CELL CYCLE
2022, VOL. 21, NO. 15, 1651–1661
https://doi.org/10.1080/15384101.2022.2060655

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RESEARCH PAPER

Pannexin1 inhibits autophagy of cisplatin-resistant testicular cancer cells by mediating ATP release

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ABSTRACT

Pannexin1 (Panx-1) is a gap junction channel protein that mediates the release of intracellular ATP during autophagy, and thus plays an important role in tumor cell apoptosis and chemo-resistance. However, the role of Panx-1 in cisplatin-resistance of testicular cancer cells remains unclear. We found that cisplatin-resistant I-10 testicular cancer cell lines (I-10/CDDP) autophagy-associated proteins (p62, p-mTOR, mTOR and LC3) exhibited high levels of autophagy in their expression, while LC3-II expression was more significantly in the presence of lysosomal degradation blocked by chloroquine (CQ). Xenograft models using I-10/CDDP cells with knockdown ATG5 and ATG7 were established in mouse models and showed blockade of autophagic flux and inhibition of tumor growth. In addition, inhibition of Panx-1 by carbenoxolone (CBX) and probenecid (PBN), as well as shRNA-mediated knockdown promoted autophagy in the I-10/CDDP cells, which was accompanied by a decrease in the levels of extracellular ATP. In contrast, overexpression of Panx-1 decreased autophagy of I-10/CDDP cells and increased extracellular ATP levels. To further determine the effect of panx-1-mediated ATP release on the autophagy of I-10/CDDP cells, apyrase was used to hydrolyze the extracellular ATP. Apyrase promoted autophagy in I-10/CDDP cells city by decreasing extracellular ATP, regardless of Panx-1 expression. This study demonstrated for the first time that Panx-1-mediated ATP release inhibits autophagy of I-10/CDDP cells, which provides a potential therapeutic strategy for cisplatin-resistant testicular cancer.

ARTICLE HISTORY
Received 28 May 2021
Revised 11 January 2022
Accepted 28 March 2022

KEYWORDS
Pannexin-1; autophagy; testis

1. Introduction

Testicular cancer is the most common solid tumor of the urinary system, and accounts for 1% of all diagnosed cancers in men. It is generally sensitive to radiotherapy and chemotherapy, although 20–30% of the patients are resistant to standard chemotherapy [1]. Cisplatin (CDDP) is routinely used to treat testicular cancer but often leads to acquired resistance and treatment failure. Therefore, it is essential to identify novel therapeutic targets for cisplatin-resistant testicular cancer cells.

Autophagy is a "self-degradation" process that recycles damaged organelles and misfolded proteins during stress conditions such as nutritional deficiency, hypoxia, cell reconstruction etc [2]. While a basal level of autophagy maintains cell homeostasis and function, it is rapidly accelerated during cellular stress to ensure survival [3]. In addition, autophagy is essential for maintaining amino acid and ATP levels during acute amino acid starvation [4]. Recent studies have also implicated autophagy as an important factor in drug resistance [5]. Pan *et al.* demonstrated that TRIM65 mediates autophagy and cisplatin (CDDP) resistance of A549/CDDP cells by regulating the expression of miR-138-5p [6]. However, it is unclear whether ATP affects autophagy of cisplatin-resistant testicular cancer cells.

Pannexins are a newly discovered family of mammalian gap junction proteins and comprise of Panx-1, Panx-2 and Panx-3 subtypes [7]. Pannexin channels are involved in immunogenic cells death (ICD), autophagy, apoptosis, necroptosis, and proliferation, and their main function is to release ATP and other important metabolites through autocrine/paracrine signals [8]. The secretion of ATP during immunogenic cell death

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(ICD) is largely dependent on the molecular mechanism of autophagy. Furthermore, ATP release is mediated by lysosome-associated membrane protein 1 (LAMP1) and Pannexin-1 (Panx-1)-dependent liposomal exocytosis [9]. Cuervo et al. found that pannexin binds to Atg16 and inhibits autophagy [10], and knocking down Panx-1 increased the production of autophagosomes and the autophagy marker protein LC3 in mouse osteoblasts [11]. However, the molecular relationship between Panx-1-mediated ATP release and autophagy remains unclear.

In a previous study, we found that Panx-1 sensitized the cisplatin-resistant testicular cancer cells (I-10/CDDP) to apoptosis via the ATP/IP3 pathway [12]. However, the molecular mechanisms regulating autophagy in CDDP-resistant testicular cancer cells are not known. Recent studies show that extracellular ATP can increase the level of Ca^{2+} and further activate mTOR, the main regulatory factor of autophagy [13]. In addition, ATP enhances autophagy by inhibiting mTOR levels in the microglia [14]. The aim of the present study was to elucidate the potential role of Panx-1 channels and extracellular ATP levels in the autophagy of I-10/CDDP cells in order to gain new insights into the molecular mechanisms of cisplatin resistance.

2. Materials and methods

2.1 Cell lines and cell culture

The study was conducted in accordance with the BCPT policy for experimental and clinical studies. The mouse Leydig tumor cell line (I-10) was obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in F12 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and penicillin and streptomycin (100 U/ml) at 37°C under 5% CO_2. The DDP-resistant mouse testicular cancer cell line (I-10/CDDP) was established by exposing the I-10 cells to increasing concentrations of CDDP (up to 26 μM) [12].

