A diazeniumdiolate-based NO donor induces apoptosis by modulating the CIP2A/PP2A/ERK signaling axis

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Research Article

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Abstract

Cancerous inhibitor of protein phosphatase 2A(CIP2A) modulates the activity of protein phosphatase 2A (PP2A). PP2A is part of the principal regulators of MAPKs through dephosphorylating tyrosine or threonine residue, resulting in down-regulation of their activities. The present study aimed to assess the inhibition of CIP2A and dephosphorylated effect of PP2A on the ERK signaling pathway in JS-K-induced apoptosis. Cells were noticeably inhibited in a time- and concentration-dependent manner after treatment with JS-K. JS-K could cause an increase in Bax, a decrease in Bcl-2, and subsequent activation of caspase cascade through the release of NO. Meanwhile, JS-K stimulated a significant decrease of CIP2A expression whereas an enhancement of PP2A expression. Furthermore, the effects of JS-K on upregulation of PP2A, downregulation of phosphorylated ERK, and activation of cleaved-caspase-3 and cleaved-PARP were enhanced in CIP2A siRNA-interfered cells. However, overexpression of CIP2A could cancel the effects of JS-K on upregulation of PP2A, downregulation of phosphorylated ERK, and activation of cleaved-caspase-3 and cleaved-PARP. In addition, dephosphorylation of MEK, ERK, Elk-1, c-Myc, and c-Fos stimulated by JS-K was declined in PP2A siRNA cells. Conversely, overexpression of PP2A could strengthen the effects of d dephosphorylation of MEK, ERK, Elk-1, c-Myc, and c-Fos stimulated by JS-K. Simultaneously, the apoptotic cells were reduced in PP2A/C siRNA cells but increased in overexpression of PP2A cells. Taking together, JS-K as a diazeniumdiolate-based NO donor induces apoptosis by modulating the CIP2A/PP2A/ERK signaling axis.

Background

Hepatocellular carcinoma is a leading cause of cancer death worldwide. Despite recent advances in therapy, the disease remains largely incurable. While chemotherapy markedly improves symptoms and the quality of life for patients. Nitric oxide (NO), a simple molecule consisting of one nitrogen atom and one oxygen atom, is everywhere[1]. It functions as a signaling molecule in the physiological and pathological processes of the human body. Diazeniumdiolate-based NO donor, JS-K (O^2-(2, 4-dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl] diazen-1-ium-1, 2-diolate), elicits higher cytotoxicity in a variety of tumors including hepatocellular carcinoma[2], ovarian cancer[3], gastric cancer[4], non-small cell lung cancer[5], and renal cell carcinoma[6]. JS-K as a NO precursor is activated by glutathione transferase (GST) and releases NO in a controlled, and sustained way in the tumor cells, which shows a promising potential application in the treatment of cancer.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) as a novel oncoprotein modulates the activity of protein phosphatase 2A (PP2A). Overexpression of CIP2A contributed to the proliferation and progression of various human malignancies [7]. However, PP2A has the tumor-suppressing effect and apoptosis-inducing function. Elevated levels of exogenous $ONOO^-$ can nitrate and activate the catalytic subunit of PP2A[8]. These results indicate that an activated mechanism of CIP2A/PP2A might occur in JS-K-induced apoptosis.

The MAPK/ERK signaling cascade is activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (RTKs), integrins, and ion channels which then transduce the signal to adaptors which eventually activate Raf, MEK and ERK, the core components of the pathway [9]. Phosphorylation of ERK factors is balanced by specific MAPK kinases and phosphatases. PP2A is part of the important regulators of the ERK pathway through dephosphorylating tyrosine or threonine residue, resulting in down-regulation of their activities [10]. In our previous study, we found JS-K-released NO induced cell apoptosis through inhibition of Raf/MEK/ERK signaling pathway and caused the activation of PP2A[11, 12]. In the present study, we aimed to assess the inhibition of CIP2A and dephosphorylated effect of PP2A on the ERK signaling pathway in JS-K-induced apoptosis. The results demonstrated the contribution of CIP2A inhibition to the inactivation of PP2A/ERK pathway could lead to cell apoptosis, which was related to the anticancer activity of diazeniumdiolate-based NO donor against hepatocellular carcinoma.

