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ORIGINAL RESEARCH

Inhibition of ROS-mediated activation Src-MAPK/AKT signaling by orientin alleviates H_2O_2-induced apoptosis in PC12 cells

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Purpose: Reactive oxygen species (ROS) are considered a direct cause of neurodegenerative diseases (NDDs). Drugs developed to target ROS are effective for the treatment of NDDs. Orientin is a pyrone glucoside extracted from *Polygonum orientale*, and it exhibits many pharmacological activities. In this study, we aimed to determine whether orientin could relieve hydrogen peroxide (H_2O_2)-induced neuronal apoptosis and to investigate the specific target of orientin.

Materials and methods: In this study, the neuroprotective effect and its possible mechanisms of orientin in mouse pheochromocytoma cell line (PC12) cells stimulated by H_2O_2, establishing an oxidative stress model, were investigated. And we further tested the role of ROS in the neuroprotective effects of orientin.

Results: Orientin (5–100 μg/mL) did not cause toxicity in PC12 cells but significantly decreased H_2O_2-induced reduction in PC12 cell viability, cell apoptosis rates, and nuclear condensation. It also inhibited the activation of caspase-3 and degradation of poly(ADP-ribose) polymerase (PARP). Under the stimulation of H_2O_2, MAPKs (ERK, JNK, and p38), AKT, and Src signaling proteins in PC12 cells were activated in a time-dependent manner. The application of inhibitors that were specific for MAPKs, AKT, and Src effectively alleviated H_2O_2-induced cell apoptosis. In addition, the Src inhibitor decreased the activation of MAPKs and AKT signaling. More importantly, orientin effectively decreased H_2O_2-induced phosphorylation of MAPKs, AKT, and Src signaling proteins. Finally, we confirmed that orientin effectively inhibited H_2O_2-induced accumulation of ROS in cells. In addition, ROS inhibitors blocked the Src-MAPKs/AKT signaling pathway-dependent cell apoptosis stimulated by H_2O_2.

Conclusion: These results indicate that alleviation of H_2O_2-induced cell apoptosis by orientin is Src-MAPKs/AKT dependent. Overall, our study confirms that orientin alleviates H_2O_2-induced cell apoptosis by inhibiting the ROS-mediated activation of Src-MAPKs/AKT signaling.

Keywords: oxidative stress, orientin, neuroprotection, apoptosis, Src, MAPKs, AKT

Introduction

Eukaryotic cells continuously produce free radicals during metabolic processes. The antioxidant defense system eliminates free radicals to maintain the redox balance in cells. Excessive reactive oxygen species (ROS) and an imbalance in the regulation of the antioxidant defense system cause oxidative stress (OS) damage.[1] In neuronal cells, OS induced by an imbalance in redox regulation causes severe damage. This neuronal damage and death is a direct cause of Alzheimer's disease, Parkinson's disease, and Huntington's chorea. Excessive ROS attack and break nucleic acids, degrade or inactivate enzymes, induce a melting reaction in polysaccharides, and induce lipid peroxidation in

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biological membranes. Generally, ROS continue to destroy all biological macromolecules until the cells die.[2] Treatment of neurodegenerative diseases (NDDs) in clinics has usually been ineffective; therefore, the development of a treatment strategy to block OS damage in neuronal cells is urgently needed.

Polygonum orientale Linn is a herb widely distributed throughout China, excluding Tibet. In traditional Chinese medicine, *P. orientale* is used to treat rheumatoid arthritis. Orientin, a pyrone glucoside, extracted from *P. orientale* Linn (Figure 1A), has anti-inflammatory,[3–5] antitumor,[6] and anti-oxidative[7,8] properties, and alleviates ischemic and hypoxic damage to cardiomyocytes.[9,10] Recent in vivo studies have confirmed that orientin alleviates cognitive deficits and OS damage in the Aβ-induced mouse model of Alzheimer's disease.[11] Therefore, orientin has a great potential in the treatment of NDDs similar to Alzheimer's disease. Although orientin exhibits satisfactory antioxidant activity in many studies, its efficacy in a neuronal damage model stimulated by exogenous peroxides is unclear. Moreover, the specific action mechanism and anti-oxidative targets of orientin have not been elucidated.

In this study, we constructed an OS damage model using exogenous H_2O_2 and the mouse PC12 neuronal cell line to confirm whether orientin could effectively alleviate OS damage and cell apoptosis in PC12 cells. Next, we investigated the specific mechanism underlying orientin-regulated apoptosis mediated by the activated signal transduction pathways.

