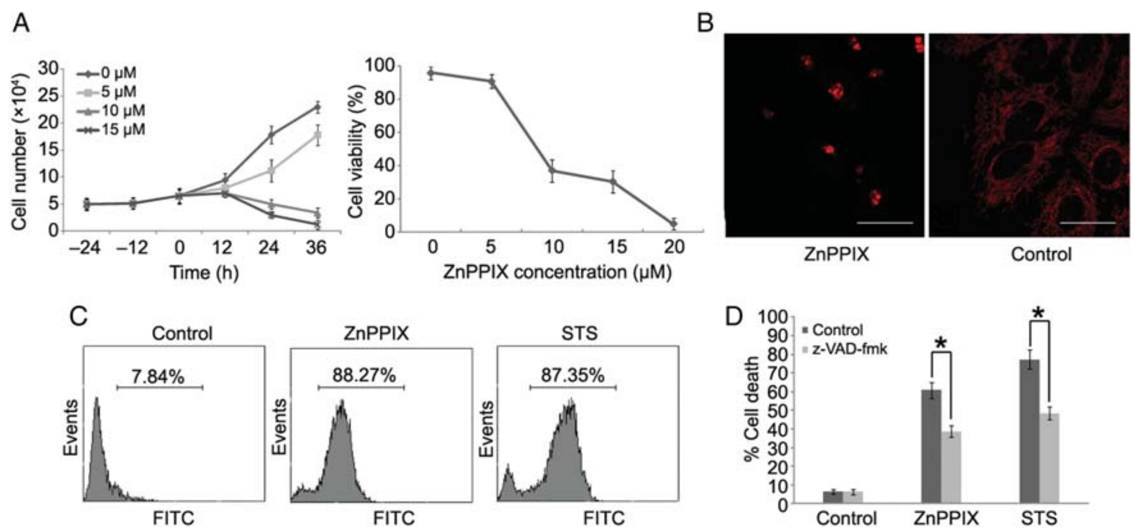

Figure 1 ZnPPIX induces cytotoxicity in HeLa cells (A) ZnPPIX inhibits proliferation and survival of HeLa cells. After exposed to different concentrations of ZnPPIX, the numbers of surviving cells were determined at different time points using blood cell counting plate (left panel) and the cell viability was analyzed using the trypan blue exclusion assay (right panel). Data are expressed as mean ± SD from at least three independent experiments. (B) Mitochondrial membrane potential is lost in ZnPPIX-treated HeLa cells. After exposed to 10 μM ZnPPIX for 48 h, cells were stained with TMRM (50 nM) for 20 min (left panel) and the untreated cells were set as control (right panel). Images with red fluorescence of TMRM were collected by confocal microscopy. Bar = 50 μm. Note: clustered red fluorescence in ZnPPIX-treated cells is from ZnPPIX itself. (C) ZnPPIX induces caspase activation in HeLa cells. CaspACE™ FITC-VAD-FMK was used to label activated caspases in cells after treatment with 10 μM ZnPPIX for 12 h. STS was used as the positive control. Caspase activity was detected by flow cytometry. (D) The caspase inhibitor z-VAD-fmk protects HeLa cells from ZnPPIX-induced cell death. After treatment with 100 μM z-VAD-fmk for 12 h, cells were incubated with 10 μM ZnPPIX for 48 h. Cells treated with STS + z-VAD-fmk were used as positive controls. The percentage of cell death was analyzed using the Trypan blue exclusion assay. Data are expressed as mean ± SD from at least three independent experiments. *$P < 0.05$.

