

Autophagy activated by the c-Jun N-terminal kinase-mediated pathway protects human prostate cancer PC3 cells from celecoxib-induced apoptosis

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Abstract. The aim of the present study was to investigate the role of autophagy in celecoxib-induced apoptosis in human hormone-insensitive prostate cancer cell line PC3 cells and to explore the underlying molecular mechanism leading to autophagic activation. A cell viability assay was applied to investigate the effect of various concentrations of celecoxib (0, 40, 60, 80, 100 and 120 $\mu\text{mol/l}$) on PC3 cells for 24 and 48 h, respectively. The 50% inhibitory concentration of celecoxib for 24 h was chosen for subsequent experiments. Annexin V-fluorescein isothiocyanate/propidium iodide double staining flow cytometry, as well as caspase 3 and poly (ADP-ribose) polymerase proteins detected by western blotting, were applied to analyze cellular apoptosis induced by celecoxib. Ultrastructural cellular changes observed by transmission electron microscopy and the level of LC-3 II and P62 detected by western blotting were used to determine the activation of autophagy. It was demonstrated that celecoxib induced apoptosis and activated autophagy in PC3 cells in a dose- and time-dependent manner. Furthermore, flow cytometry and western blotting were applied to elucidate whether the role of autophagy in celecoxib-induced apoptosis is protective

or destructive. Blockade of autophagy markedly increased apoptosis, suggesting that celecoxib-activated autophagy was cytoprotective. Additionally, c-jun-N-terminal kinase (JNK) was demonstrated to have a role in autophagic activation, and suppression of JNK was able to reduce autophagy and increase apoptosis. In conclusion, the results of the present study indicate that celecoxib induces apoptosis in PC3 cells; however, celecoxib also activates JNK-mediated autophagy, which exerts cytoprotective effects in prostate cancer PC3 cells. Blockade of autophagy via the JNK-mediated pathway may provide a promising strategy for prostate cancer therapy.

Introduction

Prostate cancer is the second most common cancer and the sixth leading cause of cancer mortality worldwide (1). According to estimates of the American Cancer Society, the expected number of new cases of prostate cancer is 220,800, which accounted for ~25% of new cancer diagnoses in 2015. However, the expected number of prostate cancer deaths in 2015 was ~27,540, which was slightly less compared with the expected mortality rate of 29,480 in 2014 (2). Although patients with hormone-sensitive localized prostate cancer may experience successful outcomes with the application of surgery, radiotherapy or hormonal therapy, the disease inevitably progresses into castration-resistant and metastatic prostate cancer, negatively affecting quality of life and markedly reducing the survival rate. Therefore, it is necessary to investigate the underlying mechanisms of the onset and progression of prostate cancer in order to develop updated therapeutics, as well as preventative strategies. Increasing research has indicated that autophagy has an important role in cancer, which may become an effective drug target in anticancer therapy (3,4).

Cyclooxygenase-2 (COX-2), an inducible iso-enzyme that converts arachidonic acid to prostaglandins, is involved in cancer angiogenesis, apoptosis and invasion (5). Celecoxib is a specific COX-2 inhibitor that competes with arachidonic acid for the active site of cyclooxygenase. Numerous trials have indicated that the regular use of nonsteroidal anti-inflammatory drugs may provide benefits against malignancies (6,7).

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Abbreviations: Bcl-2, B-cell lymphoma 2; CQ, chloroquine; COX-2, cyclooxygenase-2; FCM, flow cytometric; DMSO, dimethyl sulfoxide; JNK, c-Jun N-terminal kinase; TEM, transmission electron microscopy; LC3, microtubule associated protein 1 light chain 3; 3MA, 3-methyladenine; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene fluoride; MAPKs, mitogen-activated protein kinases; MTT, methyl thiazolyl tetrazolium; OD value, optical density value; PBS, phosphate-buffered saline; PARP, poly (ADP-ribose) polymerase

Key words: autophagy, apoptosis, cyclooxygenase-2, prostate cancer

In the present study, whether celecoxib was able to induce apoptosis and autophagy in PC3 cells, an androgen-independent cell line, was investigated. Furthermore, whether celecoxib-induced autophagy exerted protective effects in PC3 cells and the underlying mechanism for the induction of autophagy were investigated. The present study also investigated whether the inhibition of autophagy by targeting the activated molecule was able to enhance apoptosis induced by celecoxib.