Materials and methods

Antibodies and reagents

Orientin, H_2O_2, N-acetyl-L-cysteine (NAC), 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2), and 4-amino-7-phenylpyrazol [3,4-d] pyrimidine (PP3) were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies specifically targeted to caspase-3, PARP, GAPDH, JNK, p38, ERK, AKT, SRC, phospho-JNK, phosphor-p38, phospho-ERK, phospho-AKT, and phospho-Src were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorophore-labeled secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA). Inhibitors of ERK, p38, JNK, and AKT namely U0126, SB203580,

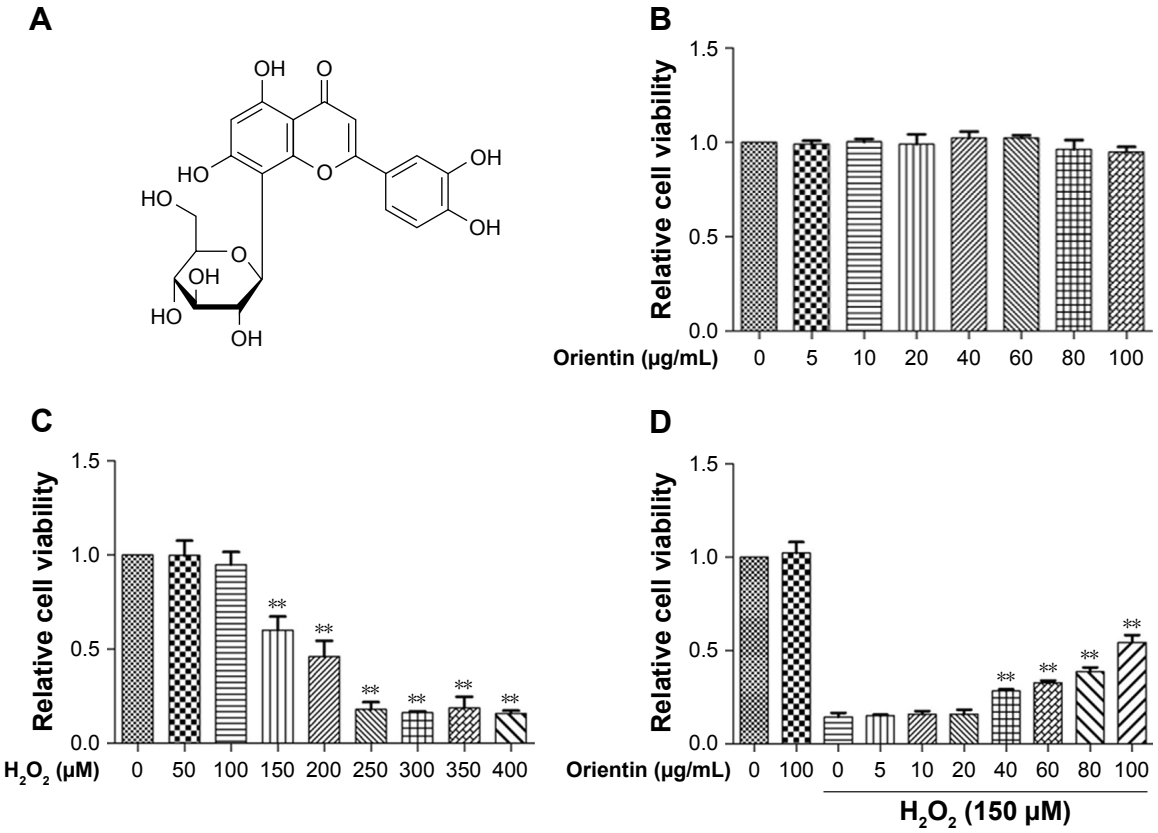


Figure 1 Orientin alleviated H_2O_2-induced reduction in PC12 cell viability.

Notes: (**A**) Chemical structure of orientin. (**B**–**D**) Relative cell viability of PC12 cells incubated with orientin (**B**), H_2O_2 (**C**), orientin, then treated with H_2O_2 (**D**) was detected by the CCK-8 assay. Data are presented as mean±SD (n=5). Significant differences are indicated with asterisks (**$P<0.01$). Compared with Control group (**B** and **C**); compared with single H_2O_2 group (**D**).