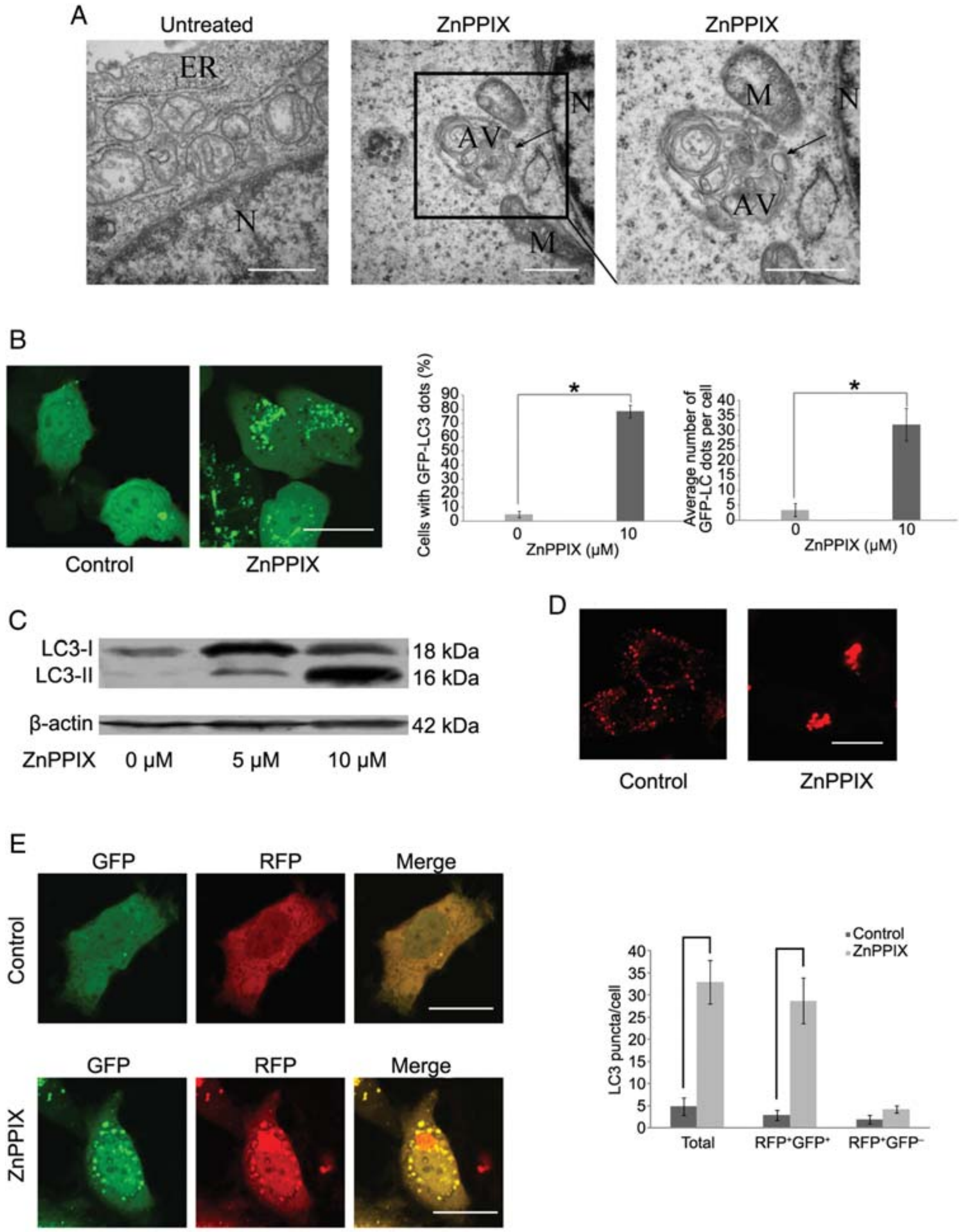

Figure 2 ZnPPIX induces autophagy in HeLa cells (A) Representative electron microscopy images of autophagosomes in 10 μM ZnPPIX-treated HeLa cells (middle and right panels) and untreated cell (left panel). Black arrow indicates the double membrane of an autophagic vacuole containing multiple membranes and partially degraded material. AV, autophagic vesicle; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus. Bar = 0.5 μm. (B) Representative fluorescent images of HeLa cells expressing GFP-LC3 treated with 10 μM ZnPPIX for 12 h, and the controls were shown (left panel). The percentage of cells with GFP-LC3 puncta (middle panel) and the number of GFP-LC3 puncta per cell (right panel) were quantified using ImageJ. Data are expressed as mean ± SD from at least three independent experiments. $^*P < 0.05$. (C) HeLa cells were incubated with different concentration of ZnPPIX for 12 h and the conversion of LC3I to LC3II was analyzed by western blotting. (D) Autophagic flux was impaired in ZnPPIX-treated HeLa cells. Cells were stained with LysoTracker. Note: clustered red fluorescence in cells is from ZnPPIX itself. (E) Autophagic flux was analyzed using mTagRFP-mWasabi-LC3 probe. Bar = 0.5 μm. The number of RFP^+GFP^+ puncta and RFP^+GFP^- puncta in ZnPPIX- treated cells was counted. Data are expressed as mean ± SD from at least three independent experiments. $^*P < 0.05$.

treated with ZnPPIX, both the percentage of cells with LC3 puncta and the average number of GFP-LC3 dots per cell were increased after 10 μM ZnPPIX treatment for 12 h [**Fig. 2(B)**].

We then examined the conversion of LC3 from LC3-I (the cytosolic form) to LC3-II (the membrane-bound form) by western blotting. The results showed that increasing concentrations of ZnPPIX were accompanied by increasing levels of endogenous LC3-II in HeLa cells, over a 12-h treatment [**Fig. 2(C)**].

