Berberine Induces NSCLC Apoptosis Via Activation of ROS/ASK1/JNK Pathway in Vitro and Vivo

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Research

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Abstract

Background

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Berberine (BBR), as an isoquinoline alkaloid, is commonly utilized in traditional Chinese medicine. Previous studies have proven that BBR possesses potential anti-tumor effect. However, the mechanism of on mitochondrial function in anti-NSCLC are still unknown.

Methods

Cell Counting Kit-8 (CCK-8), flow cytometry and western blotting were utilized to characterize the roles and relationships among BBR, ROS, ASK1, JNK, coxIV,

caspase-3, cytochrome *c* ,bcl-2 and bax in NSCLC. Immunohistochemical (IHC) analysis was built to examine their expression in vivo.

Results

In this study, we found that BBR potently suppressed NSCLC cells (A549 and PC9) growth by inducing apoptosis in a dose- and time-dependent manner. BBR induced apoptosis in NSCLC as evidenced by caspase-3 cleavage, cytochrome *c* release, and mitochondrial membrane depolarization. Furthermore, BBR induced ROS generation and ASK1 and JNK activation. To explore whether such apoptosis was linked to ROS production and ASK1 and JNK activation, we treated cells with a JNK inhibitor (SP600125), which significantly suppressed BBR-induced apoptosis. We further found that treating these cells with the anti-oxidant N-acetyl cysteine (NAC) was sufficient to both suppress ASK1 and JNK activation and to disrupt apoptotic induction.

Conclusions

Together, these data suggest that BBR induces NSCLC cells apoptosis via ROS-mediated ASK1/JNK and mitochondrial pathway activation.

Background

Lung cancer is among the most prevalent and deadly cancers, with an estimated 228,820 new cases and nearly 136,000 deaths associated with this condition being estimated to have occurred in the USA alone. Lung cancer rates have risen substantially in China in recent years, and thus represent a major threat to public health [1]. Treatment of this cancer type is typically a combination of surgical tumor resection, adjuvant chemotherapy, and adjuvant immunotherapy. However, non-small-cell lung cancer (NSCLC) is often chemoresistant, and individuals with advanced NSCLC tend to have poor outcomes owing to an absence of reliable curative treatments [2]. More work is thus necessary to identify effective approaches to treating NSCLC.

Berberine (BBR), is an isoquinoline alkaloid found in Coptidis Rhizoma, which has been shown to induce cell death in a range of cancer cell models through regulating cell cycle, autophagy, apoptosis, and the surrounding tumor microenvironment [3]. Current research found BBR trigger cell apoptosis through both the Fas-mediated extrinsic pathway and the intrinsic mitochondrial pathway, resulting in cytochrome *c* release and associated death signaling [4]. The specific mechanisms whereby BBR can drive the apoptosis of NSCLC cells, however, remain to be clarified.

Apoptosis is a key regulator of diverse physiological and pathological processes including cancer development and treatment [5]. Chemotherapeutic drugs and many different stimuli can induce apoptotic cell death, often via inducing reactive oxygen species (ROS) production and thereby driving death receptor signaling and mitochondrial pathway activation [6]. It has been shown that ROS triggers the activation of the apoptosis signal-regulating kinase 1 (ASK1)/ mitogen activated protein kinase (MAPK) signaling pathway [7]. ASK1, a serine/threonine protein kinase, participates in cell differentiation and apoptosis [8].Once activated,ASK1 dissociates from Trx-1 and induces cell death by activating the c-jun-NH2-kinase (JNK) and p38 MAPK Pathways [9]. Targeting such ROS generation is thus a promising strategy for novel anticancer drug design.

In the present study, we demonstrated BBR induced apoptosis in NSCLC via ROS generation and subsequent activation of ASK1/JNK signaling and the mitochondrial apoptosis pathway. Together, these data suggest BBR may offer value for the clinical treatment of NSCLC.

Methods

Cells and treatment

A549 and PC9 NSCLC cells from the Cell Bank of Shanghai Institute of Cell Biology, Shanghai, China. BBR and DMSO were from Sigma (MO, USA). Antibodies specific for coxIV, p-JNK, JNK, p-ASK1, ASK1, bax, bcl-2, caspase3, cytochrome *c*, and β-actin were from Abcam (MA, USA). HRP-labeled anti-mouse and anti-rabbit IgG were from Santa Cruz Biotechnology Inc. (TX, USA). RIPA buffer, Hoechst33342, and DCFH-DA were from Sigma-Aldrich Inc. (MO, USA). JC-1 was from Life Technologies Inc. (CA, USA). A Caspase-3 Colorimetric Assay Kit was from Nanjing Keygen Biotech CO. Ltd. (China). A High Pure Mitochondria Isolation Kit was from Shanghai Genmed Biotech CO. Ltd. (China). All other materials were from BioRad.

CCK-8 assays

A Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) approach was used to monitor cell proliferation. Briefly, 2×10^3 cells were added to 96-well plates with a range of BBR concentrations (0, 20, 40, 80, and 160 μM). After 24, 48, or 72 h, cells were incubated for 2 h with CCK-8 reagent at 37°C, and absorbance was then measured via spectrophotometer (BioTek, USA).

Flow cytometry

Cells were treated for 48 h with BBR (0, 40, 80 μmol/L), rinsed with pre-cooled PBS and stained for 20 min with Annexin V-FITC/PI (BD Biosciences, CA, USA) in the dark, followed by analysis via flow cytometry (BD Biosciences).

Intracellular ROS measurement

The membrane-permeable DCFH-DA probe was utilized to quantify ROS levels within cells. Exposure to intracellular esterases results in the inability of hydrolyzed DCFH to exit the cell, while exposure to peroxides results in its oxidation to yield DCF, which is fluorescent. Following treatment with 50μM of BBR, cells were treated for 30 min with DCFH-DA (50 μM). After two washes with PBS, cells were lysed and analyzed via flow cytometry (BD Biosciences).

