**Inhibition of autophagy promotes apoptosis and enhances anticancer efficacy of adriamycin via
augmented ROS generation in prostate cancer cells**

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Abstract

The interplay between autophagy and apoptosis response to chemotherapy is still a subject of intense debate in recent years. More efforts have focused on the regulation effects of apoptosis on autophagy, whereas how autophagy affects apoptosis remains poorly understood. In this study performed on prostate cancer cells, we investigated the role of autophagy in adriamycin-induced apoptosis, as well as the mechanisms mediating the effects of autophagy on apoptosis response to adriamycin (ADM). The results show that ADM not only inhibited cell viability and enhanced apoptosis, but also promoted autophagy via PI3K/Akt(T308)/mTOR signal pathway. Inhibition of autophagy by either pharmacological inhibitor chloroquine (CQ) or RNA interference of Atg5 increased ADM-induced apoptosis and enhanced the chemosensitivity of prostate cancer cells. Moreover, blockade of autophagy augmented reactive oxygen species (ROS) generation induced by ADM. Scavenging of ROS by antioxidant N-acetyl-cysteine (NAC) reversed the strengthened effects of CQ on ADM-induced apoptosis and rescued the cells from apoptosis. The results identified ROS as a potential mediator directing the modulation effects of the protective autophagy on apoptosis response to ADM. Suppression of the protective autophagy might provide a promising strategy to increase the anticancer efficacy of agents in the treatment of prostate cancer.

Abbreviations: ADM, adriamycin; ADT, androgen deprivation therapies; CQ, chloroquine; CRPA,

castration-resistant prostate cancer; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HRP, horseradish peroxidase; MTT, methylthiazole tetrazolium; NAC, antioxidant N-acetyl-cysteine; PCa, prostate cancer; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; ERK1/2, extracellular signal-regulated kinase 1/2; Akt, protein kinase B; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species.

1. Introduction

Prostate cancer (PCa) is the most common type of primary malignant tumor in men, with appropriately 15% of men suffering from PCa during their lifetime (Howlader et al. , 2014). Androgen deprivation therapies (ADT) are used for PCa initial treatment via decreasing the circulating levels of androgen. However, most patients receiving ADT will gradually progress to castration-resistant prostate cancer (CRPA), and chemotherapeutics become the main treatment strategies. Various agents including adriamycin, cisplatin, docetaxel and cabazitaxel have been applied to treat CRPA. Unfortunately, reduced drug sensitivity was commonly observed in patients initially responding sensitivity to the chemotherapy. Several key mechanisms have been identified as the reasons for decreased drug efficacy (Armstrong and Gao, 2015, Thadani-Mulero et al. , 2014, Xie et al. , 2008, Zhu et al. , 2013). How to improve the therapeutic efficacy of the agents is an urgent issue to be solved in clinic.

It is well known that apoptosis induced by chemotherapeutic drugs such as adriamycin is a pivotal mechanism of anti-cancer treatment (Amantana et al. , 2004, Tu et al. , 1995). Anti-apoptosis is one of the mechanisms by which cancer cells become resistant to anticancer drugs (Zhang et al. , 2015). Resensitizing cancer cells to the drugs is a promising approach to circumvent the chemoresistance to improve the anticancer efficacy (Semenas et al. , 2012). Recently, accumulating evidences have shown that autophagy, a cellular process contributes to the maintenance or restoration of cellular homeostasis (Mizushima and Levine, 2010, Sui et al. , 2013), plays an essential role in determining the response of cancer cells exposed to the chemotherapeutic stresses (Ouyang et al. , 2012, Xu and Zhang, 2011). Although crosstalk between autophagy and apoptosis has been reported in recent studies, the mutual

regulation between each other remains to be defined (Mukhopadhyay et al. , 2014, White, 2015). Understanding the exact role and the related mechanisms of autophagy in apoptosis may lead to develop a promising therapeutic strategy to enhance anti-apoptosis effects of chemotherapy and therefore improve the clinical therapeutic efficacy.

Adriamycin (ADM) is a chemotherapeutic agent which is widely used for treatment of multiple malignant tumors including PCa via inducing apoptosis of cancer cells. However, little is known about how autophagy contributes to apoptosis response to ADM in prostate cancer cells. In the present study, we first attempt to define the role of autophagy in the apoptosis of prostate cancer cells exposed to ADM. The mechanisms mediating the effects of autophagy on the ADM-induced apoptosis were further determined so as to provide the evidence for improving the therapeutic efficacy of agents via increasing apoptosis by autophagy modulation.