Furthermore, ZnPPIX induced lysosomal dysfunction in HeLa cells [**Fig. 2(D)**]. We also investigated autophagic flux in ZnPPIX-treated cells using mTagRFP-mWasabi-LC3 probes. Results showed that most LC3 puncta were autophagosomes (RFP^+GFP^+ puncta) instead of autolysosomes (RFP^+GFP^- puncta) [**Fig. 2(E)**]. These results indicated that ZnPPIX induced an impaired autophagic flux in HeLa cells.

ZnPPIX-induced autophagy is Beclin 1-independent

The above results prompted us to explore the formation mechanisms of autophagosomes in ZnPPIX-treated HeLa cells. In this experiment, cells treated with 10 μM Z18 (a compound previously reported to induce Beclin 1-independent autophagy) for 12 h [4] were used as positive controls, whereas cells treated with 500 nM rapamycin (a mammalian target of rapamycin inhibitor that induces PtdIns3K- and Beclin 1-dependent autophagy) [15] for 12 h were used as negative controls. In fact, the PtdIns3K inhibitor 3-MA did not show significant effect on ZnPPIX-induced autophagy [**Fig. 3(A)**]. It failed to affect both the number of cells with GFP-LC3 puncta and the average number of GFP-LC3 puncta per ZnPPIX-treated HeLa cell [**Fig. 3(B,C)**]. Western blot analysis also indicated that 3-MA did not inhibit the conversion of LC3-I to LC3-II in ZnPPIX-treated cells [**Fig. 3(D)**].

Since Beclin 1 is essential for the PtdIns3K complex, these observations prompted us to investigate the role of Beclin 1 in ZnPPIX-induced autophagy. A HeLa cell line stably expressing a Beclin 1 shRNA (Beclin KD HeLa cells) was treated with 10 μM ZnPPIX for 12 h. Western blot results confirmed that the expression of Beclin 1 in Beclin KD HeLa cells was inhibited [**Fig. 4(A)**]. Similar to the 3-MA treatment results, knockdown of Beclin 1 did not result in a significant effect on ZnPPIX-induced autophagy [**Fig. 4(B–D)**]. Therefore, ZnPPIX-induced autophagy in HeLa cells is Beclin 1-independent.

