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Mitochondrial autophagy protects against heat shock-induced apoptosis through reducing cytosolic cytochrome *c* release and downstream caspase-3 activation

Ying Yang, Da Xing *, Feifan Zhou, Qun Chen

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China

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ABSTRACT

Autophagy is an evolutionarily conserved process for bulk degradation of cytoplasmic components, including large molecules and organelles. It can either help to enhance or to resist apoptosis, depending on the circumstances. The mechanism of how autophagy impacts apoptosis and the subsequent cellular events upon heat shock remains unclear. In this study, we demonstrate for the first time that mitochondrial membrane permeability transition (MPT)-sensitive mitochondrial autophagy can protect against heat-induced apoptosis through reduction of cytosolic cytochrome *c* release and downstream caspase-3 activation. With confocal microscopy, it was revealed that as autophagosomes increased, mitochondrial content was mass decreased after heat shock. Detailed analysis shows that a single swelling mitochondrion could be entrapped into autophagosome. The depolarization of mitochondria preceded the mitochondrial loss, and both could be abolished by MPT inhibitor cyclosporine (CsA). In addition, along with the decrease of mitochondrial content, the level of total cytochrome *c* was also reduced, resulting in a reduction of its release to cytoplasm. When heat shock was combined with 3-methyladenine (3-MA), an inhibitor of autophagy, the mitochondrial loss and the reduction of total cytochrome *c* were both inhibited, and then caspase-3 activation and cell apoptosis were increased. Thus, it is reasonable to believe that, heat shock-induced cellular events can be modulated by controlling autophagy, and this may represent a novel approach to enhance the efficacy of hyperthermia.

1. Introduction

Hyperthermia as an alternative or supplemental cancer treatment modality has been developed in recent years [1]. Apoptosis is identified as a major cell death pathway after hyperthermia. Similar to many other apoptotic stimuli, hyperthermia utilizes a mitochondria dependent pathway to achieve its cytotoxicity [2]. Hyperthermia can induce mitochondrial depolarization, which results in the release of cytochrome *c* and subsequent activation of the molecular cascade leading to apoptosis.

Hyperthermia can enhance cytotoxic effects of many chemotherapeutic drugs, although its efficacy as a stand-alone modality has been challenged [1]. The weak therapeutic effect of hyperthermia alone is partially attributed to the cellular self-repairing mechanism after heating. The preferential expression of heat shock proteins helps cells to recover from thermal damages [3]. Whether cells have other self-repairing system that is independent of heat shock proteins is unclear.

Autophagy is a sub-cellular process that digests injured or surplus organelles. It is a major degradative pathway in mitochondrial turnover. Researchers use the term mitophagy to refer the degradation of mitochondria by autophagy [4]. The core autophagy machinery is evolutionarily conserved and shared among constitutive and selective autophagy [5]. Depending on the circumstances, inhibition of autophagy may result in opposite outcomes, either promoting or inhibiting apoptosis [6]. Yee et al. have demonstrated that PUMA or Bax-induced selective targeting of mitochondria for autophagy can enhance apoptosis [7]. In contrast, autophagy can delay apoptotic cell death in breast cancer cells following DNA damage [8]. Whether autophagy promotes or inhibits cell apoptosis under heat shock remains uncertain.

In this study, we demonstrate whether autophagy can be induced during the recovery phase after heat shock and its effect on apoptosis. These results may help us to better understand autophagy in response to heat shock, and provide information for future improvement of hyperthermia by modulating the autophagic process.

* Corresponding author. Fax: +86 20 85216052.
E-mail address: xingda@scnu.edu.cn (D. Xing).

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2. Materials and methods

2.1. Chemicals and plasmids

The following fluorescent probes were used: Rhodamine 123 (5 μM, Molecular Probes, Inc., Eugene, OR) to monitor mitochondrial transmembrane potential ($\Delta\psi_m$); MitoTracker Red (100 nM, Invitrogen Life Technologies, Inc.) to label mitochondria. The following reagents were used: 3-methyladenine (3-MA) (10 mM) to inhibit autophagy; cyclosporine (CsA) (5 μM) to inhibit mitochondrial membrane permeability transition (MPT); rapamycin (50 nM) to induce autophagy. The caspase-3 activity kit was obtained from Beyotime Institute of Biotechnology (Haimen, China). The plasmid, GFP-LC3, a kind gift from Professor Marja Jäättelä [9], was used to label autophagosome. DsRed-Mit, a selective fluorescence marker for mitochondria, was kindly provided by Prof. Yukiko Gotoh [10]. GFP-cyt *c* was kindly provided by Dr. G.J. Gores [11].