SP600125, and LY294002, respectively, were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

The PC12 cells were obtained from the Kunming Cell Bank of the Chinese Academy of Sciences and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (containing 10% horse serum, 5% fetal bovine serum, and 1% penicillin/streptomycin double antibiotic) at 37°C in a 5% CO_2 incubator. The culture medium was replaced every day, and cells were passaged every other day.

Cell viability assay

The PC12 cells were seeded into 96-well plates at 5×10^4 cells per well 24 hours before treatment. Following treatment with orientin and/or H_2O_2 for the indicated time periods, cells were incubated with 10 µL of CCK-8 (Dojindo, Kumamoto, Japan) for 2 hours, and absorbance values were measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

DAPI staining

The PC12 cells were seeded into six-well plates. After 24 hours, the cells were incubated with orientin and/or H_2O_2 at the indicated concentrations for the indicated time. The cells were then fixed in 4% paraformaldehyde for 30 minutes and stained with DAPI (Beyotime, Shanghai, People's Republic of China) for 1 hour. Cell morphology was observed under an Olympus IX71 inverted microscope.

Measurement of cell apoptosis rate

PC12 cells were cultured into six-well plates at 5×10^4 cells. Twenty-four hours later, the cells were handled with orientin and/or H_2O_2 as indicated in the figure legends. Cells were co-stained with Annexin V-FITC and propidium iodide (PI) (Beyotime, Shanghai, People's Republic of China) for 30 minutes, and cell apoptosis rates were measured using BD FACSVerse flow cytometry (BD Biosciences, San Jose, CA, USA).

Western blotting

Cells were washed with pre-cooled PBS and lysed in cell lysis buffer containing 1% phenylmethylsulfonyl fluoride, a protease inhibitor, on ice for 40 minutes. Cell lysates were collected and mixed with 2× loading buffer (Beyotime, Shanghai, People's Republic of China), and 20 µL of each sample was subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked in 5% nonfat milk, incubated with the corresponding primary antibodies at 4°C overnight, and then incubated with fluorophore-labeled secondary antibodies for 1 hour. The results were observed using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

ROS detection

The intracellular accumulation of ROS, including H_2O_2 and other peroxides, was monitored using the fluorescent probe CM-H2DCFDA. At the end of the treatment, 10 µM of the fluorescent probe CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA) was added and the samples were incubated at 37°C for 30 minutes in each well. Absorbance was measured at 450 nm (excitation) and 535 nm (emission) using a microplate reader.

Statistical analysis

All data are reported as mean±SD. Comparisons between two groups were performed using the *t*-test and one-way ANOVA. Statistical significance of differences was determined at $P<0.05$. Statistical analysis of data was performed using SPSS 13.0.

Results

Orientin alleviates H_2O_2-induced reduction in PC12 cell viability

To confirm that orientin is not toxic to PC12 cells, PC12 cells were incubated with 0, 5, 10, 20, 40, 60, 80, and 100 µg/mL orientin for 24 hours, and cell viability was measured using the CCK-8 method. Results showed that orientin was not toxic to PC12 cells at any of the doses tested (Figure 1B). To identify the toxic dose of H_2O_2, PC12 cells were incubated with 0, 100, 150, 200, 250, 300, 350, and 400 µM H_2O_2 for 24 hours. Measurement of cell viability showed that H_2O_2 caused significant toxicity in cells at concentrations higher than 100 µM (Figure 1C). Therefore, 150 µM H_2O_2 was used as the toxic dose in the OS damage model in PC12 cells. To evaluate the effects of orientin on the reduction of PC12 cell viability induced by H_2O_2, PC12 cells were incubated with 0, 5, 10, 20, 40, 60, 80, and 100 µg/mL orientin for 2 hours and then stimulated by 150 µM H_2O_2 for 24 hours. Measurement of cell viability showed that the reduction in H_2O_2-induced viability of PC12 cells was significantly suppressed at orientin concentrations higher than 40 µg/mL (Figure 1D). Therefore, orientin at 60, 80, and 100 µg/mL was used as a low, medium, and high dose, respectively, in subsequent experiments.