Materials and methods

Cells and cell culture. Prostate cancer PC3 cells were obtained from Chongqing Key Laboratory of Molecular Oncology and Epigenetics (Chongqing, China). Cells were cultured in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA), penicillin (100 µg/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere of 95% air and 5% CO₂.

Reagents and antibodies. Antibodies for phospho-JNK (cat. no. 4668; 1:1,000), JNK1 (cat. no. 3708; 1:1,000) and cleaved-caspase 3 (cat. no. 9664; 1:1,000) were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Anti-poly (ADP-ribose) polymerase (PARP; cat. no. 556494; 1:1,000) antibody and P62 (cat. no. 610497; 1:1,000) were purchased from BD Biosciences (San Jose, CA, USA). Anti-LC3B (cat. no. L7543; 1:1,000) antibody was purchased from Sigma-Aldrich (Merck KGaG, Darmstadt, Germany). Antibodies against β-actin (cat. no. ABM-0001; 1:1,000) were purchased from Zoonbio Biotechnology Co., Ltd. (Nanjing, China) and both secondary antibodies including goat anti-rabbit IgG-HRP secondary antibody (cat. no. ASS1006; 1:2,000) and goat anti-mouse IgG-HRP secondary antibody (cat. no. ASS1007; 1:2,000) were purchased from Abgent, Inc., (San Diego, CA, USA). The JNK inhibitor SP600125 (cat. no. s1460) and pan-caspase inhibitor z-VAD (cat. no. S7023) were purchased from Selleck Chemicals (Houston, TX, USA). Chloroquine diphosphate (CQ; cat. no. 50-63-5) was purchased from Sigma-Aldrich (Merck Millipore). The autophagy inhibitor 3-methyladenine (3MA; cat. no. sc-205596) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; 10 mM) to pretreat the cells prior to the MTT assay, flow cytometry and western blot analysis. Celecoxib (Pfizer Inc., New York, NY, USA) was dissolved in dimethyl sulfoxide (DMSO) and used within 1 month.

Cell viability assay. The effect of celecoxib on cell viability was measured using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. Cells were seeded at 5,000-10,000 cells/well in 96-well plates and incubated overnight at 37°C. Following this, different concentrations of celecoxib were added and the plates were incubated for an additional 24 or 48 h at 37°C. Subsequently, 20 µl of MTT solution (5 mg/ml) was added to each well and the cells were incubated for 4 h at 37°C. After removal of the culture medium, 100 µl dimethyl sulfoxide (DMSO) was added per well to dissolve the formazan crystals and the optical density (OD) was measured

at 460 nm by a microplate reader. Changes in percentage viability were calculated using the following formula: Cell viability (%) = (OD of the experimental sample / OD of the control group) x 100.

Measurement of apoptosis by flow cytometry. Cell apoptosis was detected using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) kit (Beyotime Institute of Biotechnology, Haimen, China). Cells were collected and suspended in 200 µl medium buffer. Subsequently, ~10 µl annexin V solution was added to the cell suspension solution and the solution was incubated for 15 min in the dark at room temperature. Following this, 300 µl medium buffer and 5 µl PI were added and the cell suspension was immediately analyzed using a flow cytometric machine. Annexin V-negative/PI-negative represented viable cells, annexin V-positive/PI-negative represented early apoptotic cells, annexin V-positive/PI-positive represented terminal apoptotic cells and annexin V-negative/PI-positive cells represented necrotic cells. Flow cytometry acquired ~10⁴ cells and the data were acquired using a FACSCanto 6-color flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using BD FACSDiva™ software version 6 (BD Biosciences).