2.2. Cell culture and transfection

Human epithelial carcinoma cells (HeLa) and human lung adenocarcinoma cells (ASTC-a-1) were used for the experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 15% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in 5% CO_2 and 95% air at 37 °C in a humidified incubator. We used Lipofectamine™ 2000 reagent (Invitrogen Life Technologies, Inc., Grand Island, NY) to transfect plasmid DNA into cells. Cells were examined 24–36 h after transfection.

2.3. Heat shock

Cells were cultured in complete medium under heat shock for 1 h at 43 °C followed by recovery at 37 °C. The heat treatment was performed in a precision water bath (±0.1 °C).

2.4. Western blot analysis

After heat shock and various recovery time at 37 °C, cells were scraped from the culture dishes, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and lysed with ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 100 mg/ml PMSF) for 30 min. The lysates were centrifuged at 12,000*g* for 10 min at 4 °C and the protein concentration was determined. Equivalent samples (30 μg protein extract was loaded on each lane) were subjected to SDS–PAGE on 12% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with primary antibody: anti-LC3/anti-β-actin at a dilution of 1:1000, followed by secondary antibodies, IR-Dye®800 anti-rabbit IgG (Rockland, Gilbertsville, PA, USA) and Alexa Fluor 700® goat anti-Mouse IgG (Molecular Probes, OR, USA). Detection was performed using a LI-COR Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE) [12].

2.5. Imaging analysis of living cells

Fluorescence emissions from GFP, DsRed-Mit, Rhodamine 123, and MitoTracker Red were monitored confocally using a commercial laser scanning microscope combination system (LSM 510/ConfoCor 2, Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3NA Oil DIC objective. The excitation wavelength and the detection filter settings for each of the fluorescent indicators were as follows: GFP and Rhodamine 123 fluorescence was excited at 488 nm with an Ar-ion laser (reflected by a beam splitter HFT 488 nm), and the fluorescence was recorded through a 500–530 nm IR band-pass filter. MitoTracker Red fluorescence was excited at 633 nm with a He–Ne laser, and the fluorescence was recorded through a 650 nm long-pass filter. Cells were stained with Rhodamine 123 and/or MitoTracker for 30 min, and rinsed three times with PBS prior to the fluorescence measurements. DsRed-Mit was excited using a 543 nm He–Ne laser. The fluorescence was recorded through a long pass 560 nm filter. For intracellular measurements, a desired measurement position was chosen in the LSM image. The cells were maintained at 37 °C and in 5% CO_2 throughout each experiment by utilizing a mini-incubator (CTI-Controller 3700, Zeiss) built on the microscope stage. To quantify the results, average emission intensities of a desired measurement position were processed with Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany).

2.6. Detection of cell apoptosis

The activity of caspase-3 was determined using the caspase-3 activity kit, based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) into a yellow formazan product *p*-nitroaniline (pNA). Lysates were centrifuged at 12,000*g* for 10 min, and protein concentrations were determined by Bradford protein assay. Cellular extracts (30 μg) were incubated in a 96-well microtitre plate with 20 ng Ac-DEVD-pNA for 4 h at 37 °C. OD_{405}, the absorbance value at 405 nm, was read with a 96-well plate reader (INFINITE M200, Tecan, Switzerland). An increase in OD_{405} indicated the activation of caspase-3.

Cells were stained with annexin V-fluorescein isothiocyanate (Annexin-V-FITC) and propidium iodide (PI) for flow cytometry analysis. Flow cytometry was performed on a FACSCanto II cytofluorimeter (Becton Dickinson, Mountain View, CA, USA) with an excitation at 488 nm. Fluorescent emission of FITC was measured at 530/30 nm and that of PI at 585/42 nm.