Materials And Methods

Materials

JS-K (Fig.1A), PP2A-Cα (siRNA) and control siRNA were available from Santa Cruz Biotechnology (San Diego, CA). Carboxy-PTIO was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Annexin V-FITC/PI kit was from BD Biosciences (NJ, San Diego, CA, USA). Apoptosis Antibody Sampler Kit, antibodies for phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), Bax, Bcl-2, CIP2A, phospho-c-Raf (Ser338), phospho-MEK1/2 (Ser217/221), phospho-c-Myc (Ser62), phospho-c-Fos (Ser32) and phospho-Elk-1 (Ser383) mouse mAb were purchased from Cell Signaling Technology (Beverly, MA, USA). The dilution ratio of CST antibodies is 1:1000. β-actin (1:5000), Histone H3, HRP-conjugated affinipure goat anti-rabbit IgG, and HRP-conjugated affinipure goat anti-mouse IgG were supplied from Proteintech Group, Inc (Wuhan, China). The methylthiazolyl tetrazolium biomide (MTT) was from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). CIP2A siRNA, CIP2A cDNA, PPP2CA cDNA (PP2A-Cα, GV230 carrier, NheI/AgeI enzymatic cutting) was obtained from Shanghai Genechem Co., Ltd.

Cell proliferation determination

HepG2 cells were seeded into 96-well cell plates at a final density of 1.0×10^4 cells/well for 12 h and incubated with different concentrations of JS-K for a specific time. Then, MTT at a concentration of 5 mg/mL was added to each well and incubated for another 4 h. DMSO was used to dissolve the formazan crystals. Finally, the absorbance was measured using a microplate reader (Molecular Devices, Sunnyvale, USA) at 490nm. Inhibition rate (%) = $[(A_{Control} - A_{Treated})/ A_{Control}] \times100\%$.

Annexin V-FITC/PI dual staining

HepG2 cells were treated with different concentrations of JS-K for 6 h, 12 h, and 24 h. Thereafter, cold PBS was used to wash the cells twice that subsequently were resuspended in 500 μL binding buffers.

Annexin-V-FITC and PI were added to binding buffers for 10 min in the dark. The stained cells were analyzed by flow cytometry and analyzed by BD CellQuest Pro software (version 2.0, BD Pharmingen, BD Biosciences).

Immunofluorescence assay

HepG2 cells were grown on a 48-well plate and resuspended with PBS in the absence or presence of JS-K. Then the cells were fixed with 4% paraformaldehyde and permeabilized with cold methanol for 15 min. Next, cells were aspirate methanol and rinsed in PBS for 5 min each, followed by incubating cells in 5% normal goat serum for 60 min. Subsequently, cells were incubated with the primary antibody at 4°C overnight. Then the cells were washed three times with PBS and incubated with Alexa Fluor 594-conjugated anti-rabbit IgG antibody for 2 h at room temperature in a humidified chamber. Cells nuclei were stained with DAPI and visualized by a fluorescence microscope.

Western blot analysis

HepG2 cells were washed with PBS and lysed for 30 min. Then cells were collected and centrifuged at 12,000 g for 40 min. The proteins of total cell lysis, nuclear proteins were obtained, respectively. Protein was separated by a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h and then transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, USA) for 2 h. 5% non-fat milk in TBST (Tris-buffered saline and 1% Tween 20) was used to block the membranes for 2 h. After blocking, cells were cultured with primary antibodies (diluted at 1:1000) at 4°C overnight. HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG were cultured for 2 h at a dilution of 1:10000. The proteins were visualized with ECL assay through a Bio-Rad gel imaging and analysis system.

RNA and DNA interference assay

RNA interference Cells were collected and re-suspended in a DMEM medium. A single-cell suspension was subsequently seeded in 12-well plates (2×10^5 cells/well). Cells were infected or transfected with CIP2A or PP2A in the serum-free medium the next day. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect cells (2×10^5 cells/well) with CIP2A or PP2A-C siRNA for 48 h before treatment with JS-K for 24 h. Then the cells were collected to determine the expression of proteins.

DNA interference Cells were infected or transfected with CIP2A or PP2A in the serum-free medium the next day. For transfection, 2.5μg of DNA was used for each transfection using lipofectamine 3000 reagent for 4 h. Then the infection medium was removed and replaced with a complete medium for 48 h. Next, cells were treated with JS-K for 24 h to observe the expressions of proteins by Western blot analysis. Negative control plasmid was treated according to the above methods.

Statistical analysis

Quantitative data were expressed as mean±SD and analyzed by one-way ANOVA. Multiple comparisons between the groups were performed using Tukey's test. Statistical significance was set at a level of $*P<$

0.05 or $^{**}P < 0.01$ level.

Results

Effect of JS-K on the proliferation and apoptosis in hepatocellular carcinoma cells

The effect of JS-K on the proliferation of HepG2, PLC5, Huh-7, and L02 cells was assessed by MTT assay. As shown in Fig 1B, JS-K noticeably inhibited three hepatocellular carcinoma cells for 24 h, especially HepG2 cells. JS-K with a concentration of up to 20 μM did not show significant cytotoxicity against human normal liver cell line (L-02). As shown in Fig.1C, JS-K caused a time- and concentration-dependent manner in cell reduction when the cells were treated with different concentrations of JS-K (0, 1.25, 2.5, 5, 10 or 20 μM) for a specified time, indicating that JS-K exhibited potent inhibition effect on hepatocellular carcinoma cells.