2. Materials and methods

2.1. Materials

Adriamycin (ADM) was purchased from Sangon Biotech (Shanghai, China). The viability assay reagent MTT was purchased from Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Lipofectamin[®]RNAiMAX were purchased from Invitrogen (Carlsbad, CA, USA). AnnexinV-FITC/PI apoptosis kit was purchased from MultiSciences (Hangzhou, China). Chloroquine (CQ) was the product of Sigma-Aldrich (St. Louis, MO, USA). The polyvinylidenedifluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA). The primary antibodies including anti-phospho-Erk1/2(T202/Y204) (cat no. 4370), anti- Erk1/2(T202/Y204) (cat no. 4695) anti-phospho-Akt(S473) (cat no. 4060), anti-phospho-Akt(T308) (cat no. 9275), anti-phospho-Akt (cat no. 4691), anti-phospho-p70S6K(T389) (cat no. 9234), anti-LC3B (cat no. 3868), anti-p62 (cat no. 5114), anti-Atg5 (cat no. 9980), anti-Becclin1 (cat no. 3495), anti-GAPDH (cat no. 2118), and horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Reactive Oxygen Species Assay Kit and N-acetyl-L-cysteine (NAC) were purchased from Beyotime (Shanghai, China). The mitochondrial ROS-specific fluorescent probe MitoSOX Red was the product of Invitrogen (Carlsbad, CA, USA).

Atg5 siRNA (5'-GAGACAAGAAGACAUUAGU-3') and the scramble siRNA (sc siRNA) were synthesized by Ribobio (Guangzhou, China).

2.2. Cell culture

TRAMP-C2 is a transgenic mouse cell line of prostate origin containing SV40 viral DNA sequences. LNCaP is a human prostate cancer cell line derived from left supraclavicular lymph node. Both cell lines were kept by the Institute of Tissue Transplantation and Immunology of Jinan University, cultured in DMEM containing 10% FBS and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.3. RNA interference

TRAMP-C2 and LNCaP cells were allowed to grow to 40%-50% confluence, and transfected with Atg5 siRNA or the sc siRNA using Lipofectamin[®]RNAiMAX reagent. After transfection for 48 h, the cells were harvested for western blot analysis of the knockdown efficiency.

2.4. Cell viability assays

TRAMP-C2 and LNCaP cells were seeded in 96-well plates at a density of 3×10^3 and 5×10^3 cells per well, respectively, and allowed to attach overnight. The cells were treated with serial dilutions of ADM, or CQ combined with ADM for 48 h. For Atg5 siRNA group, cells were transfected with siRNA 6 h prior to ADM treatment. Each group was set with three parallel wells. The cell viability was determined by the methylthiazole tetrazolium (MTT) colorimetric assay. Briefly, MTT reagent was added to each well and cultivated for 4 h. After the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) for 10 min, the number of viable cells was determined by detection of the absorbance at 570 nm.

2.5. Apoptosis assay by flow cytometry

TRAMP-C2 and LNCaP cells were plated in 12-well plates at densities of 7×10^5 and 1.2×10^6 cells per well, respectively, in DMEM media with 10% FBS and were allowed to attach overnight. The cells were treated with serial dilutions of ADM, or CQ plus with ADM for 48 h. For Atg5 siRNA group, cells were transfected with siRNA 6 h prior to ADM treatment. The cells were then washed twice with ice cold PBS, resuspended in 500 µL of binding buffer, and stained with AnnexinV-FITC and propidium iodide (PI) as described by the manufacturer's instructions. After incubation for 5 min in the dark at room temperature, the cells were subjected to flow cytometric analysis. The percentages of the

apoptotic cells were analyzed using FCS Express Version 3 software.