3. Results

3.1. Real-time detection of autophagosome under heat shock

GFP-LC3 is designed specifically to detect autophagosome accumulation [9]. It is reported that the number of GFP-LC3 puncta is directly related to the autophagy activity [13]. The GFP-LC3 expressing cells in the standard control group (no treatment) were exposed to room air at 37 °C for 8 h. The results showed that there was no obvious accumulation of GFP-LC3 puncta during an 8 h recording period. The measurement process itself had negligible effects on GFP-LC3 puncta (Fig. 1A, control). Upon heat shock, an apparent increase in the number of GFP-LC3 puncta was observed at 2 h of recovery and reached its maximum at 3–4 h of recovery in both ASTC-a-1 and HeLa cells (Fig. 1A, heat shock). The average number of GFP-LC3 puncta per cell was quantified after heat shock (Fig. 1B). Taken together, we found that heat shock (43 °C, 1 h) could stimulate autophagy in both ASTC-a-1 and HeLa cells.

To further confirm the results obtained from the fluorescence imaging, western blot assay was used to monitor the conversion of LC3-I to LC3-II (the phosphatidylethanolamine-conjugated form) after heat shock in ASTC-a-1 cells [13]. The conversion of LC3-I to LC3-II became obvious at 3 h than that at 30 min of recovery (Fig. 1C). The results from the western blot experiments were in complete agreement with that from the fluorescence imaging study.

3.2. Heat shock induced mitophagy

Because heat shock can destroy mitochondria [14], we therefore considered the possibility that heat shock-induced autophagy may degrade the damaged mitochondria. In the control group, the mitochondrial red fluorescence was relatively stable, and there was no formation of green GFP-LC3 puncta (Fig. 2A). However, in the heat shock group, the accumulation of autophagosome was in-