To verify whether the inhibition effect of JS-K in hepatocellular carcinoma cells (HepG2 cells) was caused by apoptosis, Annexin V-FITC/PI was conducted for the indicated time. As shown in Fig 1D and E, the apoptosis rate was raised in a time-dependent manner in JS-K-treated cells. Apoptotic cells including early-stage apoptosis and late-stage apoptosis could reach approximately 80% with JS-K (10μM) treatment for 24 h. These results confirmed that JS-K could induce cell apoptosis in hepatocellular carcinoma cells.

Effect of JS-K on the expressions of related apoptotic proteins in HepG2 cells

To further verify whether the apoptotic effect induced by JS-K was related to intracellular NO levels, related apoptotic proteins were determined. As shown in Fig 2A and B, cells exposure to JS-K resulted in a down-regulation of Bcl-2, an up-regulation of Bax in a time-dependent manner. Simultaneously, JS-K caused activation of cleaved-caspase-9, cleaved-caspase-3, and cleavage of PARP. Carboxy-PTIO as a NO scavenger weakened the changes of Bcl-2, Bax, cleaved-caspase-9/3, and cleaved-PARP in JS-K-treated cells (Fig 2C and D), which demonstrated that JS-K induced cell apoptosis via NO release.

Effect of JS-K on the expression of CIP2A in HepG2 cells

To further evaluate the relationship between NO and CIP2A, an immunofluorescence assay was performed. As shown in Fig3, JS-K could decrease the intracellular fluorescence intensity of CIP2A in HepG2 cells while Carboxy-PTIO declined the intracellular fluorescence intensity of CIP2A in JS-K-treated HepG2 cells, which suggested NO released from JS-K could inhibit the expression of CIP2A.

Effect of JS-K on the inhibition of CIP2A/PP2A/ERK pathway in HepG2 cells

To certify the essential effect of the CIP2A/PP2A/ERK pathway, HepG2 cells were transfected with CIP2A plasmid and CIP2A expression was determined using western blotting. JS-K decreased the expression of CIP2A in a concentration-dependent manner whereas Carboxy-PTIO could partially weaken it (Fig.4A and B). As shown in Fig 4C, CIP2A depletion significantly upregulated the expression of PP2A, downregulated

the phosphorylation of ERK, and caused a marked rise in caspase-3 and PARP cleavage. However, CIP2A overexpression significantly downregulated the expression of PP2A, upregulated the phosphorylation of ERK, and caused a marked decline in caspase-3 and PARP cleavage (Fig.4D). These results confirmed that CIP2A was involved in the inhibition of PP2A/ERK pathway and the induction of cell apoptosis caused by JS-K.

Effect of PP2A on the inhibition of MEK/ERK pathway in JS-K-treated HepG2 cells

As shown in Fig 5A, the effects of downregulation of phosphorylated MEK and ERK caused by JS-K were abolished in PP2A siRNA interfered-cells, but no obvious change was determined in control siRNA cells. Similarly, JS-K-caused dephosphorylation of nuclear Elk-1, c-Myc, and c-Fos was abolished in PP2A siRNA interfered-cells. Conversely, overexpression of PP2A could strengthen the effects of dephosphorylated MEK and ERK caused by JS-K (Fig.5B). The same results of JS-K were also observed in dephosphorylation of nuclear Elk-1, c-Myc, and c-Fos in overexpression of PP2A cells. The dephosphorylation of c-Raf induced by JS-K did not exhibit obvious changes in RNA or DNA interference cells. The results suggested that JS-K suppressed ERK signaling pathway through dephosphorylation effects of PP2A. Simultaneously, the apoptotic cells were reduced in PP2A siRNA cells but increased in overexpression of PP2A cells (Fig.5C and D).

Discussion

JS-K as a diazeniumdiolate-based NO donor has apoptotic induction effects on hepatocellular carcinoma cells[2, 13]. In the present study, JS-K treatment triggered downregulation of CIP2A, accompanied by the inhibition of the PP2A/ERK signaling pathway, suggesting a novel mechanism of the NO donor.

Escape from apoptosis often exists in the occurrence and development of tumor cells, and the activation of apoptosis is a tumor treatment strategy[14]. Our results indicated that JS-K treatment inhibited cell viability in a time-dependent way in three hepatocellular carcinoma cells, especially HepG2 cells, which further validated the previous results. Additionally, JS-K induced cell apoptosis in a time-dependent way. Moreover, NO was released from JS-K in a time-dependent manner, which resulted in the modulation of related-apoptosis proteins including Bax/Bcl-2 modulation, cleaved-caspase-9/3, and cleaved-PARP activation. Therefore, the apoptotic induction effect on HepG2 cells caused by JS-K was associated with NO release.