Mitochondrial membrane potential analysis

Mitochondrial membrane potential (MMP) was assessed with the JC-1 probe. Briefly, cells were initially treated for 48 h with a range of BBR concentrations (0, 40, 80 μM), followed by a 20 min incubation with JC-1 (10 μM) at 37°C in the dark. Cells were then washed prior to analysis via flow cytometry (BD Biosciences, USA), with JC-1 aggregates (red) being detected at 590nm, and JC-1 monomers (green) being detected at 529 nm. The resultant ratio of red to green fluorescence was then reported.

Mitochondrial isolation

Cellular mitochondria were collected with a High Pure Mitochondria Isolation Kit following a 48 h BBR treatment based on provided instructions. Briefly, 10^7 cells were rinsed using chilled reagent A, after which they were lysed on ice with the prepared lysis reagent. Samples were then spun at 800g at 4℃ for 10min, after which the supernatants were spun again at 13,000g at 4℃ for 10min to collect mitochondria.

Western blotting

RIPA buffer containing protease inhibitors (NCM Biotech) was used to lyse cells, after which a BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to quantify protein levels. Equal protein amounts from each sample were then separated via 10% SDS-PAGE (Haochen Biotechnology Co., Ltd., Shanghai, China) and transferred to nitrocellulose membranes. Blots were blocked for 1 h with 3% BSA (Google Bio), after which they were probed overnight with anti-β-actin (1:2000), anti-bax, anti-bcl-2, anti-caspase-3, anti-p-JNK, anti-JNK, anti-cytochrome *c*, and anti-coxIV (1:1000) at 4°C. After three washed in PBST, blots were probed with secondary antibodies for 1h at room temperature and visualized with an Odyssey scanner (LI-COR Biosciences, USA).

Immunohistochemical (IHC) analysis

IHC testing technology was applied to detect the expression level of the candidate target gene. The samples were dewaxed, paraffin-embedded, and incubated in 3% hydrogen peroxide for 30 min to suppress endogenous peroxidase activities. Citrate buffer was used to infiltrate these sections, followed by a 10-min heating process in a microwave oven to retrieve the antigen. Afterwards, these sections underwent an overnight-incubation in primary antibodies (1:2000) at 4°C, rinsed with PBS, treated with

peroxidase-labeled goat anti-mouse secondary antibodies at room temperature for 1h, stained with hematoxylin and 3'-diaminobenzidine tetrahydrochloride (DAB), and finally visualized.

Statistical analysis

Data are given as mean ± SD, and were compared via one-way ANOVAs with posthoc least significant difference tests. The significance threshold for this study was $p \leq 0.05$.

Results

BBR inhibited proliferation and induced apoptosis in NSCLC cells

A549 and PC9 cells were treated with 0–160 μM of BBR, and the cell viability was assayed after 24, 48 and 72 h using the CCK8 assay. BBR markedly induced cell death in A549 and PC9 cells in a concentration- and time-dependent manner (Fig. 1a). IC50 was determined to be 80–100 μM at 48 h of exposure to BBR. These cells also exhibit marked morphological changes following a 48h treatment with BBR (Fig. 1b). Based on these experiments, we selected a BBR dose of 80μM and a treatment time of 48 h for subsequent experiments to study the mechanistic basis for these changes in NSCLC cell viability. To explore whether BBR-induced cell growth inhibition is related to apoptosis, we performed Annexin V-FITC/PI double staining. The result revealed A549 and PC9 cells underwent significant apoptosis after treatment with increasing concentration of BBR over a 48h period, with roughly 7% of A549 and 21% of PC9 cells treated with the 80μM dose exhibiting signs of apoptosis as opposed to just 1.3% and 1.5% of the respective control cell groups (Figs. 1c–d). Furthermore, to determine the mechanism of BBR-induced apoptosis, we examined the expression of apoptosis-related proteins. Western blotting revealed dose-dependent increases in caspase-3 and bax levels as well as dose-dependent reductions in bcl-2 levels, which led to an increase in the proapoptotic/antiapoptotic (Bax/Bcl-2) ratio in A549 and PC9 (Fig. 1e–f). Taken together, these data indicated that BBR induced cell growth inhibition by apoptosis.

BBR activated ASK1/JNK and mitochondrial apoptotic pathway in NSCLC cells

Next, we explored how BBR induces the apoptosis of NSCLC cells. As we all acknowledged, the MAPK signaling pathway is one of the most important apoptotic pathway and ASK1 is known as a member of the MAPK kinase kinase. We thus tested the ability of BBR-induced ASK1 and JNK activation in A549 and PC9 cells, revealing a marked increase in the phosphorylation of the Thr 845 residue of ASK and JNK following a 48h treatment with 80 μM BBR in both cell types (Figs. 2a–b). Moreover,we examined A549 and PC9 cells to establish whether such apoptosis was associated with changes in mitochondrial phenotypes. We found that BBR induced a loss of mitochondrial membrane potential (MMP) in a dose-dependent fashion in both tested cell lines (Figs. 2c–d). We further found that high-dose (40 μM and 80 μM) BBR treatment for 48 h resulted in a reduction in mitochondrial cytochrome *c* levels and a

concomitant increase in cytosolic cytochrome *c* levels (Figs. 2e–f). This suggests that mitochondrial dysfunction plays a role in the apoptotic death of BBR-treated NSCLC cells.