Western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed in lysis buffer (1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 2 µg/ml aprotinin) and quantified using bicin-chonic acid (BCA) assay kit (Beyotime Institute of Biotechnology). Equal amounts of denatured protein lysates (40 µg/lane) were separated by 12% SDS-PAGE and transferred to a nitro-cellulose membrane (cat. no. CS011-0001; ExCell Biology, Inc., Shanghai, China). Following this, the membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 buffer (TBST; 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween) at room temperature for 2 h and the blots were incubated with the indicated primary antibodies (anti-phospho-JNK, JNK1, LC3B, P62, cleaved-caspase 3, PARP, β-actin; 1:1,000) overnight at 4°C. After three 10 min washes with TBST buffer, the membranes were incubated with secondary antibodies (goat anti-rabbit IgG-HRP secondary antibody, goat anti-mouse IgG-HRP secondary antibody; both 1:2,000) for 30 min at room temperature. Following the use of HRP-conjugated IgG secondary antibodies, proteins were detected with an enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA) and visualized using an electrophoresis gel imaging analysis system.

Transmission electron microscopy (TEM). For visualization of the cellular ultrastructure using TEM, celecoxib-treated cells were treated with trypsin, rinsed twice with warm PBS (37°C) and fixed for 1 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 1% sucrose. After washing with PBS, the cells were fixed in 1% osmium tetroxide and embedded in Epon resin. Sections of 0.1-mm thickness were cut and stained with uranyl acetate/lead citrate and visualized under a Hitachi-7,500 TEM (Hitachi, Ltd., Tokyo, Japan).

Statistical analysis. Statistical analysis was conducted using SPSS v.16.0 (SPSS, Inc., Chicago, IL, USA). Data was

confirmed in three independent experiments and expressed as the mean + standard deviation. Differences between groups were evaluated using Student's t-tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Celecoxib decreases cell viability in a dose-dependent and time-dependent manner. PC3 cells were exposed to celecoxib for 24 and 48 h, respectively, and cell viability was measured using an MTT assay. Results demonstrated that cytotoxicity in PC3 cells was induced by celecoxib in a dose-dependent and time-dependent manner, with all doses of celecoxib resulting in a significant decrease in cell viability compared with the untreated control group at 24 and 48 h ($P < 0.05$; Fig. 1). The 50% inhibitory concentration of celecoxib for 24 h was 100 $\mu\text{mol/l}$, which was chosen for subsequent experiments.

Celecoxib induces autophagy in PC3 cells. To explore whether celecoxib was able to induce autophagy in PC3 cells, the conversion of LC3-I to LC3-II and the degradation of P62 was detected by western blot analysis. Results demonstrated that the conversion of LC3-I to LC3-II increased following treatment with celecoxib; however, P62 degraded gradually as PC3 cells were incubated with celecoxib (100 $\mu\text{mol/l}$) for 0, 2, 4 and 8 h (Fig. 2A). In order to determine if the observed change in LC3 was due to autophagy, CQ, a lysosome inhibitor for LC3-II turnover, was combined with celecoxib to detect autophagy flux. Results demonstrated that combined exposure to celecoxib and CQ induced a marked increase in LC3-II expression levels compared with celecoxib or CQ alone (Fig. 2B). Furthermore, TEM was used to detect autophagosomes (autophagic vacuoles), which is considered to be the morphological hallmark of autophagy. Results demonstrated that celecoxib induced marked autophagosome formation compared with the control group (Fig. 2C). These results demonstrated that autophagy was induced by celecoxib treatment.

Celecoxib induces apoptosis in PC3 cells. In order to determine whether celecoxib-induced cytotoxicity was due to activated apoptosis, western blotting was performed to evaluate expression levels of PARP and caspase 3. Compared with the control group (0 h), the expression levels of PARP and caspase 3 markedly increased in a time-dependent manner (Fig. 3A). To further elucidate whether PC3 cell apoptosis was induced by celecoxib, flow cytometry was applied to compare the following groups: Control group; celecoxib-treated for 8 h; and celecoxib plus the pan-caspase inhibitor zVAD. Flow cytometry indicated that celecoxib was able to induce significant apoptosis ($P < 0.05$) compared with the control group, and zVAD was able to significantly inhibit ($P < 0.05$) celecoxib-induced apoptosis (Fig. 3B and C, respectively).