2.2 Establishment of mouse tumor model

Fifteen male BALB/c-nu (nude) mice (Experimental Animal Center of Bengbu Medical College) were randomly divided into the normal control (NC), Atg5-knockdown (shAtg5) and Atg7-knockdown (shAtg7) groups. The mice were subcutaneously injected with 3.8×10^6 blank plasmid, shAtg5 or shAtg7 I-10/CDDP cells (0.2 ml suspension) as appropriate into their right axilla. The tumor volume in each group was measured weekly, and the mice were euthanized 28 days later. The tumors were removed, weighed and measured to calculate the volume.

2.3 Western blotting

Cells were seeded and exposed to 100 μ m CBX (Sigma Aldrich), 200 μ m PBN (Sigma Aldrich), 10 μmol CQ (Sigma Aldrich) or 25 U/ml apyrase (Sigma Aldrich). Western blotting was performed as per standard protocols. The following primary antibodies were used: rabbit anti-Pannexin1 (1:1000; ab139715; Abcam, Cambridge, MA, USA), anti-LC3 (1:1000; 12,741 T), anti-p62 (1:1000; 5114S), anti-p-mTOR (1:1000; 5536S), anti-ATG5 (1:1000; D5F5U), anti-ATG7 (1:1000; D12B11) (all from Cell Signaling Technology, Danvers, MA, USA), and mouse anti-GAPDH (1:5000; 60,004-1-lg; Proteintech, Chicago, IL, USA). After incubation with HRP-conjugated secondary antibody, the bands were developed using chemiluminescence (Millipore, Billerica, MA, USA), and visualized by the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.4 Luminescence assay

The extracellular ATP concentration was detected using an ATP assay kit (Beyotime biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, the Cells were seeded into 12-well plates at the density of 8×10^3 cells per well in a medium containing 15 μM CDDP (Sigma–Aldrich), and cultured till ~50% confluency. The medium was collected at 24 hours and centrifuged at 400 × g for 1 minute at 4°C. The supernatant (20 μl) was mixed with ATP detection mixture

(100 µl) in an opaque 96-well plate, and incubated at 20°C for 3–5 minutes Luminescence was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Cell transfection

I-10/CDDP cells were transfected with Panx-1-specific shRNA (shRNA1, 5′-GCCACTTCAAGTACCCAATCG-3′; shRNA2, 5-GCATGTATCTACTTGAGCTAT-3′) or scrambled control shRNA (negative control/NC) (5′-GTTCTCCGAACGTGTCACGT-3′) (Genepharma Co. Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). A mock control only treated with Lipofectamine 2000 was also included. In addition, the cells were also transfected with a vector expressing full-length mouse Panx-1 (mPanx-1, GCT AGC GCT ACC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ATC GCC ACC ATG GTG) (Genepharma Co. Ltd., Shanghai, China) or empty vector (NC).

2.6 Transfection with mCherry-GFP-LC3B

The autophagy flux was observed by transfecting the cells with the mCherry-GFP-LC3B plasmid expressing the red fluorescent protein m-Cherry and the green fluorescent protein (GFP) and LC3 fusion protein in target cells [15]. mCherry is a monomer red fluorescent protein from mushroom coral, which can be co labeled with green fluorescent protein (GFP) and LC3 fusion protein. GFP was quenched and mCherry remained stable under lysosomal acid conditions, and then mCherry-GFP-LC3B protein was fused and expressed. After autophagy, autophagosomes fuse with lysosomes, and finally appear in the form of red spots due to partial quenching of GFP fluorescence. Briefly, the cells were seeded into 6-well plates at the density of 5×10^4 cells per well and then transfected with mCherry-GFP-LC3B (Genepharma Co. Ltd., Shanghai, China) plasmid using Lipofectamine 2000. After 48 hours, the cells were treated with CBX and PBN or re-transfected with mPanx-1 or shRNA. GFP-LC3 fluorescence was observed 12 h later under a confocal microscope.

2.7 Transmission electron microscopy

Autophagosomes were observed by transmission electron microscopy. Briefly, the cells were seeded into 6-well plates at the density of 1×10^5 cells per well and treated as above. After washing with phosphate-buffer saline, the cells were fixed with 2.5% glutaraldehyde for 2 h and stored at 4°C.

2.8 Statistical analysis

All data are presented as mean ± standard deviation (SD). SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses and GraphPad Prism 5.0 (GraphPad, CA, USA) was used for plotting graphs. Two-tailed Student's t-test was used to compare two groups, whereas multiple groups were compared by Student–Newman–Keuls (SNK, q value) test and one way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Autophagy blockade in I-10/CDDP cells inhibits ectopic tumor formation in vivo

Our previous findings showed that panx-1 expression was reduced in testicular cancer cell which is resistant to cisplatin [12]. In addition, autophagy has also been correlated with chemoresistance in cancer [16]. Autophagy is a "mobile" process and LC3I and LC3II are dynamic processes of production and degradation. When mTOR is phosphorylated, p-mTOR can inhibit autophagy. Chloroquine can inhibit the degradation of LC3II by inhibiting lysosomal function. As shown in Figure 1, i-10 CDDP cells expressed lower levels of p-mTOR/mTOR and higher levels of p62 and LC3II compared to I-10 cells, while LC3II showed higher levels of expression with CQ treatment (Figure 1(a)), indicating enhanced autophagy in CDDP-resistant testicular cancer cells. To elucidate the relevance of increased autophagy, we knocked down Atg5 and Atg7 in the I-10/CDDP cells using shRNAs (Figure 1(b-c)), and injected