BBR-induced NSCLC cell apoptosis via the activation of ASK1/JNK pathway

To test whether the activation of ASK1/JNK pathway played a role in BBR-induced NSCLC cell apoptosis, we next treated these cells with the JNK inhibitor SP60012. SP600125 partially reduced in BBR-induced p-JNK, CL-caspase-3 and bax/bcl-2 levels (Figs. 3a–b), and this coincided with ablation the death of A549 and PC9 cells treatment with BBR (Fig. 3c). These data thus suggested that the activation of ASK1/JNK pathway is associated with the BBR-induced apoptosis of NSCLC cells.

BBR-induced NSCLC cell apoptosis via the activation of ROS/ASK1/JNK pathway

The JNK signaling pathway is known to be sensitive to intracellular redox state and ROS production. To establish the impact of BBR treatment of ROS generation, we incubated A549 and PC9 cells with DCFH-DA, revealing that exposure to BBR (80 μM) for 48 h resulted in elevated ROS production to 81.3% and 75.3%, respectively (Figs. 4a–b). Such ROS induction was also dose-dependent. The above data suggested that ROS may play a key role in BBR-induced ASK1/JNK activation and consequent apoptosis in NSCLC cells. To test this possibility, we treated cells with NAC, which significantly reduced BBR-induced ROS production in both tested cell lines (Figs. 4c–d). We further found that NAC treatment reduced BBR-induced ASK1 and JNK phosphorylation in these NSCLC cells, indicating that such ASK1/JNK activation is ROS-dependent in both tested cell lines while simultaneously suppressing BBR-mediated caspase-3 and bax/bcl-2 upregulation, suggesting that ROS induction controls mitochondrial dysfunction (Figs. 4e–f). As such, BBR induces the apoptosis of A549 and PC9 cells via a ROS/ASK1/JNK pathway that promotes mitochondrial dysfunction.To test this hypothesis (Fig. 6), Immunohistochemical analysis of the tumor tissues, which embedded by previous experiment, was performed, showing that BBR increased the levels of phosphorylation-ASK1, phosphorylation-JNK and caspase-3.In addition, BBR inhibited the activity of bax in the tumors, a finding consistent with observations in vitro (Fig. 5).

Discussion

Our Previous study had shown that BBR-induced NSCLC apoptosis is related to phosphorylation of JNK [10]. To clarify the mechanisms upstream of JNK, we demonstrated that BBR can effectively sustained generation of the ROS and overproduction of ROS induced resulted in ASK1/JNK activation, which caused cell apoptosis in NSCLC.

ROS are known as important upstream molecules in the progression of cell death and survival. Therefore, induction of ROS production is an effective anti-cancer strategy. Some ROS-inducing agents such as cisplatin, cyclophosphamide, resveratrol [11], have been reported to induce apoptosis through DNA

toxicity by the induction of intracellular ROS. For example, Chlorpyrifos (CPF) could trigger oxidative stress and induce apoptosis and necroptosis in fish liver cells by regulating the ROS/PTEN/PI3K/AKT axis [12]. Moreover, ROS increase can also activate ASK1/JNK [13], PI3K / AKT / mTOR [14], AMPK/p53 [15] and other apoptosis signaling pathway. In addition, it has been well proved that BBR promoted tumor cell apoptosis by production of ROS in melanoma [16], breast cancer [17], pancreatic cancer [18] and so on. Although Fan et al found BBR increase ROS production in NSCLC cell [19], the mechanisms by which ROS exert their anti-tumor effect have not been completely elucidated so far. In light of this, the ROS/ASK1/JNK signaling pathway could be considered as a possible mechanism for BBR-dependent anti-apoptotic effect in NSCLC.

In this research, we demonstrate that BBR effectively inhibited the proliferation of A549 and PC9 cells in vitro by induced apoptosis. Annexin V and PI staining reveled that BBR was able to induce cellular apoptosis in A549 and PC9 cells and the effects of BBR on apoptosis-related genes in A549 and PC9 cells were also analyzed. It was found that BBR up-regulated the expression of the apoptosis-promoting gene bax while inhibiting the expression of the anti-apoptotic gene bcl-2. Increased expression of bax usually leads to increased mitochondrial membrane permeability, resulting in the release of pro-apoptotic factors such as cytochrome *c* from mitochondria to cytosol, which initiates caspase cascade activation and promotes apoptosis progression [20]. As a result, the experiment further analyzed the cytochrome *c* protein levels in mitochondria and cytoplasm, and found that BBR treatment significantly promoted the transfer of cytochrome *c* from mitochondria to cytoplasm, and this trend was positively correlated with drug concentration.

Next, we found that BBR-induced apoptosis was dependent upon the dose-dependent phosphorylation of the ASK1 and JNK. ASK1/JNK signaling is indeed a key regulator of apoptosis [21], and we found that the JNK inhibitor SP600125 was able to prevent BBR-induced NSCLC cell apoptosis. This is in line with prior data from colon cancer cells where in BBR treatment drove dose-dependent MAPK phosphorylation and apoptosis that could be prevented via the specific inhibition of these signaling proteins [22]. Meanwhile, we measured the level of intracellular ROS in A549 and PC9 cells after BBR treatment, and the results showed that BBR significantly increases ROS levels. Pretreatment of NSCLC cells with NAC was sufficient to suppress ROS generation and associated apoptosis following BBR treatment, indicating that such ROS production is necessary for programmed cell death induction, consistent with prior data generated using prostate cancer cells, which NAC treatment prevented BBR-induced ROS generationin PC-3 cells, thereby interfering with their apoptosis [23]. In fact, our observation supported initial hypothesis that NAC-mediated ROS ablation being sufficient to largely ablate BBR-induced ASK1/JNK phosphorylation and associated cell death. Consistent with this, Xie et al. [17] previously found BBR to induce breast cancer cell death via the ROS/ASK1/JNK pathway. Therefore, these results indicated that treatment with BBR induced the apoptosis of NSCLC cells via ROS/ASK1/JNK pathway.