Autophagy has a cytoprotective role in celecoxib-induced apoptosis. To investigate the role of autophagy in celecoxib-induced apoptosis in PC3 cells, cells were pretreated with 3-methyladenine (3MA) to inhibit autophagy before celecoxib exposure (80, 100 and 120 $\mu\text{mol/l}$). MTT assay results demonstrated that cell viability was significantly reduced ($P < 0.05$) by inhibition of autophagy compared with

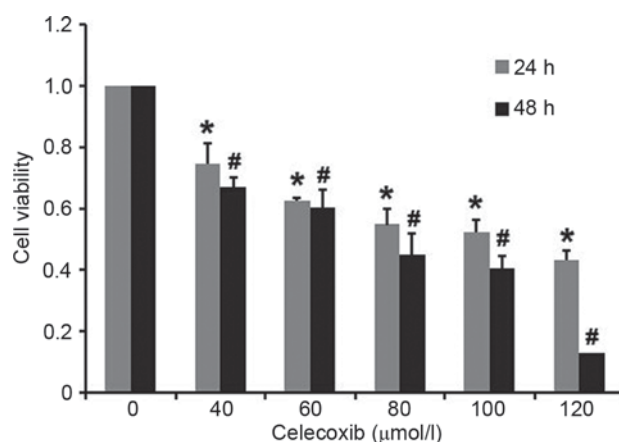


Figure 1. Effects of various concentrations of celecoxib on prostate cancer PC3 cell viability evaluated by MTT assay. Data are presented as the mean + standard deviation ($n=3$). * $P < 0.05$ vs. the control group at 24 h; # $P < 0.05$ vs. the control group at 48 h.

the celecoxib-only group (Fig. 4A). Furthermore, flow cytometry was used to detect cell apoptosis following pretreatment with 3MA and the results were consistent with the MTT assay results (Fig. 4B). Altogether, these results demonstrated that autophagy may have a protective role in PC3 cells under celecoxib-induced apoptosis.

Celecoxib-induced apoptosis is mediated by JNK activation. JNK activation has been demonstrated to be involved in autophagy due to various stresses (8). To investigate whether JNK was activated in celecoxib-induced autophagy, western blot analysis was conducted to detect the phosphorylation of JNK in PC3 cells following exposure to celecoxib (Fig. 5). JNK was activated into pJNK following incubation with celecoxib (100 $\mu\text{mol/l}$) for 0, 1, 2 and 4 h (Fig. 5A). Furthermore, SP600125, a specific inhibitor of JNK, was able to inhibit the conversion of LC3-I to LC3-II (Fig. 5B) and reduce cell viability by increasing cell apoptosis (Fig. 4). These results demonstrated that JNK was related to celecoxib-induced apoptosis.

Discussion

In the present study it was demonstrated that celecoxib was able to induce apoptosis and autophagy in PC3 cells. COX-2, an enzyme involved in cancer angiogenesis, apoptosis and invasiveness, has been associated with poor prognosis in various types of cancer, including rectal and cervical cancer (9,10). Celecoxib is a specific COX-2 inhibitor that is widely used to treat acute pain, rheumatoid arthritis and familial adenomatous polyposis. Previous studies have reported that celecoxib may exert anticancer effects in various human neoplasms, such as lung cancer, breast carcinomas, colorectal cancer and prostate cancer (11-13). A meta-analysis of 11 randomized clinical trials by Chen *et al* (14) reported that celecoxib was beneficial in the treatment of various types of advanced cancer. Considering the significant role of COX-2 in the regulation of cancer angiogenesis, apoptosis and invasiveness, the molecular mechanisms of COX-2 have been intensively studied in prostate cancer (15,16). A study by Khor *et al* (17) investigated