2.6. Western blot analysis

For protein analysis, the treated cells were washed twice with cold PBS, and lysed in 1× SDS-PAGE loading buffer. The lysate was clarified by centrifugation at 12,000 g for 10 min at 4 °C to remove the insoluble components, and subjected to separation by 10% SDS-PAGE. The proteins were subsequently transferred to a PVDF membrane, which was further blocked at room temperature for 1 h in TBST (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1 % Tween-20) containing 5% non-fat dry milk, and incubated with anti-phospho-Erk1/2 (T202/Y204), anti-phospho-Akt (S473), anti-phospho-Akt (T308), anti-p70S6K (T389), anti-LC3B, anti-p62, anti-Atg5, anti-Becclin1, and anti-GAPDH primary antibodies separately at 4 °C overnight. The membranes were washed three times with TBST, and incubated with goat anti-rabbit IgG, HRP-linked antibody for 1 h at room temperature. The bands were visualized by an ECL detection kit according to the manufacturer's procedure. The intensity of the bands was quantified by quantity one 4.6 software to determine the relative ratio. GAPDH was used for loading control. The protein concentrations were detected by the method of Bradford (Bradford, 1976).

2.7. Intracellular and mitochondrial ROS detection

TRAMP-C2 and LNCaP cells were treated with ADM, or CQ combined with ADM for 36 h. For removal of the ROS, cells were pretreated with NAC for 1 h before the addition of ADM or CQ plus with ADM. The 2', 7'-dichlorofluorescein diacetate (DCFH-DA, 10 μM) kit and the mitochondrial ROS-specific fluorescent probe MitoSOX Red (2 μM) were respectively applied to determine the intracellular and mitochondrial ROS levels. Briefly, cells were incubated with DCFH-DA for 20 min or with MitoSOX Red for 30 min at 37 °C in the dark, washed with serum-free medium for three times, and subjected to flow cytometric assay. FCS Express Version 3 software and FlowJo 7.6.1 were used to analyze and determine the intracellular and mitochondrial ROS levels, respectively.

2.8. Statistical analysis

The results were analyzed using Graph Pad Prism software 5.01. The student's t-test was used to compare data between two groups, and an oneway ANOVA followed by Tukey's multiple comparison test was used for multiple comparison data. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. ADM reduces cell viability and increases apoptosis in prostate cancer cells

The capability of ADM to influence cell viability of prostate cancer cell lines TRAMP-C2 and LNCaP was evaluated by MTT assay. The results showed that ADM decreased cell viability in a dose-dependent manner. The calculated IC_{50} value of ADM for TRAMP-C2 and LNCaP cells was 406 ng/ml and 1371 ng/ml, respectively (Figure 1A).

To further determine whether the inhibition effect of ADM on cell viability was relevant to apoptosis, AnnexinV-FITC/PI staining combined with flow cytometric analysis was used to detect the influence of ADM on apoptosis. Compared with the control, ADM treatment resulted in a significant increase in apoptotic cell percentage, with the apoptosis rate increased from 6.22% to 33.29% in TRAMP-C2 cells triggered by 200 ng/ml ADM, and from 1.72% to 22.97% in LNCaP cells stimulated with 400 ng/ml ADM (Figure 1B).

3.2. ADM induces autophagy via PI3K/Akt(T308)/mTOR cascade

Autophagy is triggered in response to various stimuli including serum starvation and certain anti-cancer agents. To confirm whether ADM treatment induced autophagy in prostate cancer cells, the expression levels of two major molecular markers of autophagy, LC3B and p62 (Jiang and Mizushima, 2015), were examined by western blotting. Similar to the group treated by serum starvation, ADM at the detected concentrations was found to drive the conversion of LC3B from the cytoplasmic form LC3B-I into the autophagosomic form LC3B-II, and decrease the expression levels of p62 in TRAMP-C2 and LNCaP cells (Figure 2A), demonstrating that ADM induces autophagy in both prostate cancer cell lines.

Since PI3K/Akt/mTOR and MEK/Erk signal pathways are the key axis involved in autophagy regulation by anticancer drugs (Ciuffreda et al. , 2010, Zhao et al. , 2012), we further investigated the effects of ADM on the activation of the signal molecules Akt and p70S6K in PI3K/Akt/mTOR cascade and Erk1/2 in MEK/Erk cascade (Aoki et al. , 2007, Fu et al. , 2016, Hennessy et al. , 2005, Jung et al. , 2010, Laplante and Sabatini, 2009). As shown in Figure 2B, ADM dose-dependently inhibited the basal activation levels of Akt (T308) and p70S6K (T389) in TRAMP-C2 and LNCaP cells, whereas no alteration of p-Erk1/2 (T202/Y204) level was found in cells treated with ADM. Moreover,