Previous studies have indicated that high CIP2A expression was highly associated with poor prognosis in several cancers[15, 16]. Therefore, CIP2A as an important target molecule is used for the tumor treatment strategy[17, 18]. The present study revealed the expression of CIP2A was significantly downregulated in JS-K-treated HepG2 cells. Transient NO depletion from JS-K significantly upregulated the expression of CIP2A, suggesting NO release from JS-K was related to the downregulation of CIP2A. Using siCIP2A or pcDNA CIP2A, the effect of CIP2A knockdown or overexpression in HepG2 cells was analyzed. Transient CIP2A depletion was able to enhance the effects on activation of PP2A, inhibition of ERK, and cleavage of caspase-3 and PARP while CIP2A overexpression had suppressed these effects, suggesting that CIP2A

was involved in cell apoptosis caused by JS-K. Thus, JS-K could inhibit CIP2A to reactivate PP2A and induce apoptosis.

The dynamic balance between the protein kinases and phosphatases regulates protein phosphorylation, which plays an important role in various biological processes[19]. We further investigated the inhibitory function of PP2A toward the ERK signaling pathway. The dephosphorylation of the ERK signaling pathway was widely regulated by PP2A[20]. The Ras-Raf-MEK-ERK mitogen-activated protein kinase (MAPK) pathway is activated by growth factors, mitogens, and antigen receptors [21]. Ras directly interacts with and activates Raf. Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERKs[22]. The present results indicated JS-K stimulated a significant enhancement of PP2A activity. Several studies indicate that PP2A can dephosphorylate and inactivate both MEK and ERK proteins while PP2A inhibitors OA can induce MEK and ERK1/2 phosphorylation [23–25]. The present results demonstrated that PP2A suppression could antagonize the inhibition effect of JS-K on the ERK signaling pathway including dephosphorylation of MEK, ERK, Elk-1, c-Myc, and c-Fos. In contrast, high PP2A expression enhanced the inhibitory effect of the ERK signaling pathway caused by JS-K. These findings demonstrated that JS-K inhibited MEK/ERK signaling pathways leading to cell apoptosis via PP2A-regulated signaling pathways. In addition, low PP2A expression caused a decrease in apoptotic rate while high PP2A expression caused an increase apoptotic rate in JS-K-treated HepG2 cells. Previous studies have shown CIP2A-mediated increases in the ERK pathway in the inactivation of PP2A[26, 27]. Several compounds that target the CIP2A protein have displayed potential effects on a variety of tumors[17, 28]. Therefore, CIP2A/PP2A/ERK pathway may serve as an alternative mechanism that underlies the effects of JS-K.

Conclusion

In summary, the present study revealed that CIP2A inhibition might increase the activation of PP2A, accompanied by an inactivated ERK pathway. Therefore, JS-K as a diazeniumdiolate-based NO donor induces apoptosis by modulating the CIP2A/PP2A/ERK signaling pathway axis.

Declarations

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Ling Liu conceived the study, its design, and coordination, Yihao Xing, Jinglei Xu, and Huaxia Xie performed the whole experimental work. Yile Hu participated in parts of the experiments and analysis of the data. Ling Liu wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figures