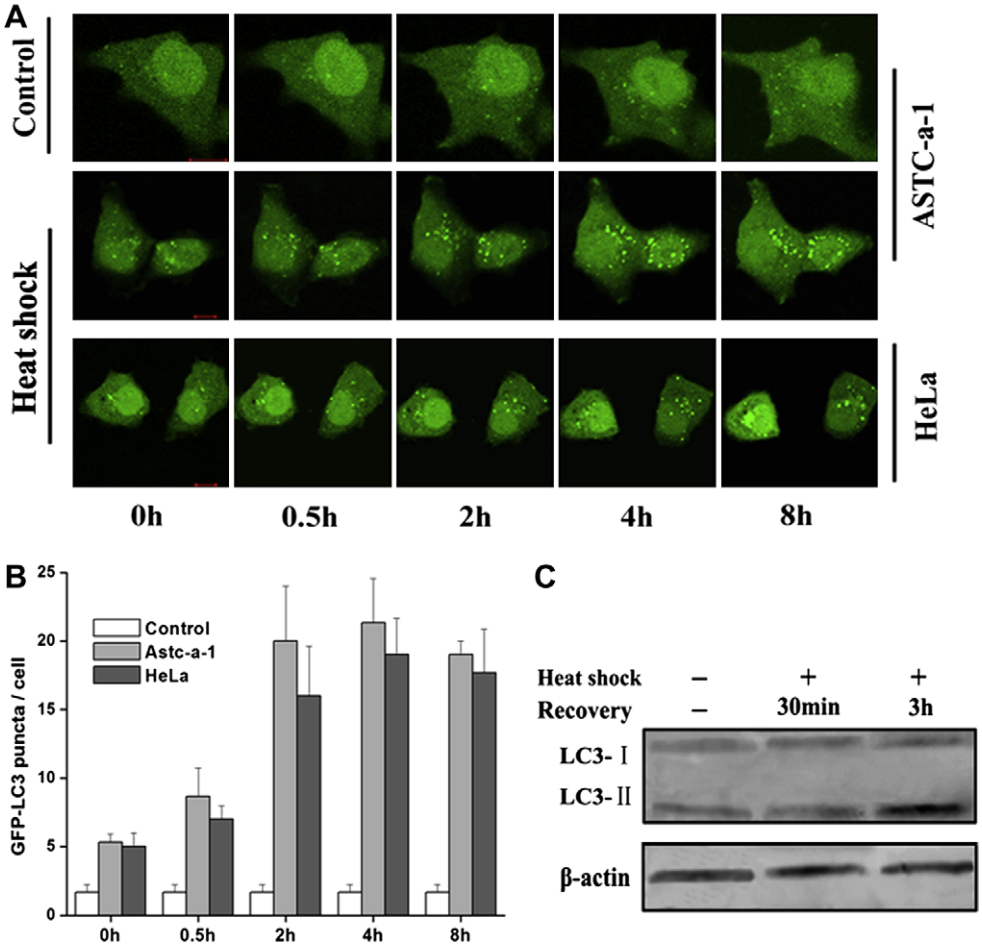

Fig. 1. Real-time detection of autophagosome after heat shock. (A) Heat shock: 43 °C, 1 h; Control: no treatment. Autophagosome formation was indicated with accumulation of GFP-LC3 puncta in HeLa and ASTC-a-1 cells. The time after heat shock was indicated in each part. Bar = 10 μm. (B) The average number of GFP-LC3 puncta per cell (each data point represents a total of 30 cells from five independent experiments for each protocol, mean ± SEM). (C) Western blot analysis of LC3 level in ASTC-a-1 cells. Loading control: β-actin.

creased, along with the decrease of mitochondrial content during recovery time (Fig. 2B). Quantitative analysis of the relative DsRed-Mit fluorescence emission intensity showed the mitochondrial content began to lose as soon as autophagosome accumulation became obvious at 2 h after heat shock. A significant decrease of mitochondrial content was observed at 150 min after heat shock, when autophagy was at its peak (Fig. 2D). These results suggest that mitochondrial loss occurred concurrently with or very rapidly after the onset of autophagy. To investigate the relationship between mitochondrial loss and autophagosome formation, cells were pre-treated with 3-MA for 12 h to inhibit autophagy. The result clearly shows that when autophagy was inhibited the mitochondrial loss did not occur (Fig. 2C). These results indicate that heat shock- induced autophagy results in the mitochondrial loss.

By co-expressing GFP-LC3 and DsRed-Mit, a single swelling mitochondrion entrapped into an autophagosomal vacuole was observed. As shown in Fig. 2E, at 0 min (4 h after heat shock), the red fluorescence of swelling mitochondria (pointed by arrows in A and B) began to superimpose with the green fluorescence of GFP-LC3. This superimposition became successively stronger over the next 2 min. After approximately 7 min, the DsRed-Mit-labeled mitochondria were completely entrapped within the GFP-LC3-labeled autophagosomes. These images directly show the transformation of a single swelling mitochondrion into an autophagosomal vacuole. The fact that the fluorescence of DsRed-Mit not co-localized with GFP-LC3 did not change during the entire 7 min period (Fig. 2E, as pointed by the arrow in C) indicates that the decreased mitochondrial content was not caused by the loss of DsRed-Mit fluorescence emission intensity but by autophagic digestion. Thus, co-localization of GFP-LC3 and DsRed-Mit during recovery time represents mitophagy.

3.3. MPT initiated mitophagy under heat shock

Recent evidence suggests a possible involvement of MPT in mitophagy [15]. In order to monitor the effect of MPT on mitophagy under heat shock, cells were stained with Rhodamine 123 and MitoTracker. In the control group (no treatment), mitochondria maintained $\Delta\psi_m$ and had no change in its content during an 8 h recording period (Fig. 3A). In comparison, heat shock caused a significant reduction in $\Delta\psi_m$ and the content of mitochondria (Fig. 3B). However, pre-treating the cells with 3-MA inhibited the decrease of mitochondrial content, but had no inhibitory effect on mitochondrial depolarization as shown in Fig. 3C. To further study the relationship between mitochondrial depolarization and mitophagy, cells were subjected to heat shock in the presence of CsA. The result clearly shows that the mitochondria did not depolarize and its content remained stable (Fig. 3D). These evidences supported that the depolarization of mitochondria played a critical role in mitophagy under heat shock.