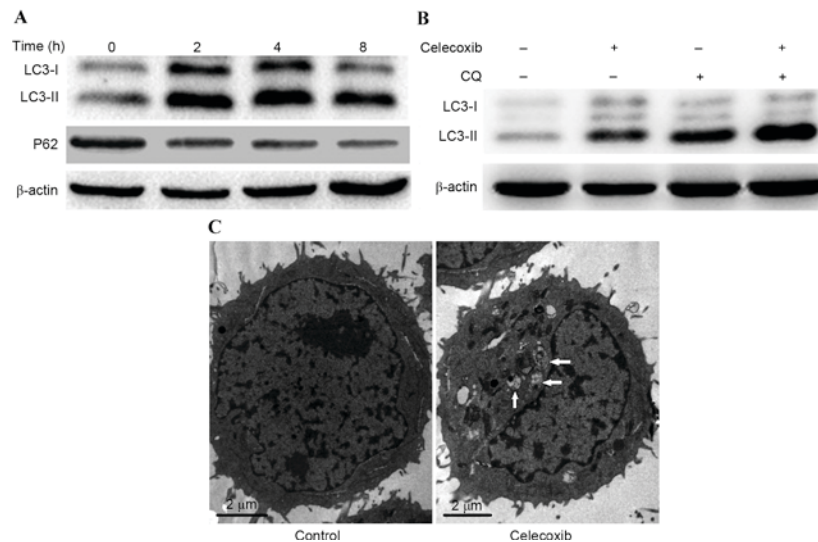


Figure 2. Celecoxib-induced autophagy in prostate cancer PC3 cells. (A) PC3 cells were incubated with celecoxib (100 $\mu\text{mol/l}$) for 0, 2, 4 and 8 h. LC3-I, LC3-II and P62 were detected using western blotting and β -actin was detected as a loading control. (B) PC3 cells were pretreated with CQ (20 μM) for 30 min and incubated with celecoxib (100 $\mu\text{mol/l}$) for 4 h. LC3-I and LC3-II were detected using western blotting and β -actin was detected as a loading control. (C) Ultrastructural changes ($\times 10,000$) in PC3 cells following treatment with 100 $\mu\text{mol/l}$ celecoxib for 8 h (stain, uranyl acetate/lead citrate). Autophagosomes were observed in the celecoxib treatment group compared with the control group. CQ, chloroquine. White arrows indicate autophagosomes.