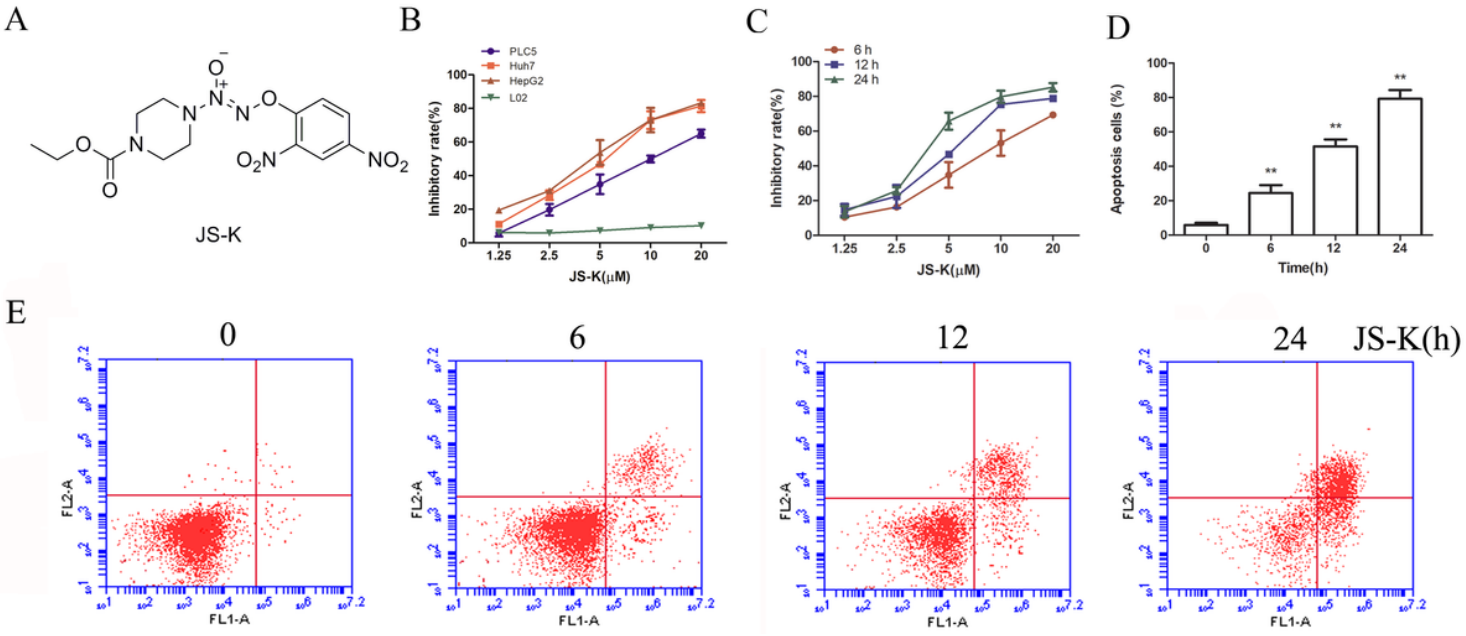

Figure 1

Effects of JS-K on cell survival and cell apoptosis in hepatocellular carcinoma cells. **A** The chemical structure of JS-K. **B** The inhibitory effects of JS-K on hepatocellular carcinoma cells and human normal liver cells. The cells were exposed to JS-K at the indicated concentrations for 24 h. **C** The inhibitory effects of JS-K on cell survival in HepG2 cells. The cells were exposed to JS-K at the indicated concentrations for 6, 12, and 24 h. **D-E** Effects of JS-K on cell apoptosis for 6, 12, and 24 h. The cells were stained with Annexin-V-FITC/PI. Data are mean ± SD. n=3 for each concentration. **P*<0.05, ***P*<0.01, vs. control group

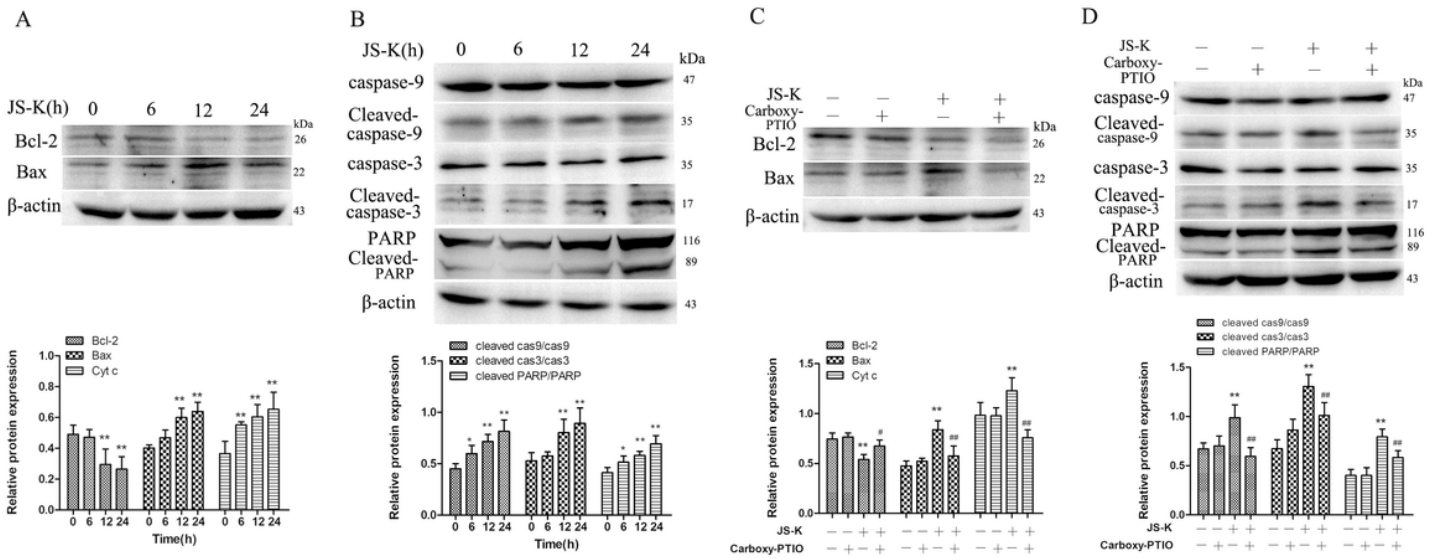

Figure 2

NO was involved in the apoptosis induced by JS-K. **A-B** Western blotting analysis of Bcl-2, Bax, cleaved caspase-9/-3, and cleaved PARP. The cells were exposed to JS-K at 10μM for 6, 12, and 24 h. **C-D** Effects of Carboxy-PTIO on the apoptotic-related protein in JS-K-treated cells. The cells were treated with NO scavenger Carboxy-PTIO (50 μM) before treatment with 10 μM JS-K for 24 h and the protein expressions of Bcl-2, Bax, cleaved-caspase-9/3, and cleaved-PARP were assessed by Western blotting analysis. Data are mean ± SD. n=3 for each concentration. *P<0.05, **P<0.01, vs. control group. $^{\#}$ P<0.05, $^{\#\#}P$<0.01, vs. JS-K-treated group