The temporal relationship between mitochondrial depolarization and mitophagy after heat shock was quantified by analyzing the relative fluorescence emission intensities from contemporaneous experiments. As shown in Fig. 3B, right, Rhodamine 123 fluo-

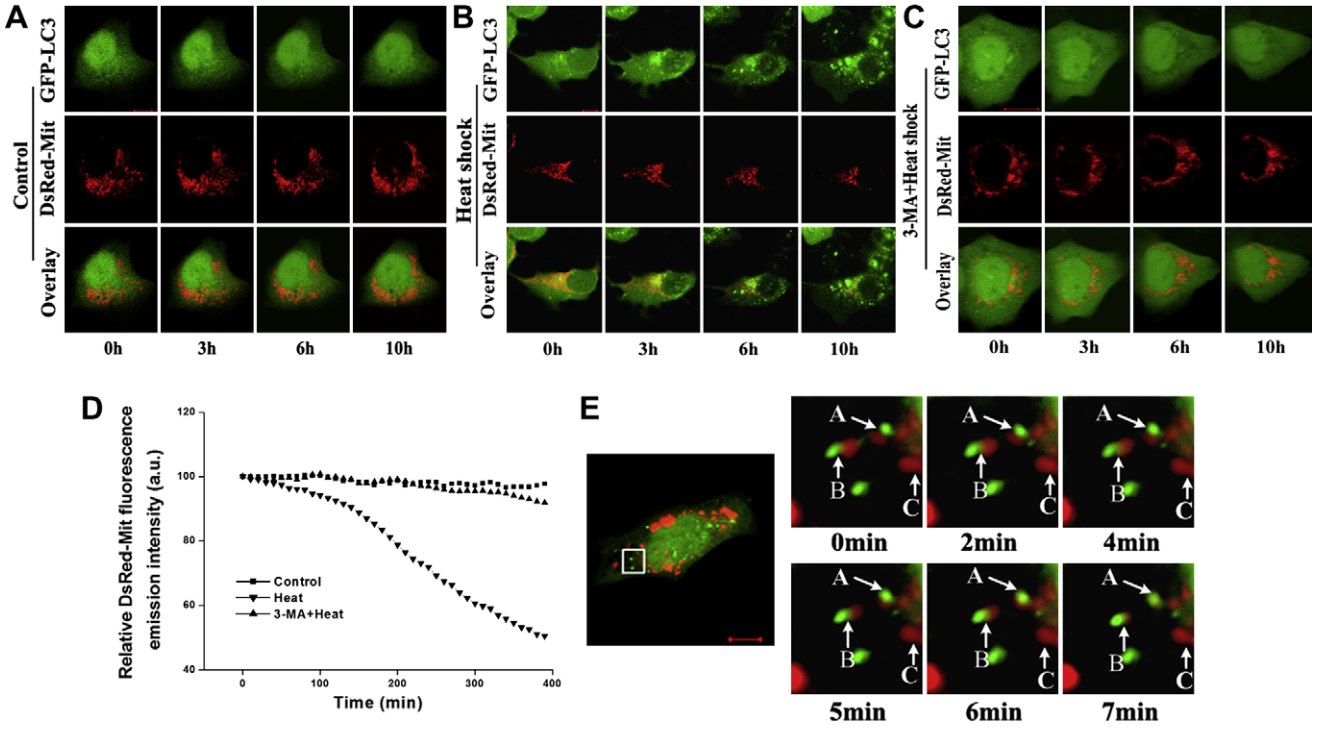

Fig. 2. Heat shock induced mitophagy. (A–C) ASTC-a-1 cells were co-transfected with GFP-LC3 and DsRed-Mit for 24 h. The time after heat shock was indicated in each part. Bar = 10 μm. (A) Control: no treatment. (B) Heat shock: 43 °C, 1 h. (C) Heat shock in the presence of 3-MA (10 mM, pre-treated for 12 h). (D) Quantitative analysis of the relative DsRed-Mit fluorescence emission intensity from contemporaneous experiments above. (E) Autophagic processes of GFP-LC3 puncta engulfed mitochondria at 4 h after heat shock. Arrows (A and B) identify superimposition of autophagosomal vacuoles (green) with mitochondria (red). Arrow (C) indicates the loss of mitochondria was not caused by the loss of DsRed-Mit fluorescence emission intensity. Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