p38 MAPK regulates autophagy in ZnPPIX-treated cells

The p38 MAPK pathway is often activated by stressful stimuli and cytokines, leading to diverse cell type-specific responses, such as cell survival, autophagy or apoptosis [16–18]. Whether p38 activity regulates autophagy in ZnPPIX-treated HeLa cells was investigated. First, we examined the activation of p38 MAPK in ZnPPIX-treated HeLa cells. Western blot analyses revealed that 10 μM ZnPPIX treatment for 12 h significantly increased p38 phosphorylation [**Fig. 5(A)**]. As SB203580 specifically inhibits p38 activity [17], we next examined autophagy activity in ZnPPIX-treated HeLa cells pre-incubated with 30 μM SB203580 for 6 h. The results showed that, compared with cells treated with ZnPPIX alone, both the number of cells with GFP-LC3 puncta and the average number of GFP-LC3 puncta per cell in ZnPPIX plus SB203580-treated cells were decreased [**Fig. 5(B)**]. SB203580 also inhibited the conversion of LC3-I to LC3-II in ZnPPIX-treated cells [**Fig. 5(C)**]. These data indicated that the p38 pathway regulates ZnPPIX-induced autophagy.

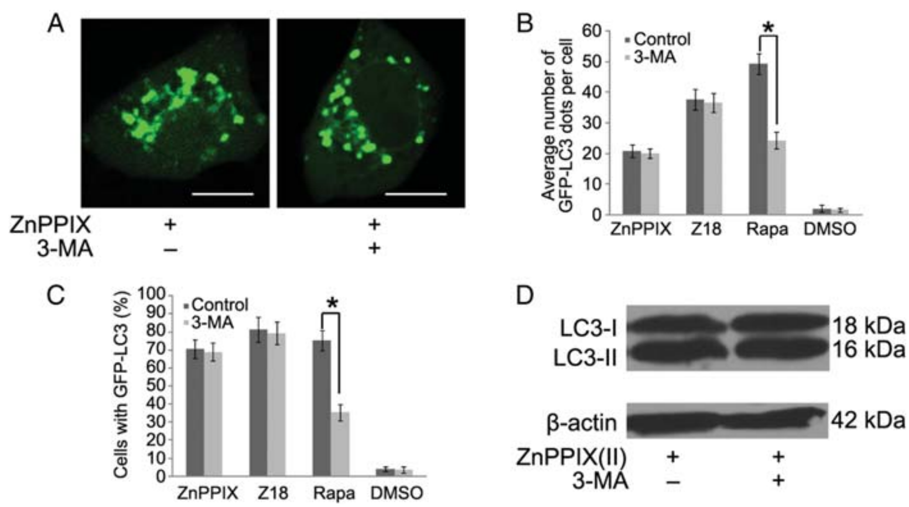

Figure 3 3-MA does not inhibit the formation of autophagosomes induced by ZnPPIX HeLa cells were pre-incubated with either 0.1% DMSO (control) or 10 mM 3-MA for 2 h before treatment with either ZnPPIX (10 μM), Z18 (10 μM), or rapamycin (500 nM). (A) After combination treatment for 12 h, autophagy activity was detected by A1R-si laser scanning confocal microscopy. There was no difference in ZnPPIX-treated cells with or without 3-MA. (B,C) The average number of GFP-LC3 puncta per cell (B) and the percentage of cells with an obvious accumulation of GFP-LC3 puncta (C) after ZnPPIX treatment were quantified using ImageJ. There was no difference in ZnPPIX-treated cells with or without 3-MA. Data are expressed as mean ± SD from at least three independent experiments. $^*P < 0.05$. (D) Western blot results showed that, after combination treatment for 12 h, 10 mM 3-MA has no effect on the conversion of LC3-I to LC3-II in ZnPPIX-treated HeLa cells.