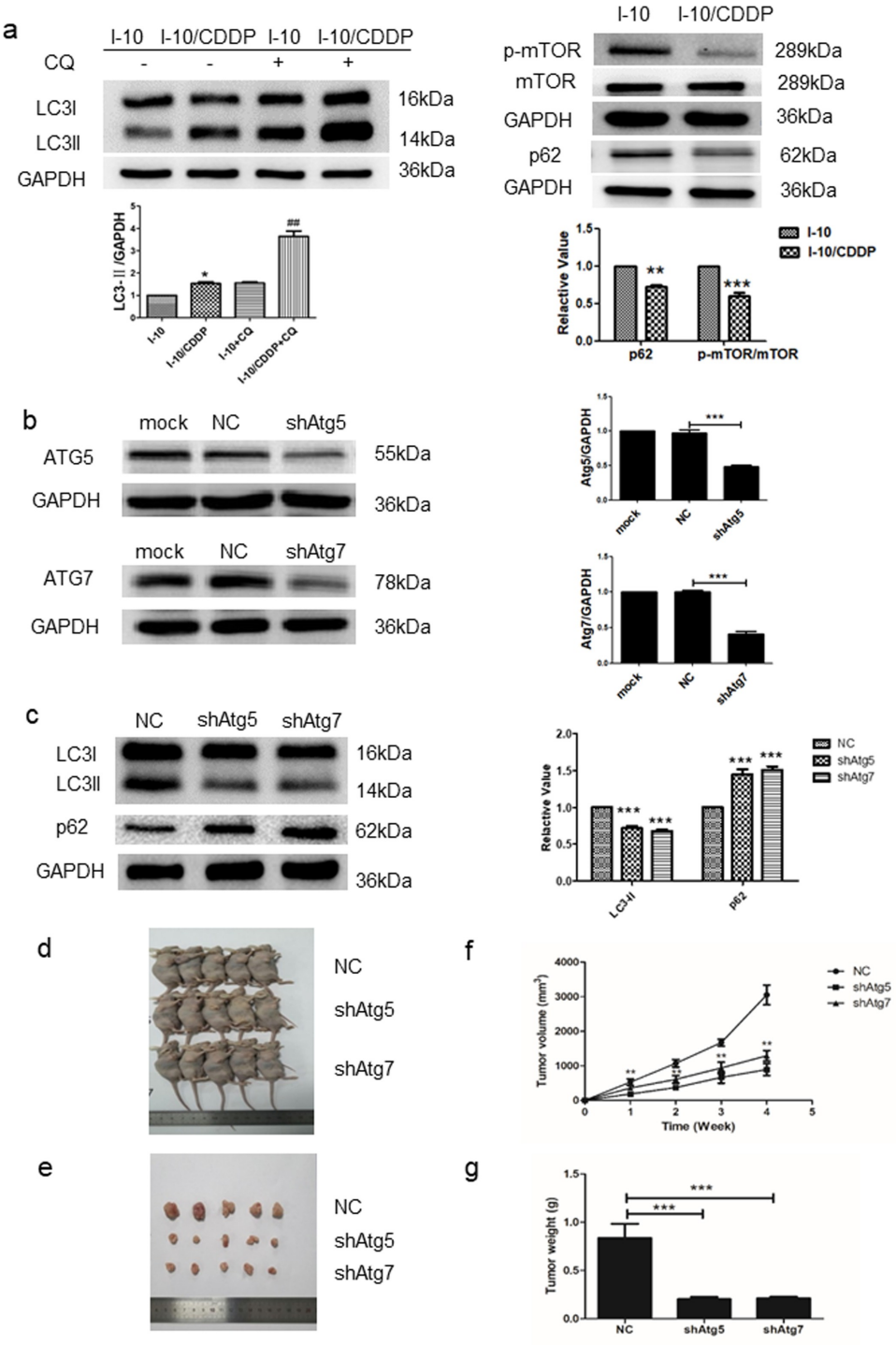

Figure 1. Inhibition of autophagy in I-10/CDDP cells retards tumor growth *in vivo*. (a) Western blots showed expression of p62, p-mTOR, and mTOR in I-10 and I-10/CDDP cells and LC3 in the presence of CQ (10 μmol). (b,c) After silencing atg5 and atg7, the expression levels of ATG5,ATG7, LC3 and p62 in I-10/CDDP cells.(d) Representative images showed nude mice injected with NC, shAtg5 and shAtg7 I-10/CDDP cell lines. (e) Representative images showed tumors isolated from the indicated groups. (f) The 28-day growth curve of tumors in the indicated groups. (g) Tumor weights in the indicated groups. Data are the mean ± SD for 3 experiments each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* I-10 cells and $^{\#\#}P < 0.01$ *vs* I-10+ CQ group.

the stable cell lines into nude mice. Compared to the NC group, the ectopic tumor volume and weight in mice injected with shAtg5 and shAtg7 I-10/CDDP cells were significantly lower (Figure 1 (d-g)). Taken together, CDDP resistance in the I-10 cells is accompanied by increased autophagy, which in turn has a pro-tumorigenic effect.