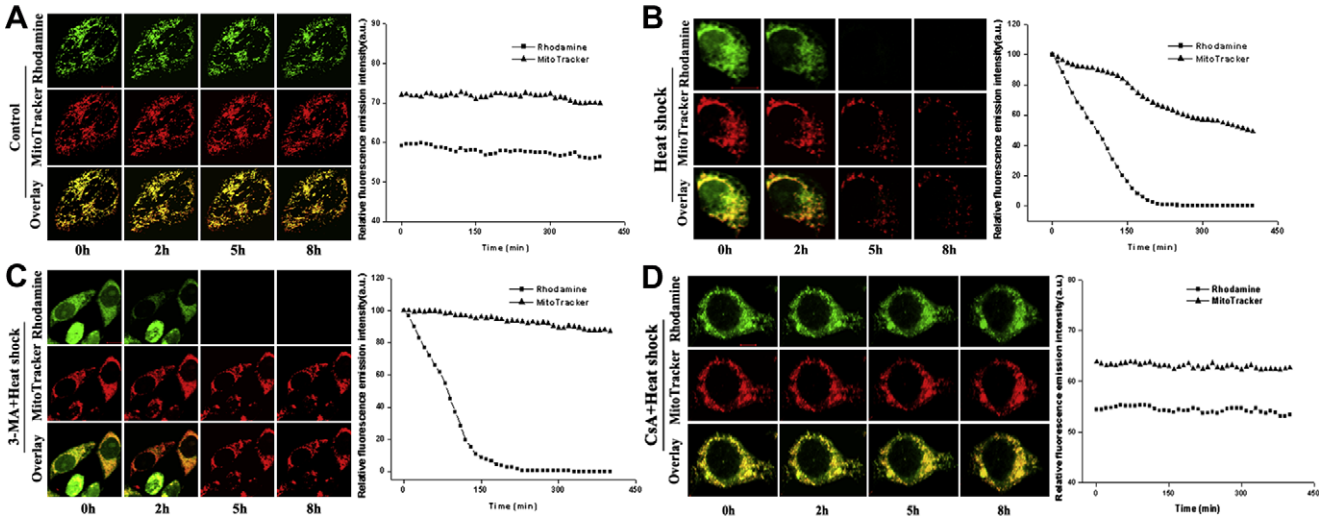

Fig. 3. MPT initiated mitophagy under heat shock. (A–D) ASTC-a-1 cells were stained with Rhodamine 123 (green) and MitoTracker (red). The time after heat shock was indicated in each part. Bar = 10 μm. (A) Control: no treatment. (B) Heat shock: 43 °C, 1 h. (C) Heat shock in the presence of 3-MA, (10 mM, pre-treated for 12 h). (D) Heat shock in the presence of CsA (5 μM, pre-treated for 6 h). Quantitative analysis of the fluorescence emission intensity is presented next to the corresponding image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rescence fell below 90% of its initial level within 150 min when the fluorescence of MitoTracker began to decrease rapidly. Mitophagy was increased markedly after mitochondrial dysfunction. It suggests that mitochondrial depolarization occurred prior to mitophagy.

Taken together, mitochondrial depolarization initiates the selective mitochondrial autophagy under heat shock. Inhibition of autophagy does not affect mitochondrial membrane potential, while inhibition of MPT prevents the emergence of mitophagy. Therefore we considered that MPT is an underlying mechanism of mitophagy under heat shock.

3.4. Mitophagy protected against heat shock-induced apoptosis through reducing cytosolic cytochrome c release and downstream caspase-3 activation

As described above, autophagy is a route for the clearance of damaged mitochondria, the potential effects of autophagy on

apoptosis was then investigated. Cells were transfected with GFP-cyt *c* for monitoring the dynamic of cytochrome *c* and stained with MitoTracker to label mitochondria. In the control group, (no treatment), spatial distribution of cytochrome *c* was relatively unchanged in mitochondria (Fig. 4A). When cells were subjected to heat shock, the content of mitochondria and the level of mitochondrial protein cytochrome *c* were both reduced (Fig. 4B). The results of the quantitative analysis show that both GFP-cyt *c* and MitoTracker fluorescence emission intensities were decreased significantly at about 150 min after heat shock. This was in concurrence with the peak of autophagy. Therefore, autophagy could be able to degraded not only mitochondria but also mitochondrial protein cytochrome *c*, which caused decreased release of cytochrome *c* to cytosol. When cells were pre-treated with 3-MA, the release of cytochrome *c* from mitochondria to cytosol was enhanced at approximately 3 h after heat shock, and the two fluorescence emission intensities remained stable like that in control group (Fig. 4C). 3-MA did not influence cytochrome *c* release, but could inhibit the mitochondrial loss and the reduction of its protein cytochrome *c*. These data indicate that mitophagy could reduce the level of mitochondrial protein cytochrome *c* and its release to cytosol under heat shock.