while ADM at high concentration (200 ng/ml) suppressed Akt phosphorylation at serine 473 in TRAMP-C2 cells, little inhibitory effect of ADM even at the concentration of 400 ng/ml was observed on the activation of Akt(S473) in LNCaP cells. Because LNCaP cells are more resistant to ADM treatment than TRAMP-C2 cells evidenced by the IC₅₀ value of ADM for LNCaP and TRAMP-C2 cells was 1371 ng/ml and 406 ng/ml, respectively, we speculated that ADM with the concentration up to 400 ng/ml may be insufficient to inhibit Akt activation at serine 473. However, further investigations are needed to determine the reasons for the different response to ADM treatment between the two cell lines. The results suggested that inhibition of PI3K/Akt(T308)/mTOR signal pathway may contribute to the ADM-induced autophagy in prostate cancer cells.

3.3. Inhibition of autophagy enhances the chemosensitivity of prostate cancer cells

Previous studies have suggested that autophagy in response to chemotherapeutic drugs triggers either pro-survival or pro-death (Jain et al. , 2013), which results in decrease or increase of the cell chemosensitivity. In order to determine the exact role of ADM-induced autophagy in the chemosensitivity of prostate cancer cells, the viability of ADM-treated cells at the presence of CQ was assessed by MTT assay. CQ acts as an autophagy inhibitor at the late phase of autophagic flux by affecting the degradation of proteins wrapped in the autophagosome via increase of the lysosomal pH, leading to the accumulation of LC3B-II and p62. The results of western blotting shown in Figure 3A indicated that CQ treatment significantly inhibited the degradation of the basal and ADM-induced LC3B-II and p62, confirming CQ suppresses both basal and ADM-triggered autophagy in TRAMP-C2 and LNCaP cells. Moreover, compared with ADM treatment alone, CQ combined with ADM significantly reduced cell survival. The results suggested that autophagy in response to ADM exerts pro-survival action in prostate cancer cells.

Consistent with the effects of CQ treatment on cell survival, knockdown of Atg5 in cells exposed to ADM expectedly reduced autophagy and decreased cell survival (Figure 3B). Thus, inhibition of autophagy by either pharmacological or genetic approach may augment the anticancer efficacy of ADM in prostate cancer cells via increase of drug sensitivity.

3.4. A blockade to autophagy promotes ADM-induced apoptosis

The survival capacity of cancer cells in response to chemotherapeutic agents is closely related to the

resistance ability of cells against drug-induced apoptosis. To evaluate whether modulation of autophagy affected apoptosis triggered by ADM, Annexin V-FITC/PI double staining was used to examine the effects of autophagy inhibition by either pharmacological or genetic method on apoptosis of cells exposed to ADM. As shown in Figure 4, compared with ADM treatment alone, co-treatment of CQ and ADM enhanced apoptotic population of TRAMP-C2 and LNCaP cells from 18.20% to 30.95% and 13.95% to 21.77%, respectively. Similarly, Atg5 knockdown significantly elevated ADM-induced apoptosis of both cell lines, with the apoptotic population of TRAMP-C2 and LNCaP cells increasing from 32.02% to 57.64% and 15.25% to 24.27%, respectively (Figure 5). The results suggested that suppression of autophagy could be an efficient approach to increase ADM-induced apoptosis, which may reduce the survival capacity of the prostate cancer cells in response to ADM treatment.

3.5. ROS mediates the modulation effects of autophagy on apoptosis response to ADM

In order to determine whether reactive oxygen species (ROS) induced by ADM functioned as the mediator directing the modulation effects of autophagy on apoptosis response to ADM, we first evaluated the effects of autophagy on ADM-induced intracellular and mitochondrial ROS. As shown in Figure 6A-6D, ADM treatment remarkably enhanced ROS levels in TRAMP-C2 and LNCaP cells, and addition of the autophagy inhibitor CQ further strengthened the increase effects of ADM on the intracellular and mitochondrial ROS generation. The free radical scavenging agent NAC significantly reduced the ROS levels triggered by either ADM alone or ADM combined with CQ. We then tested whether modulation of autophagy affected apoptosis response to ADM via alteration of ROS levels. The results displayed in Figure 6E revealed that corresponding to the effects of autophagy on ADM-induced ROS generation, inhibition of autophagy by CQ significantly strengthened the increase effects of ADM on apoptosis in both cell lines, scavenging ROS by NAC not only decreased apoptosis induced by ADM alone, but also counteracted the enhanced effects of CQ on ADM-triggered apoptosis.