Orientin alleviates H_2O_2-induced apoptosis in PC12 cells

OS damage causes cells to undergo programmed apoptosis.[12] Therefore, we evaluated the function of orientin in the process of H_2O_2-induced apoptosis in PC12 cells. PC12 cells were preincubated with 60, 80, and 100 μg/mL orientin for 2 hours and then stimulated by 150 μM H_2O_2 for 24 hours. Subsequently, PC12 cells were stained with DAPI, and nuclear morphology was observed under an inverted fluorescence microscope. The results showed that H_2O_2 induced DNA condensation and breakage in the nuclei of PC12 cells; however, orientin significantly improved the nuclear morphology and reversed the change induced by H_2O_2 (Figure 2A). PC12 cells were co-stained with Annexin V-FITC and PI, and apoptosis and survival rates of PC12 cells were measured using flow cytometry. Results showed that orientin significantly decreased the H_2O_2-induced apoptosis rates in PC12 cells (Figure 2B). Caspase-3 and PARP are indispensable for cell apoptosis. Caspase-3 is the executor of programmed apoptosis, whereas PARP is the substrate of caspase-3. After pre-incubation of PC12 cells with orientin (60, 80, and 100 μg/mL) and stimulation by H_2O_2 for 24 hours, changes in the level of caspase-3 and PARP proteins were evaluated using Western blotting. The results showed that H_2O_2 stimulation downregulated caspase-3 precursor (35 kDa), upregulated the cleaved caspase-3 protein (17 kDa), and cleaved PARP protein (117 kDa) into 116 and 85 kDa proteins (Figure 2C). Additionally, orientin reversed caspase-3 activation and PARP degradation stimulated by H_2O_2 (Figure 2C). These results indicate that orientin effectively blocks H_2O_2-induced apoptosis in PC12 cells.

Orientin inhibits MAPK and AKT signaling

The serine/threonine kinase AKT, also known as protein kinase B (PKB), represents an important intersection in multiple signaling pathways; AKT regulates cytokines, growth factors, and oncogenic Ras-activated cell survival signals.[13] The mitogen-activated protein kinase (MAPK) signal transduction pathway is deeply involved in the regulation of cell proliferation, differentiation, stress, apoptosis, and survival.[14] To verify the activation of signaling pathways in PC12 cells after H_2O_2 stimulation, PC12 cells were continuously stimulated by 150 μM H_2O_2 for indicated times, and the phosphorylation of AKT, ERK, JNK, and p38 was measured using Western blotting. The results showed that H_2O_2 stimulation of PC12 cells led to AKT activation in a time-dependent manner, with the maximum activation at 4 hours (Figure 3A). Activation of MAPKs (ERK, JNK, and p38) was the highest at 1.5 hour. To determine whether orientin decreased the activation of AKT and MAPKs induced by H_2O_2, PC12 cells were preincubated with 60, 80, and 100 μg/mL orientin for 2 hours, and phosphorylation of AKT and MAPKs was measured after 4 and 1.5 hours of H_2O_2 stimulation, respectively. The results showed that orientin decreased the H_2O_2-induced phosphorylation of AKT, ERK, JNK, and p38 in a dose-dependent manner (Figure 3B). Next, PC12 cells were preincubated with inhibitors of AKT (LY294002; 10 μM), ERK (U0126; 10 μM), JNK (SP600125; 50 μM), and p38 (SB203580; 20 μM) for 2 hours and then stimulated by H_2O_2 for 24 hours. Measurement of the cleavage conditions of caspase-3 and PARP revealed that inhibitors of AKT and MAPKs significantly inhibited H_2O_2-induced caspase-3 activation and PARP degradation (Figure 3C). These results indicate that the inhibition of H_2O_2-induced apoptosis by orientin was achieved through the downregulation of AKT phosphorylation and MAPK signal transduction pathways or their upstream signaling.

Orientin inhibits the activity of Src protein

The Src protein family plays important roles in cellular OS.[15,16] Our previous studies show that Src protein regulates the activation of AKT/ERK signaling in HeLa cells after stimulation by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[17] To investigate the upstream signaling related to the suppression of AKT and MAPK signaling activation by orientin, PC12 cells were continuously stimulated by 150 μM H_2O_2 for 6 hours, and the phosphorylation of Src was evaluated using Western blotting. Following H_2O_2 stimulation, Src was activated in a time-dependent manner, with maximum activation at approximately 45 minutes (Figure 4A). To determine whether orientin decreased the activation of Src signaling, PC12 cells were preincubated with 60, 80, and 100 μg/mL orientin for 2 hours and then stimulated by H_2O_2 for 45 minutes. Analysis of Src phosphorylation revealed that orientin decreased the H_2O_2-induced phosphorylation of Src in a dose-dependent manner (Figure 4B). Next, PC12 cells were preincubated with PP2 (Src inhibitor; 10 μmol/L) or PP3 (negative inhibitor) for 2 hours, and the phosphorylation of AKT and MAPKs was measured after 4 and 1.5 hours of H_2O_2 stimulation, respectively, and the cleavage conditions of caspase-3 and PARP were measured after 24 hours of H_2O_2 stimulation. The results showed that PP2 significantly inhibited H_2O_2-induced activation of AKT and MAPK signaling (Figure 4C). Furthermore, AKT/MAPK-dependent caspase-3