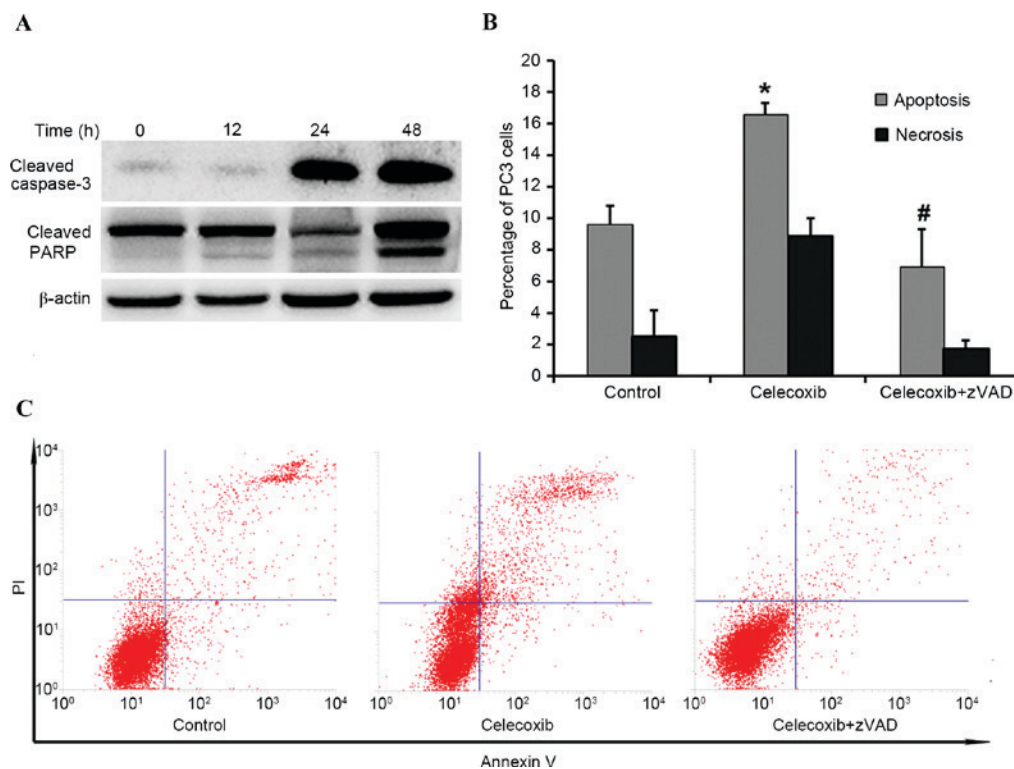


Figure 3. Celecoxib-induced apoptosis in prostate cancer PC3 cells. (A) PC3 cells were incubated with celecoxib (100 $\mu\text{mol/l}$) for 0, 12, 24 and 48 h. Cleaved caspase 3 and PARP were detected by western blotting and β -actin was detected as a loading control. (B) Percentage of apoptotic and necrotic PC3 cells in the control, celecoxib-treated and celecoxib plus zVAD-treated groups. Data are presented as the mean + standard deviation. (C) Flow cytometry was applied to demonstrate apoptosis of PC3 cells treated with celecoxib (100 $\mu\text{mol/l}$) for 8 h. Representative dot plots of apoptosis in the control, celecoxib-treated and celecoxib plus zVAD-treated groups are presented. * $P < 0.05$ vs. the control group; # $P < 0.05$ vs. the celecoxib-treated group. PARP, poly (ADP-ribose) polymerase; zVAD, pan-caspase inhibitor.

the relationship between COX-2 overexpression and outcomes in patients with prostate cancer following radiation treatment and the results demonstrated that COX-2 expression levels were positively associated with biochemical failure and distant metastasis. Furthermore, a study by Patel *et al* (6) revealed that

celecoxib possesses different COX-2-independent anticancer properties, which exert synergic effects with COX-2-dependent effects against prostate cancer growth.

Autophagy, an evolutionarily conserved adaptive cellular process, enables cells to engulf dysfunctional cellular