Conclusions

In conclusion, the results of the present study indicated that BBR mediated generation of ROS, which activated the ASK1/JNK pathway, subsequently resulted in loss of MMP and eventually triggered the A549 and PC9 cell apoptosis. Based on the study, we proposed a model by which BBR induced apoptosis in NSCLC via ROS/ASK1/JNK pathways, which may provide an insight into the therapeutic potential of BBR for NSCLC patients.

Abbreviations

NSCLC: Non-Small Cell Lung Cancer

BBR: Berberine

ROS: Reactive Oxygen Species

ASK1: Apoptosis Signal-regulating Kinase 1

JNK: c-Jun N-terminal Kinase

P-jnk: Phosphorylation Of c-Jun N-terminal Kinase

SP600125: JNK Inhibitor

NAC: Anti-Oxidant N-acetyl Cysteine

CCK-8: Cell Counting Kit-8

IHC: Immunohistochemical

MMP: Mitochondrial Membrane Potential

cyto-C: Cytochrome *c*

PC-3: Prostate Cancer Cells-3

MAPKs: Mitogen Activated Protein Kinases

Declarations

Ethics approval and consent to participate

This research program was approved by the Ethics Committee of Shanghai 10th People's Hospital of Tongji University. All participants signed the informed consent forms.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Qianqian Chen; Administrative support: Ming Li, Lihong Fan; Provision of study materials: Qianqian Chen, Yaqin Hou, Zhou Ding; Collection and assembly of data: Yaqin Hou, Bingjie Hao; Data analysis and interpretation: Dan Li, Qing Xia; Manuscript writing: Qianqian Chen, Ming Li; Final approval of the manuscript: All authors.

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Not applicable.

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Figures

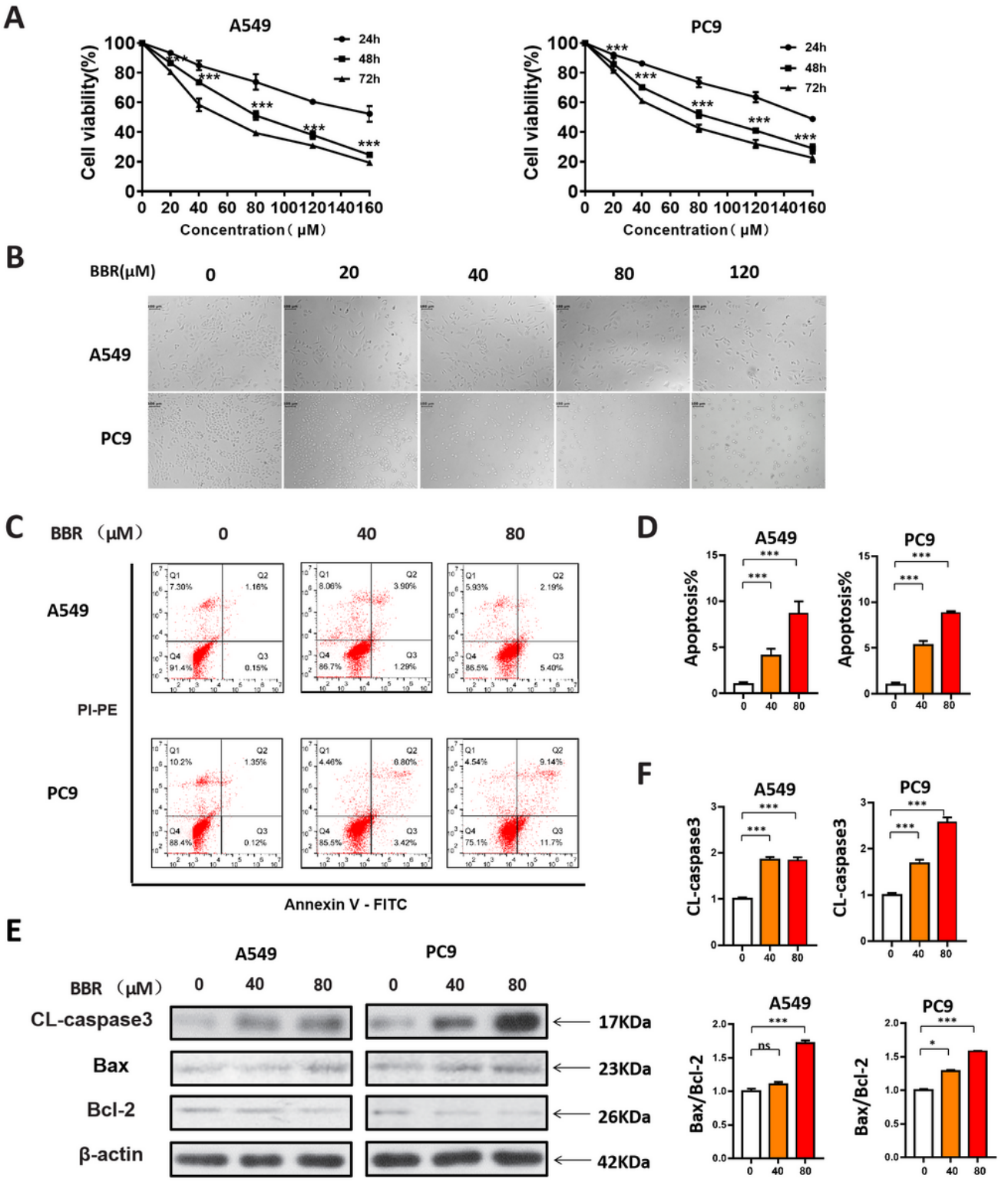

Figure 1

BBR inhibited proliferation and induced apoptosis in NSCLC cells. (a) A549 and PC9 cells were treated with various concentrations of BBR for 24, 48, and 72 h. Cell viability was analyzed by CCK8 assay. (b) Morphological changes were assessed via microscopy after a 48h treatment (20×). (c) A549 and PC9 cells were treated using a range of BBR concentrations (0, 40, 80μM) for 48h, after which Annexin-V/PI staining was used to evaluate apoptotic death via flow cytometry. (d) apoptotic rates were quantified. (e)

After treatment as in (c), CL-caspase-3, bax, and bcl-2 in NSCLC cells were measured via western blotting. (f) Protein levels from (e) were quantified. Data represent the mean ± SD of three different experiments with triplicate sets in each assay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs BBR-untreated group.