3.2 CBX and PBN (Panx channel inhibitors) increased autophagy level in I-10/CDDP cells

CBX and PBN (Panx channel inhibitors) significantly inhibited Panx channels and reduced the growth and migration ability of melanoma cells and I-10 [17,18]. To clarify the role of pannexin 1-mediated ATP in the higher autophagy levels in I-10/CDDP cells, CBX and PBN were used to inhibit pannexin channel. As shown in Figure 2 (a), both inhibitors significantly elevated LC3II expression and reduced the expression of p62 and p-mTOR, while LC3II expression was more significantly elevated in the presence of CQ. As shown in Figure 2(b), both inhibitors significantly reduced extracellular ATP levels. Furthermore, mCherry-GFP-LC3 transfection and transmission electron microscopy (TEM) clearly showed an increase in autophagy flux and the number of autophagosomes following treatment with CBX and PBN (Figure 2(c,d)).

3.3 Autophagy levels in I-10/CDDP cells depend on Panx-1 expression

To further explore the Panx-1-induced autophagy in I-10/DDP cells, two independent shRNAs and an expression vector encoding mouse full-length Panx-1 (mPanx-1) were transfected into I-10/DDP cells. The role of Panx-1 channel on the level of autophagy was further elucidated via shRNA-mediated knockdown and full-length panx-1 gene overexpression. As shown in Figure 3(a), Panx-1 expression was decreased in the shRNA-transfected group compared to the NC group. In addition, Panx-1 knockdown significantly reduced extracellular ATP levels in I-10/CDDP cells (Figure 3(b)). Panx-1 knockdown significantly elevated LC3II expression and also significantly decreased p62 and p-mTOR expression in I-10/DDP cells, while the presence of CQ more significantly upregulated LC3II protein expression (Figure 3 (c)). Furthermore, the autophagy flux of GFP-LC3B and autophagosomes were increased in the Panx-1 knockdown cells (Figure 3(d,e)).

Consistent with the above, ectopic expression of Panx-1 (Figure 4(a)) increased extracellular ATP levels in I-10/CDDP cells (Figure 4(b)). Similarly, ectopic expression of Panx-1 significantly decreased the expression of LC3-II and increased the expression of p62 and p-mTOR, while the presence of CQ more significantly downregulated LC3II protein expression (Figure 4(c)). Furthermore, the autophagy flux of GFP-LC3B and autophagosomes were also decreased in the mPanx-1 group (Figure 4(d,e)). Taken together, autophagy of I-10/CDDP cells is regulated by the expression of Panx-1.

3.4 Apyrase increased autophagy in mPanx-1 I-10/CDDP cells

To investigate the effect of extracellular ATP on autophagy in cells overexpressing Panx-1, apyrase was used to deplete extracellular ATP while detecting the concentration of extracellular ATP with luminescence assay. As shown in Figure 5(a), overexpression of Panx-1 significantly increased extracellular ATP levels. The role of extracellular ATP in the autophagy of I-10/CDDP cells was further evaluated using the ATP hydrolase apyrase. Apyrase promoted LC3II expression and reduced p62 accumulation and mTOR expression in Panx-1 overexpressing cells. Apyrase also restored LC3-II expression in the presence of CQ (Figure 5(b)), which corresponded to higher autophagic flux and more autophagosomes in GFP-LC3B (Figure 5(c, d)). Taken together, the Panx-1 channel inhibits autophagy in I-10/CDDP cells by mediating ATP release.

4. Discussion

Chemotherapeutic drugs kill cancer cells by inducing autophagy and apoptosis. However, autophagy can also exert a protective effect and promote the survival of cancer cells [19]. Several studies have shown that chemo-resistant cancer cell lines have higher autophagy levels compared