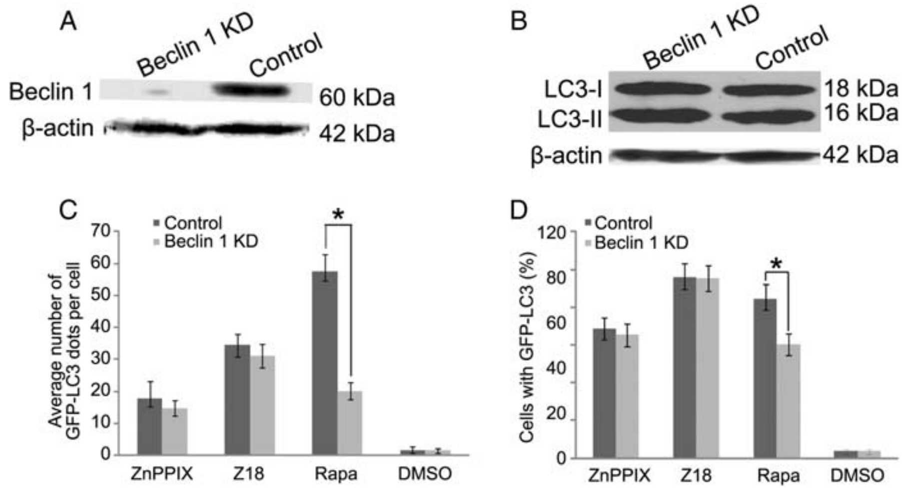

Figure 4 Knockdown of Beclin 1 has no effect on the formation of autophagosomes induced by ZnPPIX (A) Beclin 1 KD was first confirmed by western blotting. (B) The conversion of LC3-I to LC3-II in ZnPPIX-treated Beclin 1 KD HeLa cells and wild-type HeLa cells were detected using western blotting. There was no difference in ZnPPIX-treated Beclin 1 KD HeLa cells and wild-type HeLa cells. (C,D) Knockdown of Beclin 1 also has no effect on the average number of GFP-LC3 puncta per cell (C) and the percentage of ZnPPIX-treated cells with an obvious accumulation of GFP-LC3 puncta (D) after ZnPPIX treatment. Data are expressed as mean ± SD from at least three independent experiments. *$P < 0.05$.