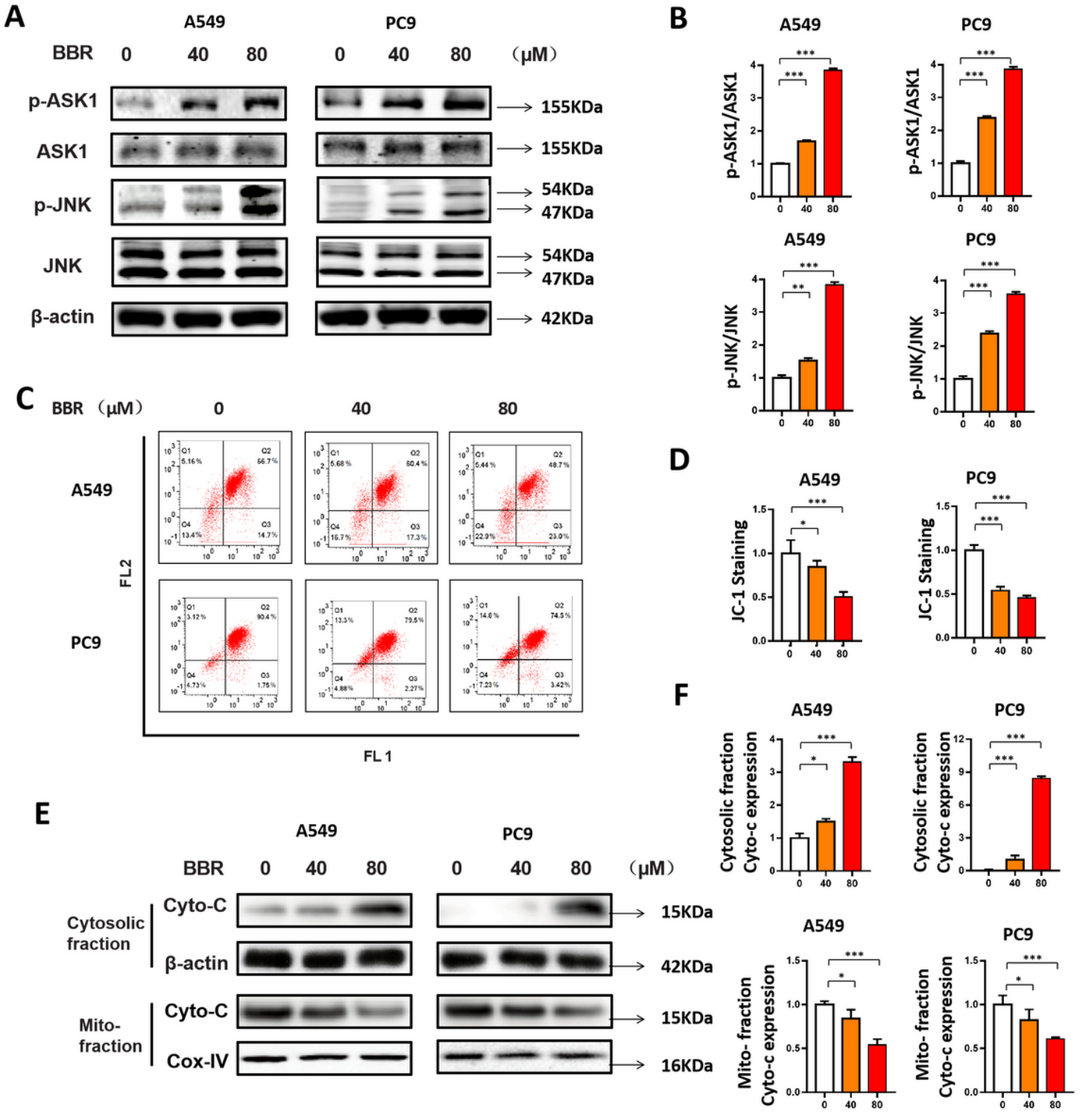

Figure 2

BBR activated ASK1/JNK and mitochondrial apoptotic pathway in NSCLC cells. (a) A549 and PC9 cells were treated with BBR (0, 40, 80 μM) for 48 h, after which ASK1,p-ASK1,JNK and p-JNK levels were

assessed via western blotting, (b) Protein levels from (a) were quantified. (c) After treatment as in (a), changes in mitochondrial membrane potential were measured in NSCLC cells. (d) Cells with low mitochondrial membrane potential are shown in Q2, and were quantified. (e) After treatment as in (a), A549 and PC9 cells were separated into mitochondrial and cytosolic fractions, and cytochrome c levels in these fractions were measured via Western blotting. (f) Protein levels from (e) were quantified. Data represent the mean ± SD of three different experiments with triplicate sets in each assay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