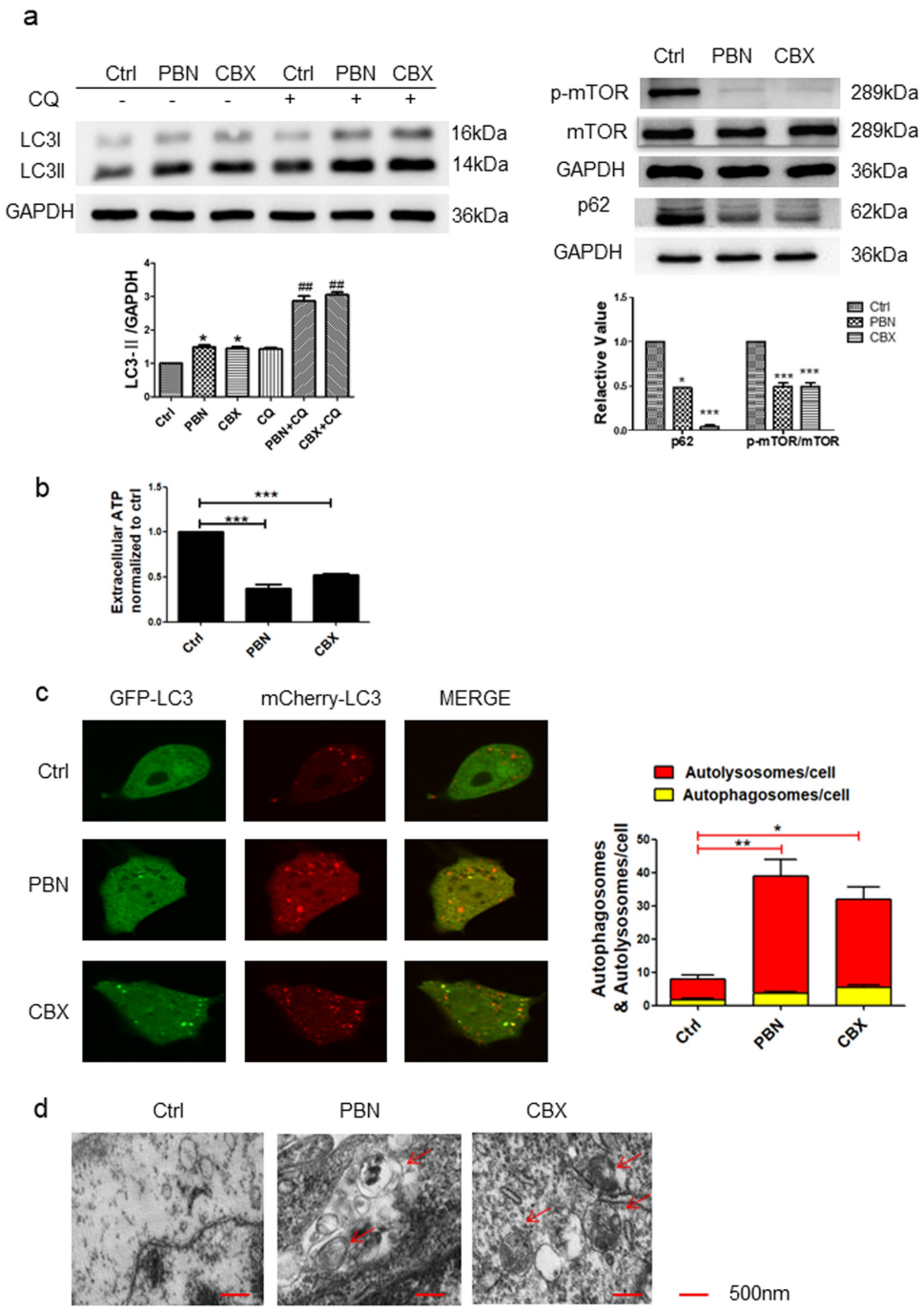

Figure 2. Pannexin-1 inhibitor (CBX and PBN) enhanced autophagy in I-10/CDDP cells. (a) Western blots showed the expression of p62, p-mTOR and mTOR proteins in I-10/CDDP cells after treatment with CBX (100 μM) and PBN (200 μM) and the expression of LC3 protein in the presence of CQ (10 μmol). (b) The extracellular ATP was assessed by Luminescence assay. (c) Representative fluorescence images showed autophagosomes and autolysosomes in the indicated groups (original magnification: × 400). (d) Representative TEM images showed autophagosomes (original magnification: × 15,000). Data are the mean ± SD for 3 experiments each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Ctrl group and $^{\#\#}P < 0.01$ *vs* CQ group.

to the chemo-sensitive cell lines, such as the gefitinib-resistant non-small cell lung cancer and cisplatin-resistant lung cancer cells versus their chemo-sensitive counterparts [20,21]. In our previous study, we showed that Panx-1 promoted the invasion and metastasis in testicular cancer cells [18], and cisplatin-resistance was related to autophagy. In this study, we demonstrated enhanced autophagy in the I-10/CDDP testicular cancer cells compared to the cisplatin-