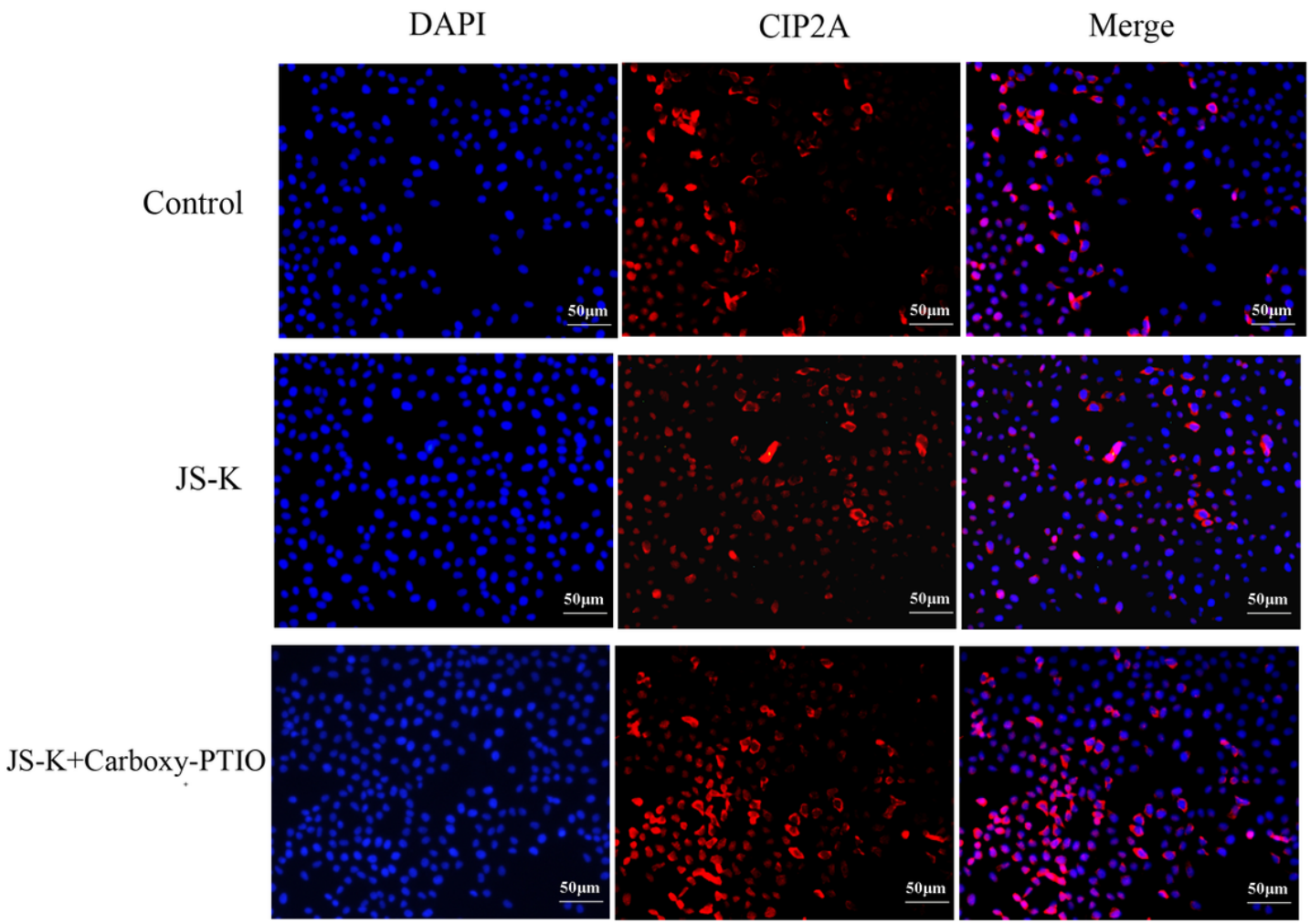

Figure 3

Effect of JS-K on the expression of CIP2A in HepG2 cells. The cells were treated with NO scavenger Carboxy-PTIO (50 μM) before treatment with 10 μM JS-K for 24 h. The expressions of CIP2A were measured by immunofluorescence assay (200×)