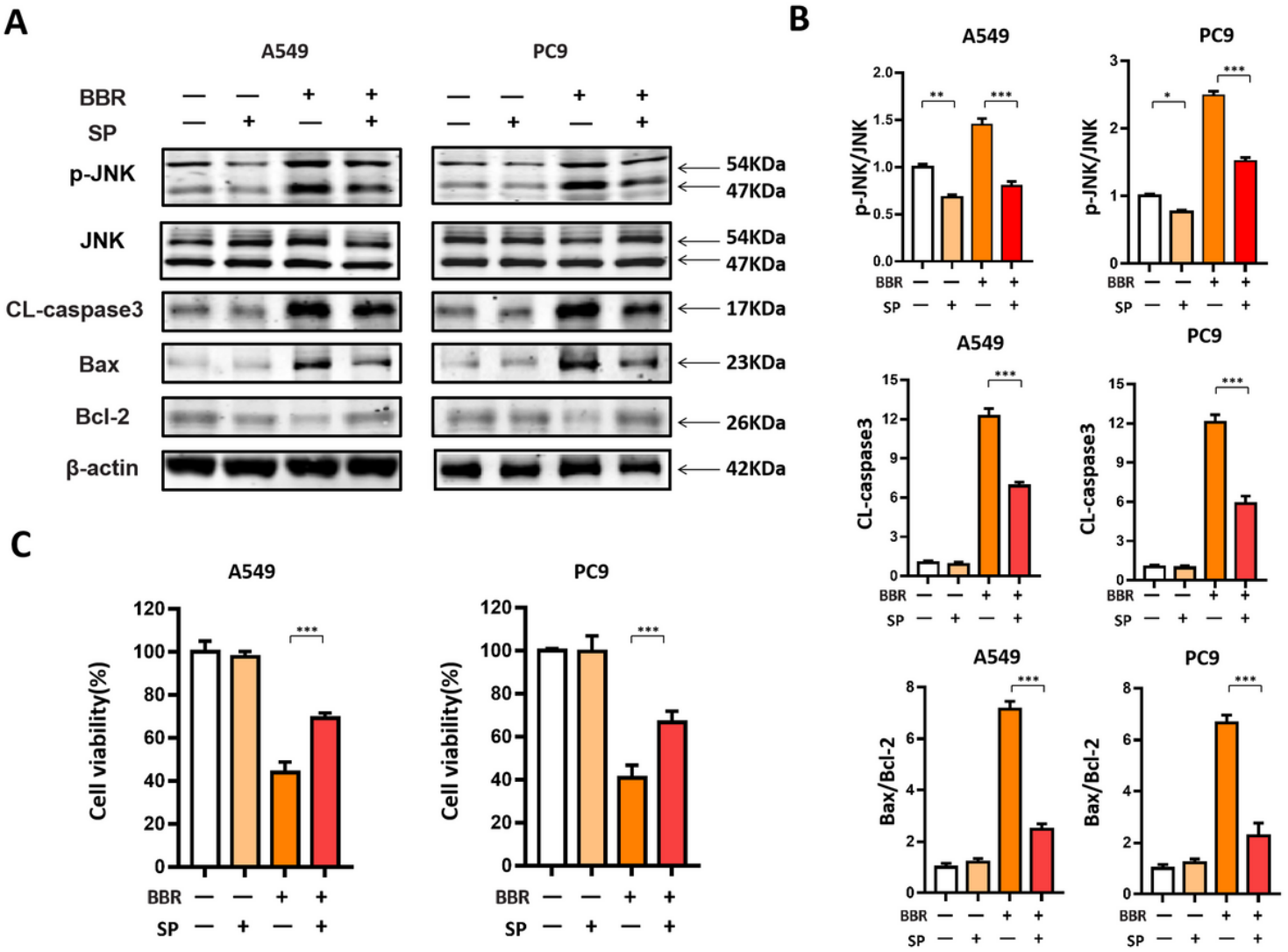

Figure 3

BBR-induced NSCLC cell apoptosis via the activation of ASK1/JNK pathway. (a) A549 and PC9 cells were treated with 40μM SP600125 (SP) for 1h prior to treatment with 80μM berberine for 48h.The protein of p-JNK, JNK, CL-caspase-3, bax, and bcl-2 in NSCLC cells were measured via western blotting. (b) Protein levels were quantified. Data represent the mean ± SD of three different experiments with triplicate sets in each assay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