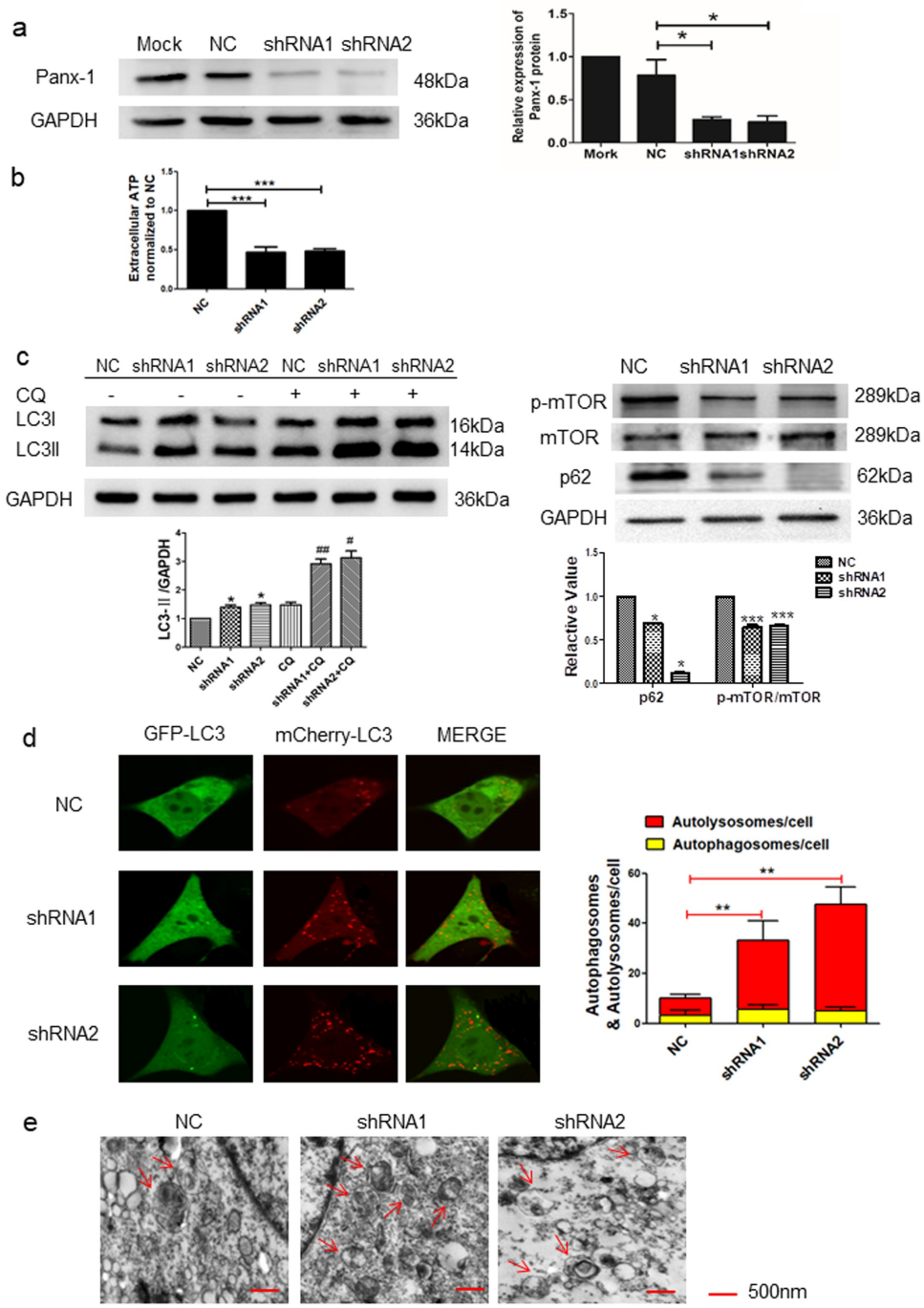

Figure 3. Knockdown of Panx-1 increased autophagy in I-10/CDDP cells. (a) Immunoblots showing Panx-1 expression in Mock, NC and shRNA-Panx-1 I-10/CDDP cells. (b) The extracellular ATP was assessed by Luminescence assay. (c) Western blots showed the expression of p62, p-mTOR and mTOR proteins after knockdown of Panx-1 and the expression of LC3 protein in the presence of CQ (10 μmol). (d) Representative fluorescence images showed autophagosomes and autolysosomes in the indicated groups (original magnification: × 400). (e) Representative TEM images showed autophagosomes (original magnification: × 15,000). Data are the mean ± SD for 3 experiments each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NC group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* CQ group.

sensitive I-10 cells, and found that Panx-1 inhibited autophagy by increasing ATP release.

ATP is an important signaling molecule involved in intercellular messaging. A decrease in intracellular ATP levels and the resulting change in ATP/AMP ratio activates AMPK, which antagonizes p-mTOR and regulates autophagy and apoptosis of A549 cells [22]. In addition, autophagy also promotes ICD by