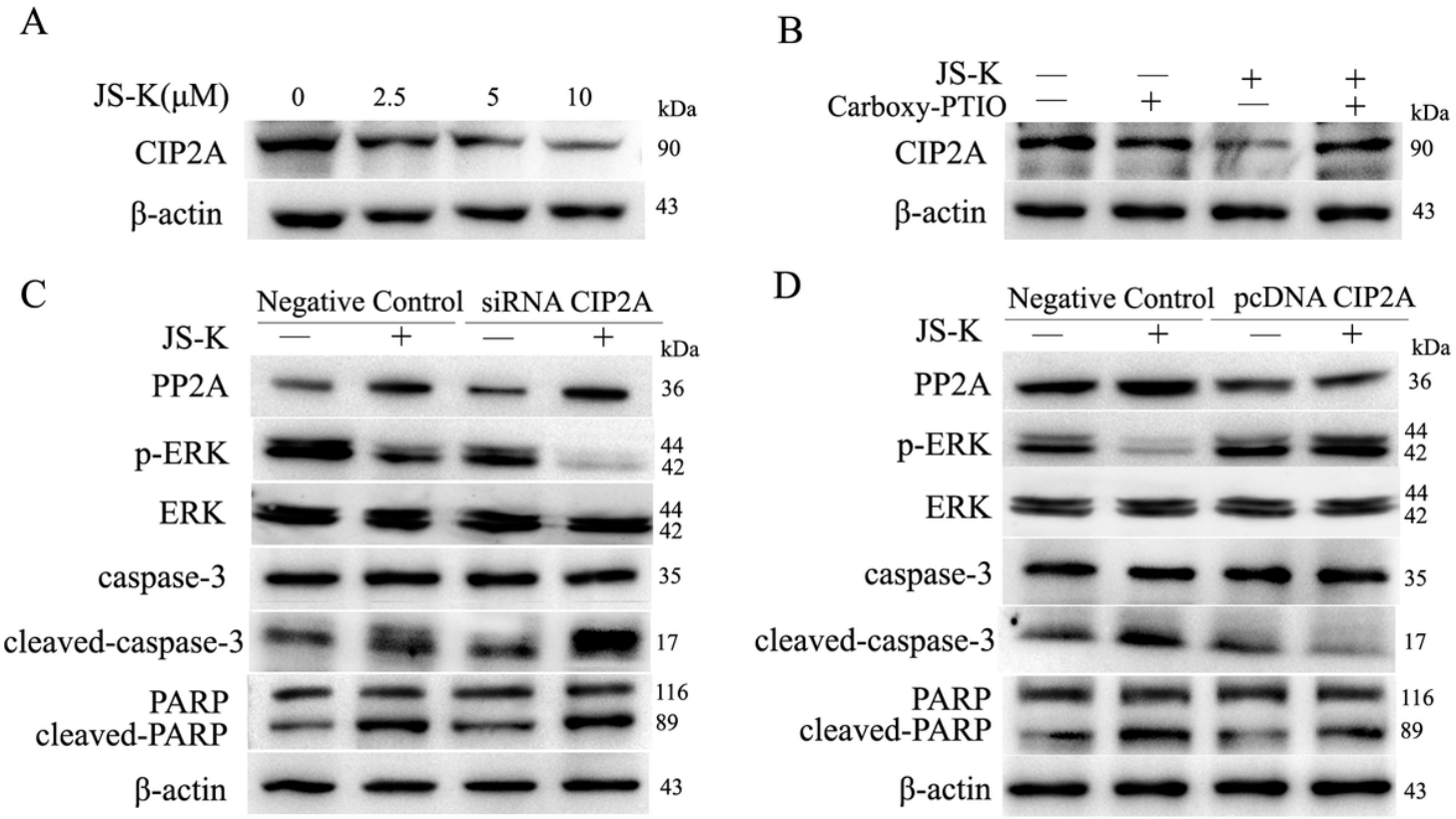

Figure 4

CIP2A inhibition was involved in the activation of PP2A, inhibitory of ERK, and cleavage of caspase pathway in JS-K-treated HepG2 cells. **A** Effect of JS-K on the expression of CIP2A. **B** Effect of Carboxy-PTIO on the expression of CIP2A caused by JS-K. **C** Effect of low CIP2A expression on the activation of PP2A, inhibitory of ERK, and cleavage of caspase pathway caused by JS-K. Cells were seeded in culture plates and transfected with CIP2A siRNA or scrambled siRNA using Lipofectamine 3000 for 4 h. Then cells were cultured with a complete medium for 48 h before treatment with JS-K for 24 h. **D** Effect of high CIP2A expression on the activation of PP2A, inhibitory of ERK, and cleavage of caspase pathway caused by JS-K