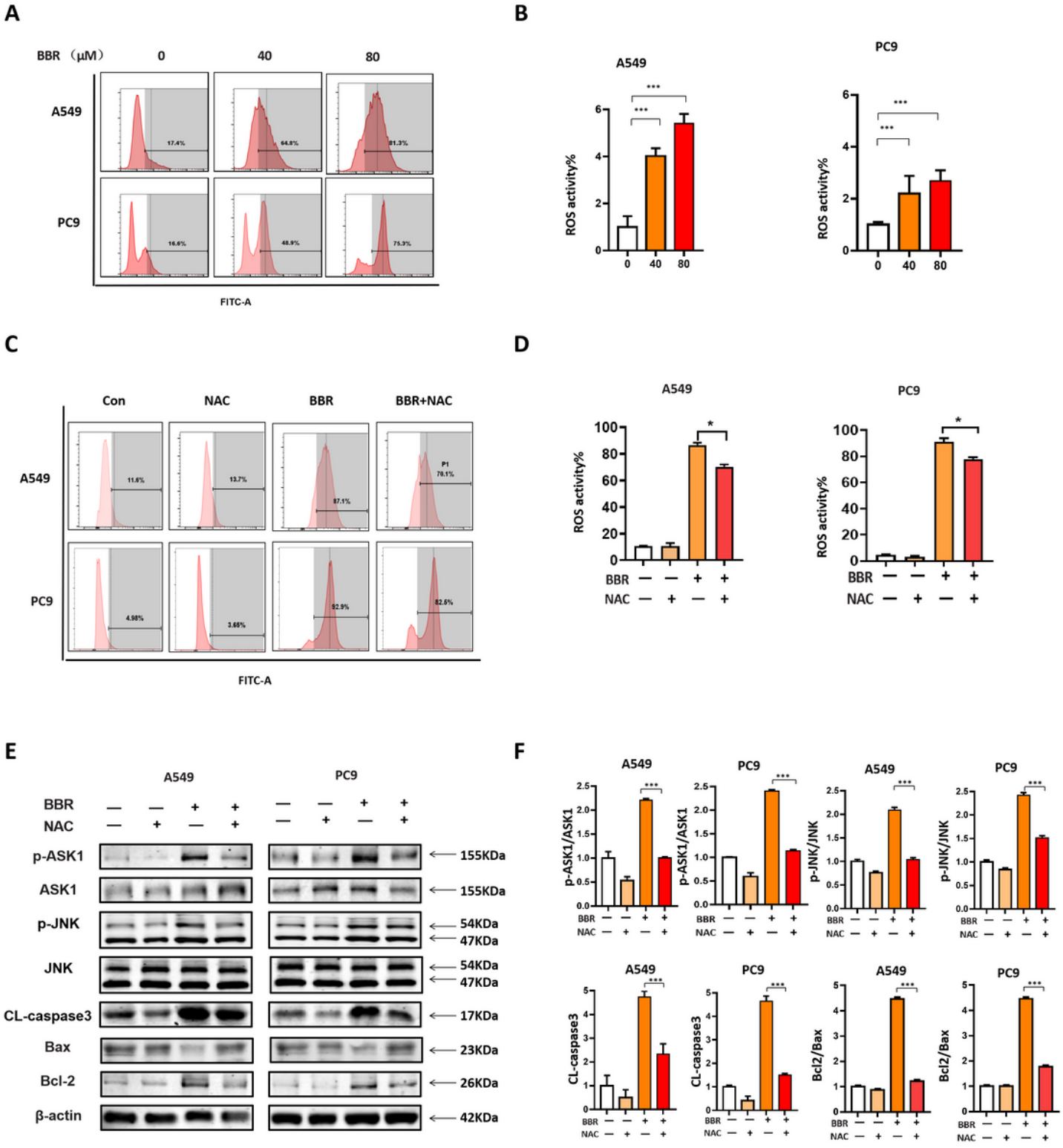

Figure 4

BBR-induced NSCLC cell apoptosis via the activation of ROS/ASK1/JNK pathway in vitro. (a) Following A549 and PC9 cells were treated with BBR (0, 40, 80 μM) for 48 h, ROS levels in A549 and PC9 cells were measured by flow cytometry. (b) Data from (a) were quantified. (c) Following a 1h pretreatment with N-acetyl cysteine (NAC; 500μM), cells were treated for 48 h with 80 μM BBR and ROS levels were then assessed via flow cytometry. (d) Data from (c) were quantified. (e) Cells were treated as in (c), and protein

levels were then assessed via western blotting. (f) Protein levels were quantified. Data represent the mean ± SD of three different experiments with triplicate sets in each assay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