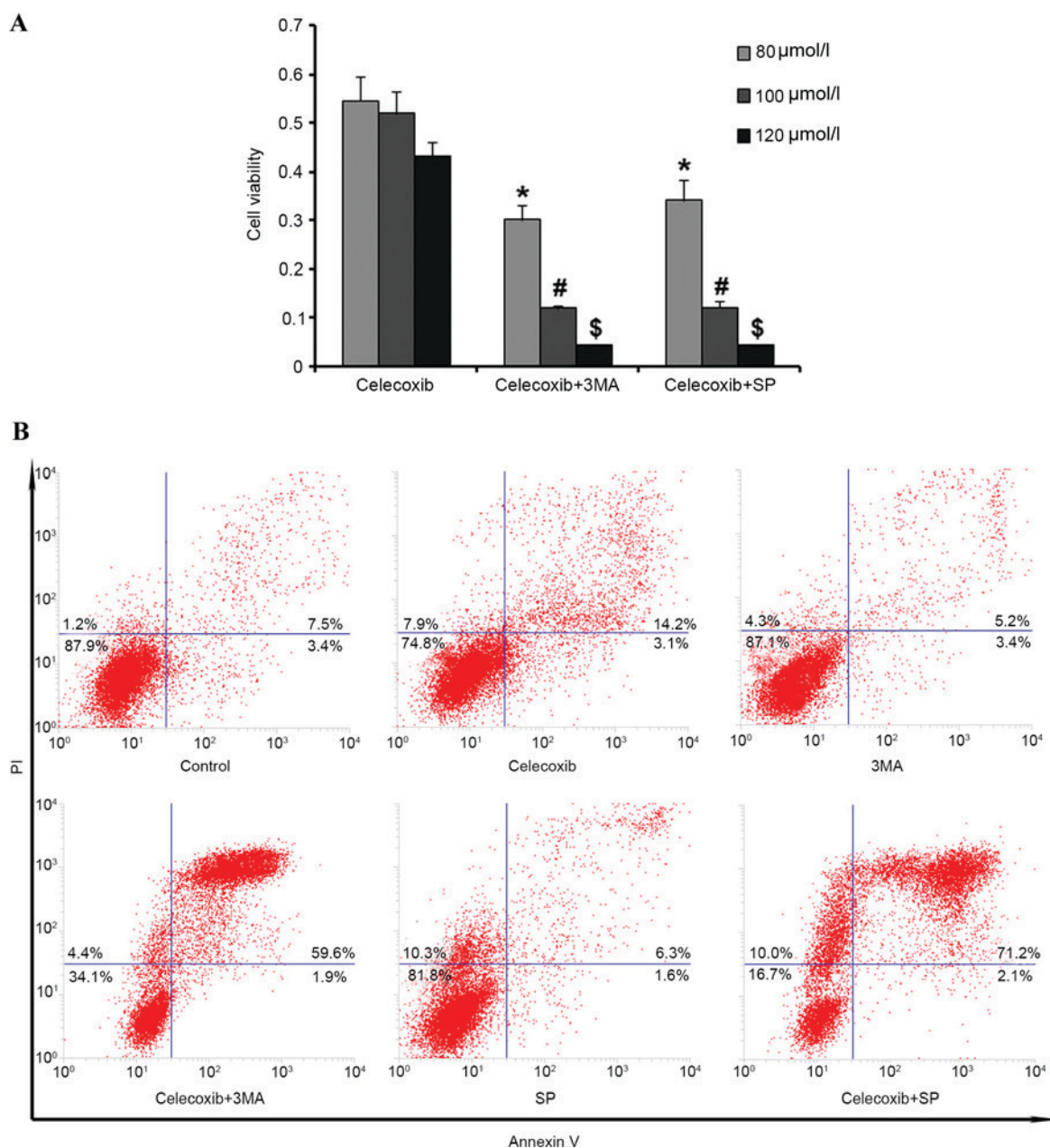


Figure 4. (A) Cell viability of celecoxib-treated (80, 100 and 120 $\mu\text{mol/l}$), celecoxib+3MA and celecoxib+SP groups. (B) Representative dot plots of apoptosis in the control, celecoxib, 3MA, celecoxib+3MA groups, SP and celecoxib+SP groups. Data are presented as the mean + standard deviation. * $P < 0.05$ vs. 80 $\mu\text{mol/l}$ celecoxib group; # $P < 0.05$ vs. the 100 $\mu\text{mol/l}$ celecoxib group; \$ $P < 0.05$ vs. the 120 $\mu\text{mol/l}$ celecoxib group. 3MA, 3-methyladenine; SP, c-Jun N-terminal kinase inhibitor SP600125.

proteins and damaged organelles, which eventually fuse with lysosomes for degradation when exposed to metabolic, toxic, hypoxic and infectious stresses (18). Accumulating evidence suggests that autophagy exerts potent anti-cancer mechanisms to suppress tumor initiation (19,20). However, at the advanced stage of cancer, autophagy enables cancer cells to enhance nutrient utilization and improve growth in the context of therapeutic intervention, and so the role of autophagy in cancer is controversial (21,22). Research on beclin 1 autophagy gene deletion in prostate cancer has indicated that autophagy suppresses prostatic tumorigenesis (23,24), whereas other studies have demonstrated that autophagy is a survival mechanism to overcome stresses in prostate cancer (3,25).

Androgen deprivation is considered the backbone of therapy for hormone-sensitive or castration-resistant prostate cancer (26). The underlying mechanism of androgen deprivation and autophagy activation has been demonstrated *in vivo* as well as *in vitro* models of prostate cancer (27,28). Specifically, AMP-dependent protein kinase activation leading to the suppression of mammalian target of rapamycin is one of the most investigated mechanisms for autophagy induction in androgen-dependent or androgen-independent models of prostate cancer (29,30).