4. Discussion

Chemotherapy represents a major treatment strategy for the prostate cancer which has progressed to the hormone-independent stage. In recent years, modulation of autophagy has been reported to be a novel approach for enhancing the chemotherapeutic efficacy (Dikic et al. , 2010, Maycotte and

Thorburn, 2011, Orzechowski et al. , 2015). Since the controversial role of autophagy either in increasing or decreasing the sensitivity of cancer cells exposed to chemotherapy via promoting or suppressing apoptosis has been proposed (Schoenlein et al. , 2009, Sun et al. , 2011), herein we aimed at clarifying the exact effects and the related mechanisms of autophagy on the sensitivity and apoptosis of prostate cancer cells responding to ADM.

In line with previous studies, ADM markedly inhibited cell viability and enhanced apoptosis in prostate cancer cells. We also found that ADM treatment promoted autophagy evidenced by elevated transformation of LC3B-I to LC3B-II and increased degradation of a specific autophagic substrate p62. AKT/mTOR signaling has been demonstrated to be essential in regulating autophagy (Santulli and Totary-Jain, 2013, Yuan et al. , 2014). Consistent with these views, we found that PI3K/Akt(T308)/mTOR rather than MEK/Erk signal pathway involved in ADM-induced autophagy. Further investigations revealed that suppression of autophagy by either the late phase autophagy inhibitor CQ or Atg5 knockdown increased ADM-induced apoptosis and enhanced the chemosensitivity of prostate cancer cells, leading to higher anticancer potency of ADM.

Autophagy and apoptosis are both essential biological processes which could be simultaneously activated by many agents (Kumar et al. , 2014, Pan et al. , 2015). But the relationship between them is complicated and how autophagy affecting apoptosis remains to be fully understood. Recent studies reported that Beclin1, acting as part of the class III phosphoinositide 3-kinase (PI3K) required for the initiation of autophagosome formation, is likely to be the convergent point linking (-)-gossypol-induced autophagy and apoptosis (Lian et al. , 2010). In Hela cells, cleavage of Beclin1 by caspase 3 reduces autophagy and promotes apoptosis (Zhu et al. , 2010). In order to identify the mediator directing the modulation effects of autophagy on apoptosis response to ADM, we first attempt to detect whether ADM triggered the cleavage of Beclin1. The results showed that little effect of ADM administration was observed on Beclin1 cleavage in TRAMP-C2 and LNCaP cells (Supplemental Figure 1), implying that Beclin1 might not contribute to the regulation effects of autophagy on apoptosis response to ADM. It seems that whether Beclin1 serves as the mediator linking autophagy and apoptosis depends on either the stimuli or the cell types.

ROS has been reported to play important roles in maintaining the intracellular homeostasis

(D'Autreaux and Toledano, 2007). Recently, a couple of reports have shown that the development of apoptosis and autophagy obviously depends on the intracellular ROS production (Duan et al. , 2016, Hui et al. , 2016, Su et al. , 2015) and suggested that ROS involves in regulation of both autophagy and apoptosis (Gilliam et al. , 2012, Kong and Zhang, 2013, Liu et al. , 2015). As ADM could induce the generation of ROS via impairing the mitochondrial morphology and function (Demant, 1984, Rutter and Pinton, 2014, Santulli et al. , 2015a, Santulli et al. , 2015b, Xie et al. , 2015), we speculated that ROS may be the mediator directing the modulation effects of autophagy on apoptosis response to ADM. As expected, ROS levels were obviously enhanced by ADM treatment. Inhibition of autophagy intensified the increase effects of ADM on ROS generation and apoptosis. Elimination of ROS with NAC reversed the intensified effects of CQ on ADM-induced apoptosis, implying that ROS might be an essential player mediating the regulation effects of autophagy on apoptosis response to ADM in prostate cancer cells. However, further investigations are required to clarify the precise mechanisms underlying autophagy affecting ADM-induced ROS generation.

In summary, the present study indicated that ADM induces protective autophagy along with apoptosis in prostate cancer cells, and inhibition of autophagy promotes apoptosis and enhances the sensitivity of cells to ADM via augmented ROS levels. Thus, suppression of protective autophagy induced by ADM using either pharmacological or genetic approach might provide a useful strategy to increase the anticancer efficacy of chemotherapy in prostate cancer.