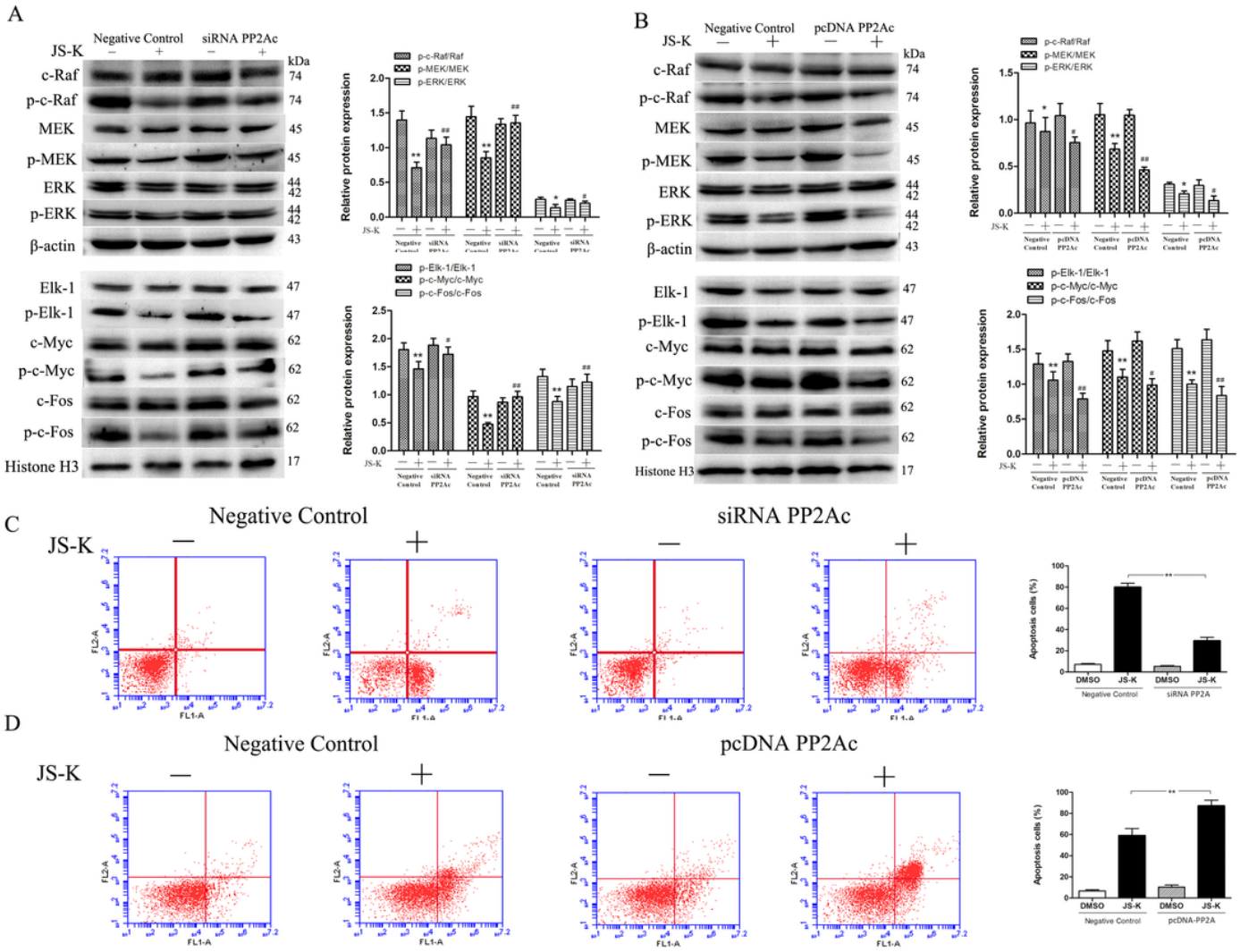

Figure 5

Effect of low PP2A expression or high PP2A expression on JS-K-treated cells. **A** Effect of low PP2A expression on ERK phosphorylation pathway caused by JS-K. Cells were seeded in culture plates and transfected with PP2Ac siRNA or scrambled siRNA using Lipofectamine 3000 for 4 h. Then cells were cultured with a complete medium for 48 h before treatment with JS-K for 24 h. **B** Effect of high PP2A on ERK phosphorylation pathway caused by JS-K. Cells were transfected with an empty expression vector, or pcDNA-PP2Ac using Lipofectamine 3000 for 4 h. Then cells were cultured with a complete medium for 48 h before treatment with JS-K for 24 h. **C-D** Effect of RNA or DNA interference on cell apoptosis caused by JS-K. Data are mean ± SD. n=3 for each concentration. **P*<0.05, ***P*<0.01, vs. control group. $^{\#}$ *P*<0.05, $^{\#\#}$*P*<0.01, vs. JS-K-treated group