JNK, a mitogen-activated protein kinase, contributes to apoptotic signal transduction when exposed to various forms of stress (31). JNK target proteins include the transcription factor c-Jun, apoptotic regulatory proteins, such as B-cell lymphoma

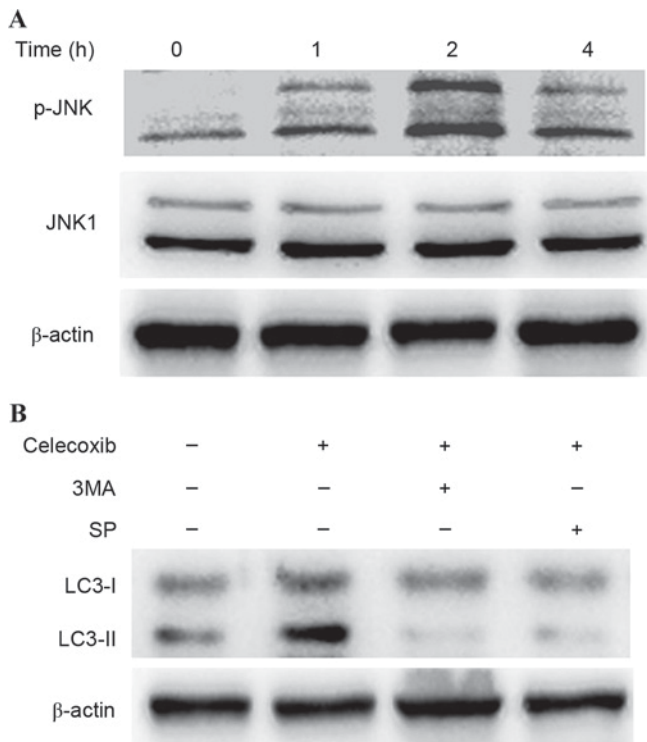


Figure 5. JNK activation is associated with celecoxib-induced autophagy. (A) Prostate cancer PC3 cells were incubated with celecoxib (100 $\mu\text{mol/l}$) for 0, 1, 2 and 4 h. JNK and p-JNK proteins were detected using western blotting and β -actin was detected as a loading control. (B) PC3 cells were pretreated with 3MA (10 $\mu\text{M/l}$) for 4 h. LC3-I and LC3-II were detected using western blotting and β -actin was detected as a loading control. JNK, c-Jun N-terminal kinase; p, phosphorylated; 3MA, 3-methyladenine; SP, c-Jun N-terminal kinase inhibitor SP600125.

2 (Bcl-2) and Bcl-2-associated X protein (Bax), and forkhead transcription factor (32). A study by Kurinna *et al* (33) demonstrated that ceramide was able to induce translocation of active JNK from the nucleus to the cytoplasm and mitochondrial fraction to promote apoptosis in lung cancer-derived A549 cells. A study by Deng *et al* (32) indicated that activation of JNK was able to upregulate acetylcholinesterase expression levels through a c-Jun-dependent mechanism during apoptosis in colon cancer cells. Furthermore, previous research has highlighted that the JNK-mediated pathway is associated with autophagy activation. A study by Wei *et al* (34) demonstrated that, during starvation, autophagy is activated by JNK-1 mediated Bcl-2 phosphorylation and disruption of the Bcl-2/beclin 1 complex. Reactive oxygen species may also induce autophagy to resist apoptosis by JNK activation in mesenchymal stem cells, while some natural agents, such as matrine, mediate crosstalk between autophagy and apoptosis via the JNK-Bcl-2/Bcl-xL-Bax/Bcl-2 homologous antagonist killer pathway to interplay with beclin 1 (6,13,35-37). In the present study, it was demonstrated that JNK is also involved in autophagy stimulation due to celecoxib treatment in hormone-insensitive prostate cancer cells. Furthermore, suppression of JNK by the specific inhibitor, SP600125, was able to inhibit autophagy and enhance celecoxib-induced cytotoxicity in PC3 cells.

In conclusion, the present study demonstrated that celecoxib induces apoptosis in PC3 cells; however, it also activates

autophagy, which exerts cytoprotective effects in prostate cancer PC3 cells. JNK is activated in celecoxib-treated cells and, when considering the dual roles of JNK in autophagy and apoptosis, further investigation is required to illustrate the precise underlying mechanisms of JNK. Meanwhile, the blockade of autophagy via the JNK-mediated pathway may provide a promising strategy for prostate cancer therapy.

Acknowledgments

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