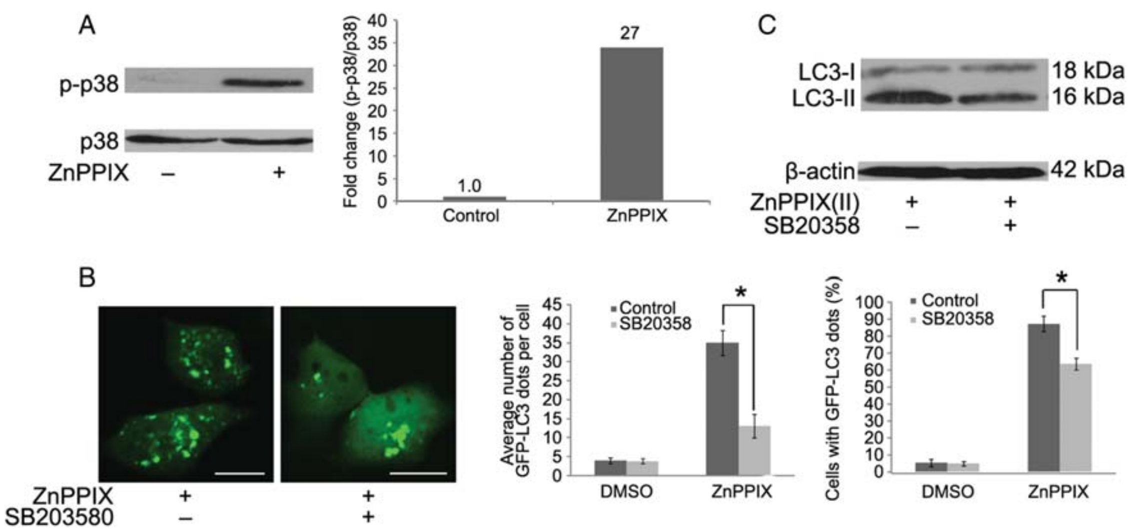

Figure 5 p38 MAPK regulates autophagy induced by ZnPPIX (A) ZnPPIX treatment significantly increased the phosphorylation of p38. After cells were treated with 10 μM ZnPPIX treatment for 12 h, p38 phosphorylation was detected by western blotting. The band intensity of phosphorylated p38 was quantified by densitometry. The densitometric values shown in the histograms are expressed as fold change relative to normal levels, which was assigned a value of 1.0. (B) p38 MAPK inhibitor SB203580 could reduce autophagy activity induced by ZnPPIX in HeLa cells. HeLa cells were pre-incubated with 30 μM SB203580 before treatment with 10 μM ZnPPIX and the autophagy activity was detected by A1R-si laser scanning confocal microscopy (left panel). SB203580 decreased the average number of GFP-LC3 puncta (middle panel) and the percentage of cells with an obvious accumulation of GFP-LC3 puncta in ZnPPIX-treated cells (right panel). Data are expressed as mean ± SD from at least three independent experiments. *$P < 0.05$. (C) Effect of SB203580 on the conversion of LC3-I to LC3-II in ZnPPIX-treated HeLa cells were detected by western blotting.

Discussion

The antitumor activity of ZnPPIX or water-soluble PEG-ZnPP IX in different cell lines has been investigated. These compounds reduced tumor growth, induced oxidative stress, consequently apoptotic death, and demonstrated potent antitumor effects. The present study has simply shown cytotoxic effects of ZnPPIX on HeLa cells. Mitochondria, as a major source of intracellular ROS, play a central part in cellular apoptotic death and mitochondrial membrane potential (Δψm) is a key indicator of cellular viability and used for assessing mitochondrial function. TMRM staining results showed a significant loss of mitochondrial membrane potential, which is a central event in the cell death and allows the release of cytochrome *c*, induces caspase activation to initiate apoptotic death. After

ZnPPIX treatment, caspases were activated in HeLa cells. It is well known that sequential activation of caspases could trigger the apoptotic pathways. z-VAD-fmk, a pan-caspase inhibitor, could effectively inhibit the induction of cell death in ZnPPIX-treated cells. These results, however, did not clarify the detailed information on the molecular mechanism of cell death, our work addressed the cytotoxicity of ZnPPIX and showed that caspase may serve as an executor in HeLa cells.

Antitumor effects of ZnPPIX on experimental tumors have been widely documented, while autophagy induction of ZnPPIX has barely been studied. It has been reported that HO-1 deficiency leads to a significant increase in autophagy levels *in vivo* [14]. Consistently, herein we have shown that ZnPPIX, a HO-1 inhibitor, has similar effects on HeLa cells. For the first time, we have demonstrated that ZnPPIX can induce autophagy. Further more, ZnPPIX increased the pH of lysosomes in cells, which inhibited the fusion of autophagosomes and lysosomes, resulting in the interruption of autophagic flux.

Our results have shown an obvious increase in punctate GFP-LC3 and mTagRFP-mWasabi-LC3 ($GFP^{+}RFP^{+}$) in ZnPPIX-treated HeLa cells. Meanwhile, electron microscopy images showed that most of the ZnPPIX-induced cells contained autophagosomes. It has been previously shown that HO-1 may reside within the endoplasmic reticulum, protecting against endoplasmic reticulum stress [19]. Based on these results, it is tempting to speculate that loss of HO-1 activity leads to the activation of autophagy through increased endoplasmic reticulum stress.

Recent findings suggested that unfolded protein accumulation and the autophagy induced by the resulting endoplasmic reticulum stress are related to the activation of p38 MAPK [16]. We have observed p38 phosphorylation in ZnPPIX-treated cells [**Fig. 5(A)**]. In addition, inhibition of p38 activity by SB203580 reduced the autophagic response [**Fig. 5(B,C)**]. These results imply that phosphorylation of p38 MAPK could be an upstream signal that may be responsible for the induction of autophagy in ZnPPIX-treated cells. Meanwhile, we found that ZnPPIX-induced autophagy was Beclin 1-independent [**Figs. 3** & **4**], indicating that p38 MAPK also participates in Beclin 1-independent autophagosome formation. Until now, little has been known about the regulation of Beclin 1-independent autophagy, although several compounds have been reported to induce this non-canonical autophagy in different cells [4–6]. Further research is needed to elucidate the mechanism(s) by which p38 MAPK regulates Beclin 1-independent autophagy.

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