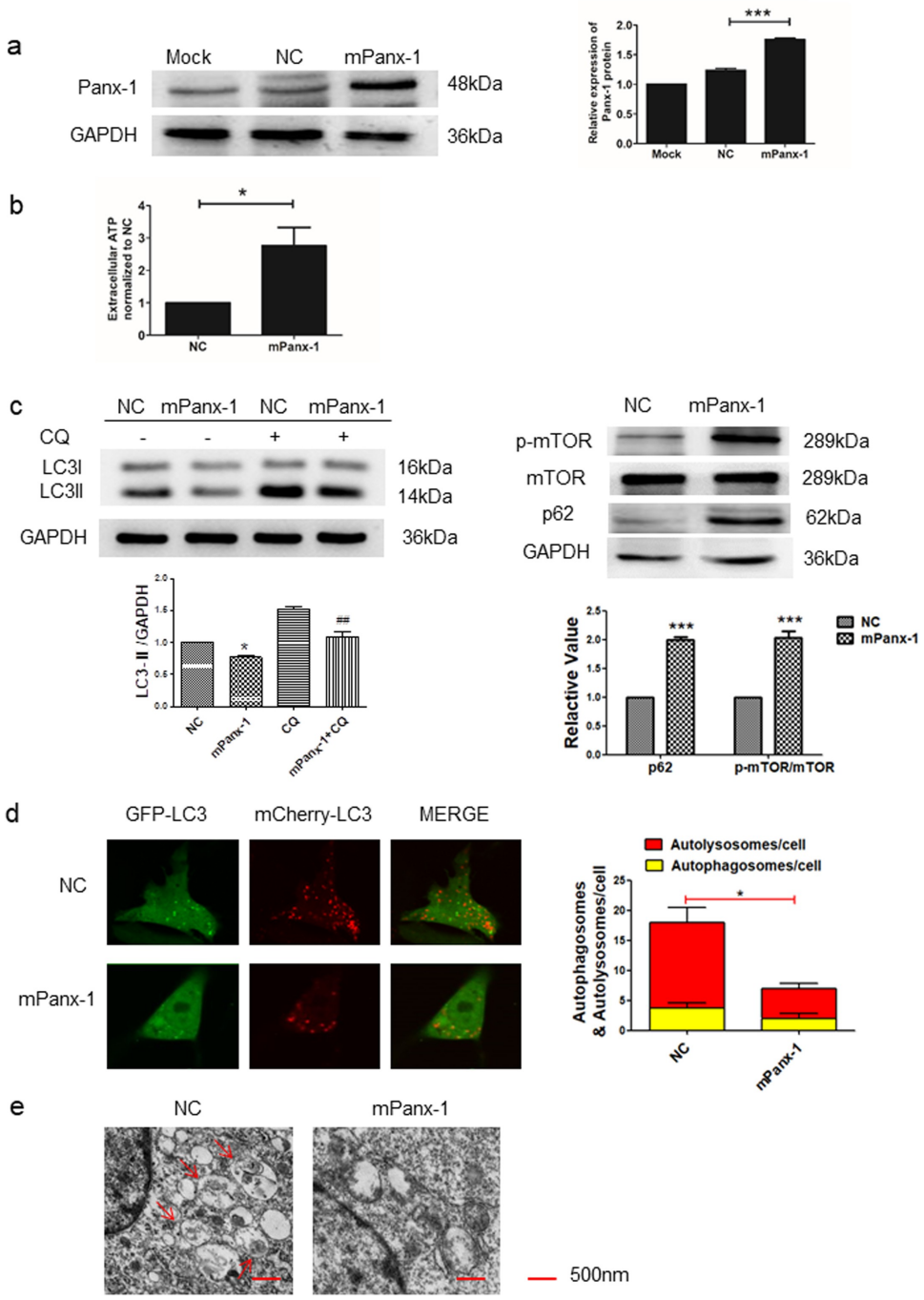

Figure 4. Overexpression of Panx-1 decreased autophagy in I-10/CDDP cells. (a) Immunoblots showing Panx-1 expression in Mork, NC and mPanx-1 I-10/CDDP cells. (b) The extracellular ATP was assessed by luminescence assay. (c) Western blots showed the expression of p62, p-mTOR, and mTOR proteins after Panx-1 overexpression and the expression of LC3 protein in the presence of CQ (10 μmol). (d) Representative fluorescence images showed autophagosomes and autolysosomes in the indicated groups (original magnification: × 400). (e) Representative TEM images showed autophagosomes (original magnification: × 15,000). Data are the mean ± SD for 3 experiments each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NC group and $^{\#\#}P < 0.01$ *vs* CQ group.

releasing the ATP stored in lysosomes [9]. Extracellular ATP is an agonist of purine receptors on the cell membrane [23]. Paola *et al.* found that extracellular ATP can stimulate P2X7 receptors on the surface of tumor cells, inhibit the mTOR pathway and enhance autophagy [24]. Bian *et al.* also reported that exogenous ATP can activate P2X7-AKT/PRAS40/mTOR signaling pathway to promote