We next examined whether the reduced mitochondrial content and the cytochrome *c* release could attenuate the mitochondria-dependent apoptotic pathway after heat shock. Caspase-3 has been shown to play a pivotal role in the execution phase of apoptosis induced by diverse stimulus [16]. The activity of caspase-3 was detected at 12 h after heat shock. It was significantly decreased when pre-treated with rapamycin and increased when pre-treated with 3-MA compared to the heat shock alone group (Fig. 4D). These results show that rapamycin inhibited caspase-3 activity due to increased autophagy which reduces damaged mitochondria. 3-MA significantly increased caspase-3 activity by preventing autophagy.

To further confirm autophagy is a survival mechanism for cells under heat shock, the effects of increased/decreased autophagy on apoptosis were evaluated. We pre-treated cells with rapamycin or 3-MA for 12 h, followed by induction of apoptosis with heat shock. With the heat alone treatment, 34.7% cells became apoptotic. With pre-treatments of rapamycin or 3-MA, the apoptotic cell populations were either lowered to 13.5% or increased to 68.3%, respectively (Fig. 4E).

Taken together, autophagy is a pro-survival pathway in our system. Furthermore, the autophagy effect on apoptosis is mediated via degradation of damaged mitochondria, which in turn reduces the release of cytochrome *c* from mitochondria to cytosol and the activity of caspase-3.

4. Discussion

We have demonstrated for the first time that MPT-sensitive mitophagy protects against heat-induced apoptosis by reducing cytochrome *c* release and downstream caspase-3 activation. Autophagy is a cytoprotective mechanism under heat shock.

It has been shown that hyperthermia alone typically has insufficient efficacy to be a primary cancer treatment modality, partially due to cells initiating their self-repairing system during recovery time after heat shock [1]. A better understanding of the self-repairing mechanism would be helpful for maximizing the efficacy of hyperthermia. Our study demonstrates a new self-repairing mechanism that contributes to heat shock protection against apoptosis. Heat shock can induce and promote autophagy to inhibit apoptosis (Figs. 1 and 4).

Depending on the circumstance, the effect(s) of autophagy on cell apoptosis can be complicated. Our study demonstrates that autophagy can protect against apoptosis under heat shock. An in-depth analysis shows that autophagy can degrade not only mitochondria but also mitochondrial protein cytochrome *c*, and subsequently reduces the release of cytochrome *c* into cytosol after heat shock (Figs. 2 and 4). Based on these results, it is believed that heat-induced autophagy can protect cells against apoptosis. This was further validated by detecting the activity of caspase-3 and apoptosis (Fig. 4).

When autophagy was inhibited by pre-treating cells with 3-MA, we observed an increased levels of cytochrome *c* release and caspase-3 activation compared to those in the cells subjected to heat shock alone (Fig. 4). To improve the efficacy of hyperthermia, clinical investigators have studied a variety of drugs to act as thermal sensitizers to improve cancer cell kill [1]. Our results