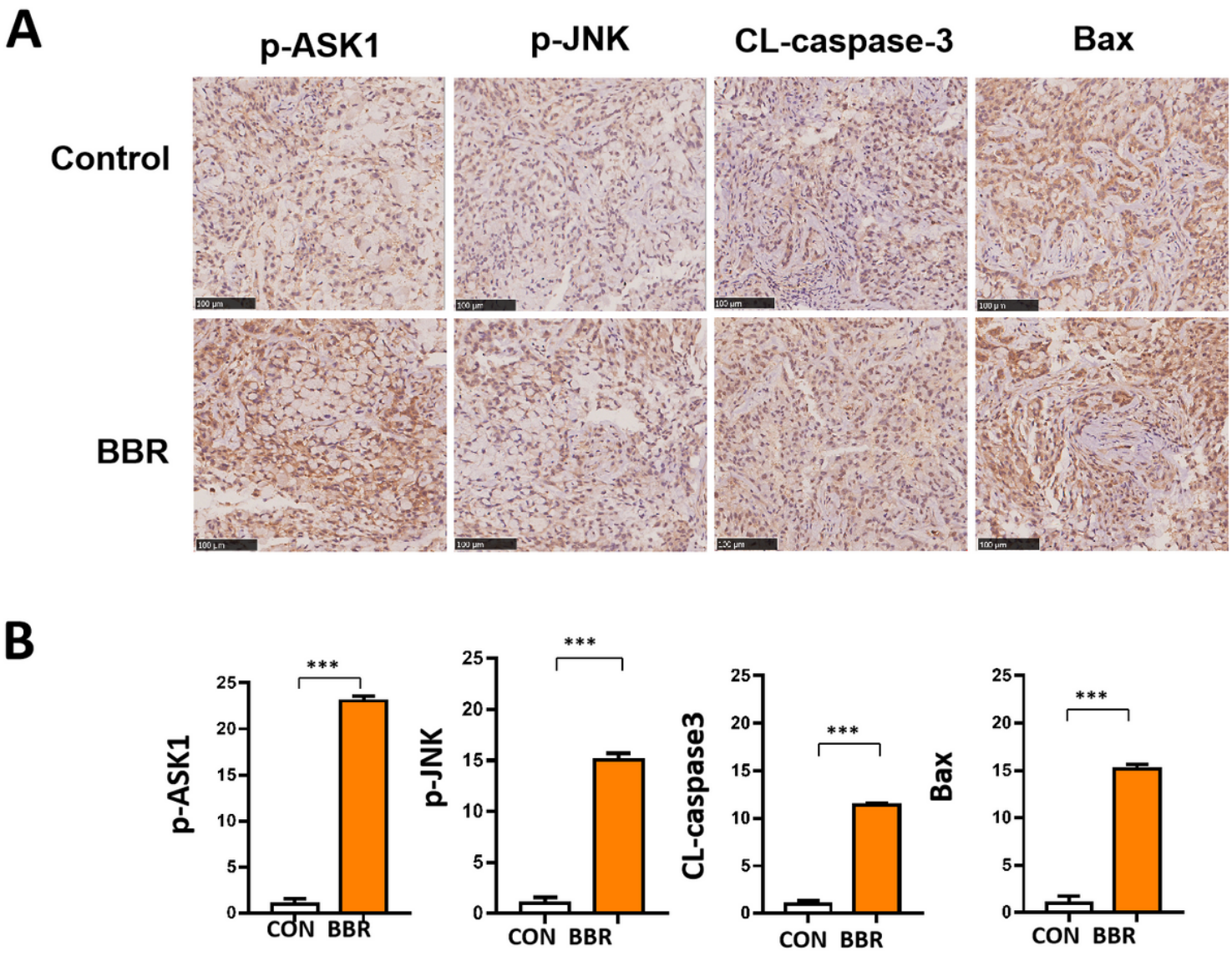

Figure 5

BBR-induced NSCLC cell apoptosis via the activation of ASK1/JNK pathway in vivo. (a) Immunohistochemistry analysis the expression of phosphorylation-ASK1, phosphorylation-JNK, CL-caspase-3 and Bax in tumor tissues treated with BBR (500 mg/kg). (b) Protein levels from (a) were quantified.

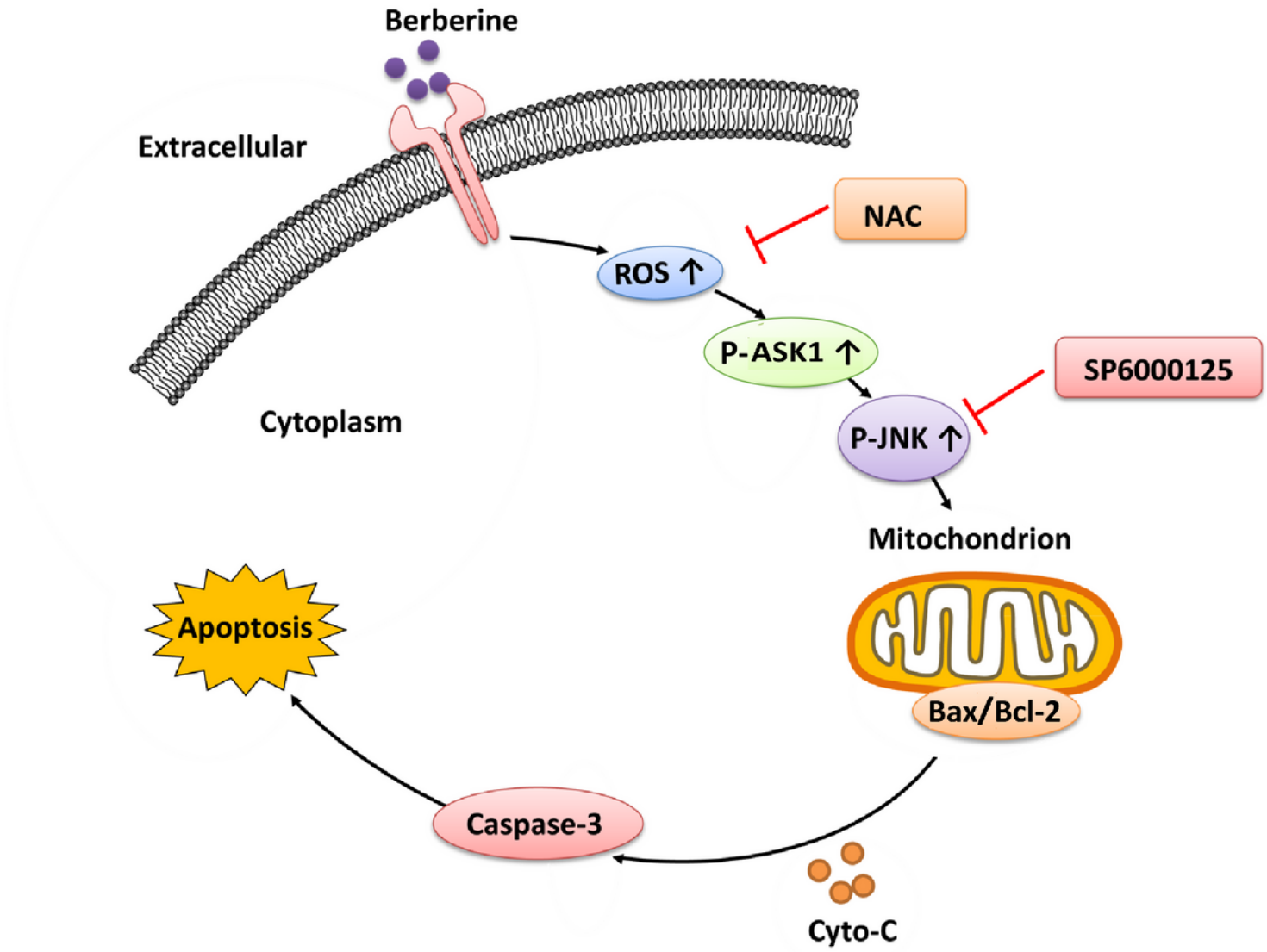

Figure 6

An overview of the BBR-induced apoptotic death of NSCLC cells. BBR-induced ROS generation promotes ASK1/JNK phosphorylation, which in turn suppresses bcl-2 family proteins, driving a loss of MMP, the release of mitochondrial cytochrome c, and consequent caspase-dependent apoptosis pathway.