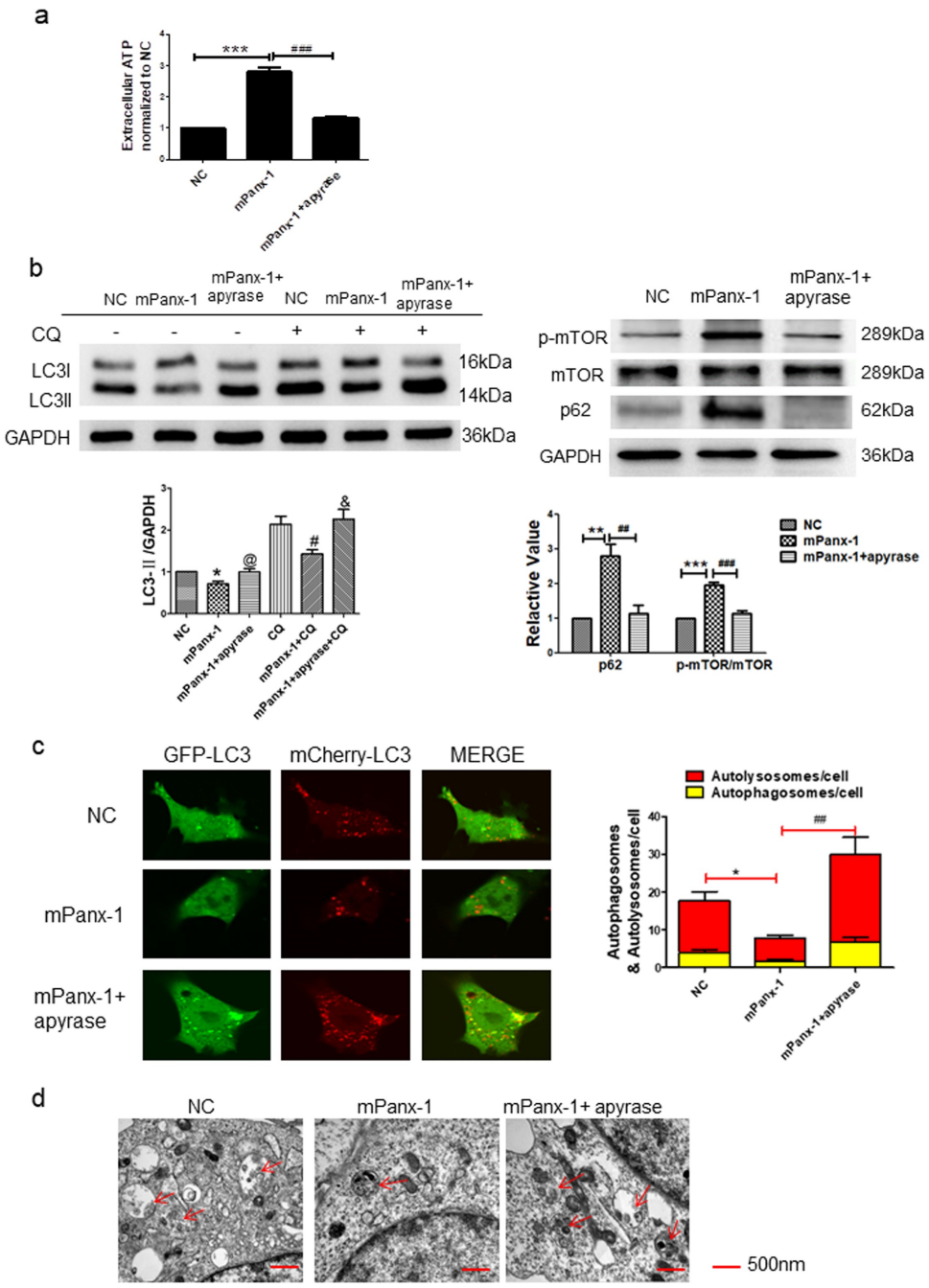

Figure 5. Apyrase increased autophagy in I-10/CDDP cells treated with mPanx-1. (a) The extracellular ATP was assessed by luminescence assay. (b) Western blots showed the expression of p62, p-mTOR and mTOR proteins after treatment with apyrase (25 U/ml) and the expression of LC3 protein in the presence of CQ (10 μmol). (c) Representative fluorescence images showed autophagosomes and autolysosomes (original magnification: × 400). (d) Representative TEM images showed autophagosomes (original magnification: × 15,000). Data are the mean ± SD for n = 3 experiments each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NC group, $^{@}P < 0.05$ *vs* mPanx-1 group, $^{\#}P < 0.05$ *vs* CQ group and $^{\&}P < 0.05$ *vs* mPanx-1 + CQ group.

apoptosis in the colon cancer MCA38 cells [25]. Increased levels of extracellular ATP also increase Ca^{2+} influx, which in turn activates mTOR [15,26]. Therefore, we hypothesized that decreasing extracellular ATP concentration can have a pro-survival effect on cancer cells.

Panx-1 is elevated in most chest tumors, colon cancer, lung cancer, urothelial cancer, gastric cancer and around 70% of melanoma cases, indicating a pro-tumorigenic role [27]. However, the expression of Panx-1 is low in normal gallbladder tissues and gallbladder adenocarcinoma [28]. One study

showed that knocking down Panx-1 in murine osteoblasts increased LC3 expression and the number of autophagosomes[11]. Similar results were observed with in astrocytes treated with the Panx-1 inhibitor CBX [29]. Body-Tressler et al study found that during chemotherapeutic drug-induced apoptosis, caspase-3 irreversibly activated Panx-1 and triggered the release of a large amount of ATP [30]. We found that the level of autophagy in testicular cancer cells was regulated by Panx-1, most likely via ATP release. In a previous study, we found that Panx-1 promoted cisplatin-induced apoptosis of I-10/CDDP cells via the ATP/IP3 pathway, which was inhibited by apyrase [12]. Consistent with this, the consumption of extracellular ATP by apyrase reversed the anti-autophagy effect of Panx-1.

In summary, Panx-1 channel-mediated ATP release regulates autophagy and cisplatin resistance in I-10/CDDP cells, which can be abrogated by ATP consumption. Our findings provide insights into the mechanisms underlying cisplatin-resistance in testicular cancer.

Acknowledgments

This research was supported by the Scientific Research Project of Anhui Province (No. 2008085MH275, No. 1808085QH269), 512 talent cultivation plan of Bengbu Medical College (by51201210), Innovative Science Research for Graduates of Bengbu Medical College (No.Byycx1924), Innovative training program for college students of Bengbu Medical College (No.201910367011).

Ethics approval and consent to participate

Bengbu Medical Ethics Committee approved all our experiments. BALB/c null mice were purchased from the Animal Center of Bengbu Medical College and raised under special pathogen-free (SPF) conditions. We follow the agreement approved by the Committee of Animal Care institutions of Bengbu Medical College.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Scientific Research Project of Anhui Province [2008085MH275]; 512 talent cultivation plan of Bengbu Medical College [by51201210]; Innovative training program for college students of Bengbu Medical College [201910367011]; Innovative Science Research for Graduates of Bengbu Medical College [Byycx1924]; Scientific Research Project of Anhui Province [1808085QH269].

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