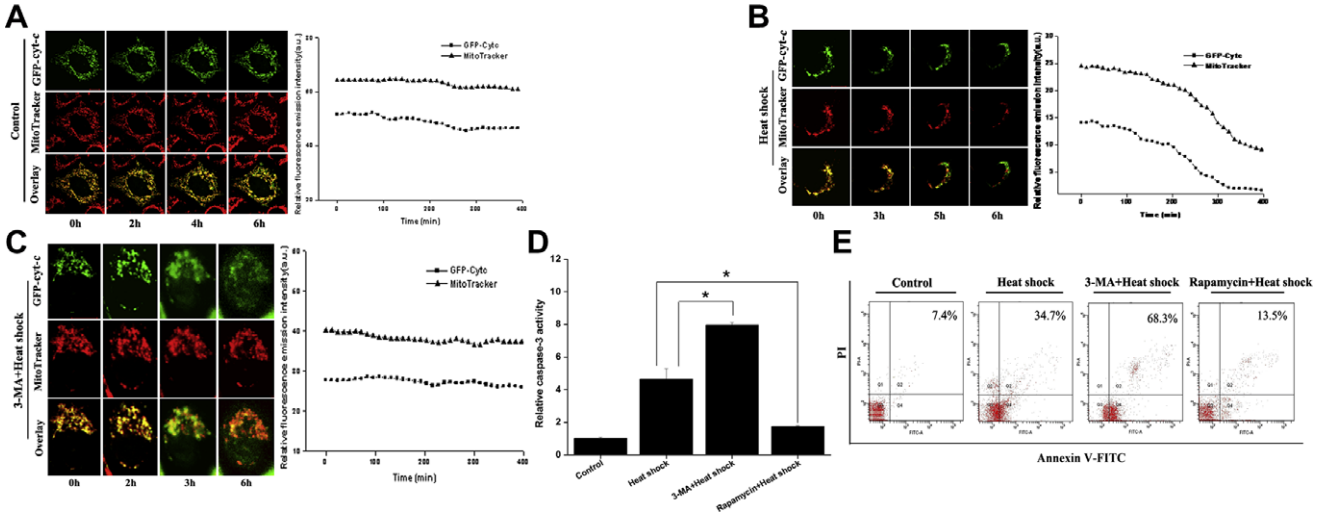

Fig. 4. Mitophagy protected against heat shock-induced apoptosis. (A–C) ASTC-a-1 cells were transfected with GFP-cyt *c* and stained with MitoTracker for mitochondrial localization. The time after heat shock was indicated in each part. Bar = 10 μm. (A) Control: no treatment. (B) Heat shock: 43 °C, 1 h. (C) Heat shock in the presence of 3-MA, (10 mM, pre-treated for 12 h). Quantitative analysis of the fluorescence emission intensity is presented next to the corresponding image. (D) Heat shock induced caspase-3 activation in ASTC-a-1 cells. Cells were pre-treated with rapamycin (50 nM) or 3-MA (10 mM) for 12 h and then treated with heat shock (43 °C, 1 h). Relative caspase-3 activity was calculated as a ratio of fluorescence intensity of treated cells to control cells, and the value of control was set to 1. Results are shown as mean ± SEM from four independent experiments for each protocol. $^{*}P < 0.05$. (E) Apoptosis of ASTC-a-1 cells were determined by flow cytometry (FCM). Cells were pre-treated with rapamycin (50 nM) or 3-MA (10 mM) for 12 h followed by heat shock (43 °C, 1 h). Cell distribution was analyzed with Annexin-V-FITC and PI uptake. Numbers indicated the percentage of the cells present in the sums of Q2 and Q4 area. Control group received no treatment.

suggest that, 3-MA may be an efficacious *in vitro* sensitizer. Clearly, its potential in clinical applications requires further detailed investigations.

Mechanisms regulating mitophagy in response to autophagic stimuli have been reported recently [17]. Yee et al. demonstrated PUMA functions through Bax to induce mitophagy. In our study, we found that MPT can induce mitophagy under heat shock (Fig. 3). How heat stimulation signals the MPT in mitochondria remains to be elucidated. Bax is a pro-apoptotic member of the Bcl-2 protein family that resides in the outer mitochondrial membrane. Bax promotes cell death directly through its activation and oligomerization on the mitochondrial outer membrane to serve as a channel protein [18]. Heat can directly induce the oligomerization of Bax and Bak [19], which may confer an ability to permeabilize mitochondria to induce cytochrome *c* release. Future studies are necessary to evaluate these hypothetical mechanisms by which heat stimulation induces MPT.

Although the existence of mitophagy has been known for some time, it remains unclear whether mitochondria are randomly or selectively degraded by autophagosomes under heat shock. In this paper, when cells were exposed to heat, MPT occurred. Swelling mitochondria were targeted for degradation (Figs. 2 and 3). Mitochondria underwent CsA-sensitive MPT prior to engulfment. This suggests that damaged mitochondria were targets for autophagosomal removal. These findings imply that mechanisms exist to specifically target dysfunctional mitochondria for removal by autophagy. The underlying pathway(s) regulating the process is yet to be explored.

In conclusion, we demonstrate for the first time that mitophagy as a new self-repairing mechanism contributes to heat shock protection against apoptosis. With further investigation, it may provide an alternative approach to improve the efficacy of hyperthermia by modulating the autophagy.

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