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Conflicts of interest

None of the authors have any relevant conflicts of interest or disclosures.

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Figure legends

Figure 1 ADM reduced cell viability and increased apoptosis in prostate cancer cells. (A) TRAMP-C2 and LNCaP cells were treated with various concentrations of ADM, and cell viability was measured 48 h later using MTT assay. (B) Cells were treated with ADM for 48 h prior to detection of the effects of ADM on apoptosis by annexinV-FITC/PI staining combined with flow cytometric analysis. Data are presented as the mean $\pm$ SD of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

Figure 2 ADM induced autophagy via PI3K/Akt(T308)/mTOR signaling pathway. (A) TRAMP-C2

and LNCaP cells were treated with ADM for 24 h before subjected to analysis of the expression of LC3B and p62 by western blotting. Inducement of autophagy by nutrient deprivation was achieved by serum starvation of cells for 24 h, which was used as the positive control group (SF: serum-free). (B) The phosphorylated levels of Akt (T308), Akt (S473), p70S6K (T389) and Erk1/2 (T202/Y204) were determined by western blot analysis. GAPDH was used as a loading control. Data are presented as the mean $\pm$ SD of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

Figure 3 CQ or Atg5 siRNA enhanced the sensitivity of the prostate cancer cells to adriamycin. (A)

TRAMP-C2 and LNCaP cells were pretreated with an autophagy inhibitor CQ for 2 h prior to incubation with ADM for 48 h, and subjected to analysis of the expression of LC3B and p62 by western blotting and cell viability by MTT assay respectively. (B) After transfected with Atg5 siRNA or sc siRNA for 6 h, TRAMP-C2 and LNCaP cells were treated with ADM for 48 h, and subjected to analysis of the expression of Atg5 and LC3B by western blotting and cell viability by MTT assay respectively. GAPDH was used as a loading control. Data are presented as the mean $\pm$ SD of three independent experiments. * p <0.05, ** p <0.01.

Figure 4 Inhibition of autophagy by CQ increased ADM-induced apoptosis. TRAMP-C2 and LNCaP cells were treated with an autophagy inhibitor CQ for 2 h prior to incubation with ADM for 48 h, and subjected to apoptotic analysis by annexinV-FITC/PI staining combined with flow cytometry. Data are presented as the mean $\pm$ SD of three independent experiments. n.s. = no significance, * p <0.05, ** p <0.01.

Figure 5 Knockdown of Atg5 augmented ADM-induced apoptosis. After transfected with Atg5 siRNA or sc siRNA for 6 h, TRAMP-C2 and LNCaP cells were treated with ADM for 48 h, and subjected to apoptotic analysis by annexinV-FITC/PI staining combined with flow cytometry. Data are presented as the mean $\pm$ SD of three independent experiments. n.s. = no significance, * p <0.05, ** p <0.01, *** p <0.001.

Figure6 ROS mediated the modulation effects of autophagy on apoptosis response to ADM. (A) & (B) TRAMP-C2 and LNCaP cells were treated with CQ for 2 h or NAC for 1 h prior to exposure to ADM for 36 h, and subjected to analysis of the ROS generation by DCFH-DA (10 μ M) or MitoSox (2 μ M) Red probe staining. The fluorescence was imaged with a fluorescence microscope (200 $\times$). (C) &

(D) The intracellular and mitochondrial ROS levels were determined by DCFH-DA (10 μ M) (C) and MitoSox Red (2 μ M) (D) staining followed by flow cytometric analysis, respectively. (E) TRAMP-C2 and LNCaP cells were treated with CQ for 2 h or NAC for 1 h prior to exposure to ADM for 48 h, and subjected to apoptotic analysis by AnnexinV-FITC/PI staining combined with flow cytometry. Data are presented as the mean $\pm$ SD of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

Supplemental Figure 1 Effects of ADM on the cleavage of Beclin1. TRAMP-C2 and LNCaP cells were treated with ADM for 48 h followed by detection of the cleavage of Beclin1 by western blotting. GAPDH was used as a loading control.

Supplemental Figure 2 Effects of ADM on the generation of ROS. TRAMP-C2 and LNCaP cells were exposed to ADM for 12 h, 24 h, 36 h and 48 h, and subjected to flow cytometric analysis of ROS generation by DCFH-DA probe staining.

figure 1

figure 2

figure 3

figure 4

figure 5

figure 6