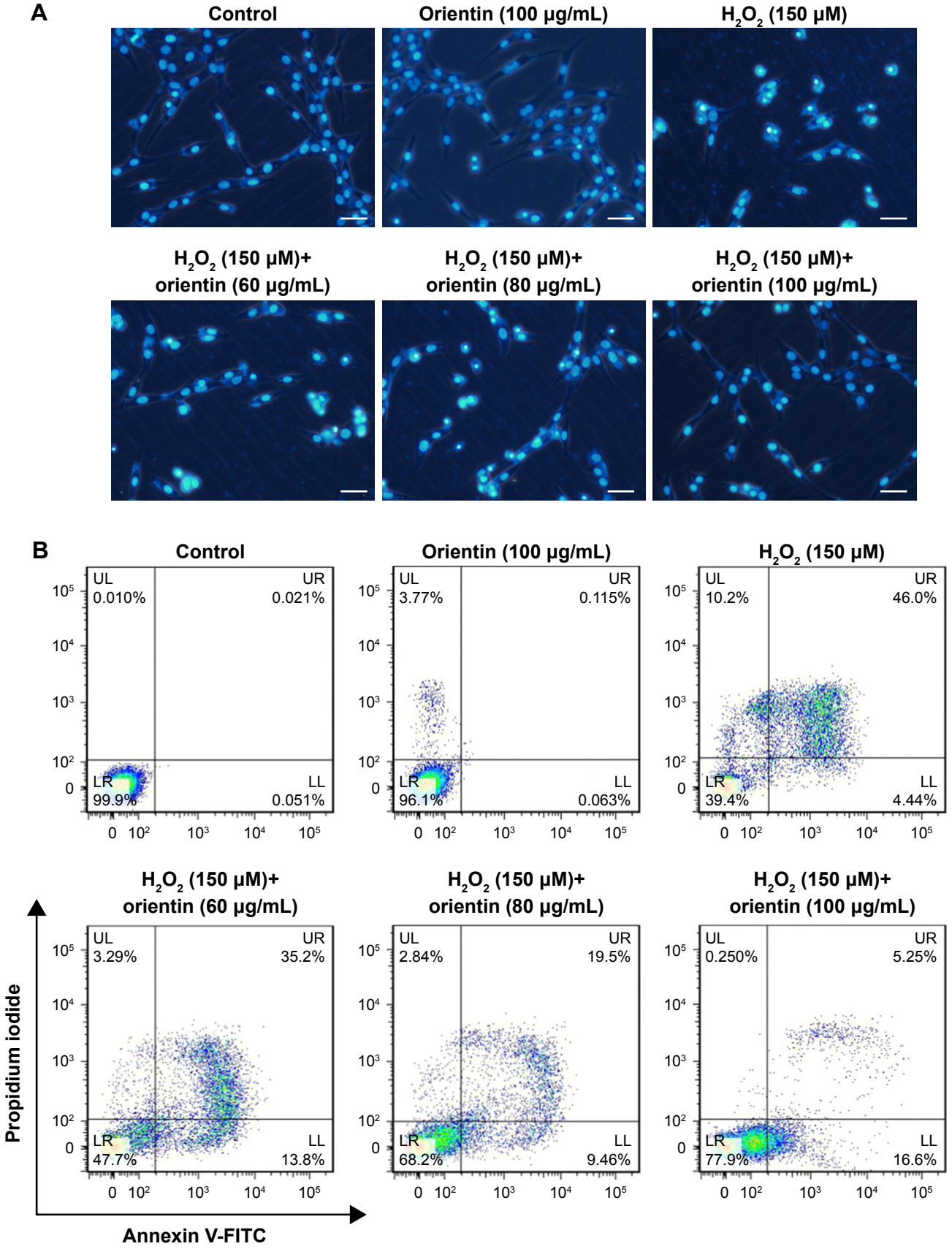


Figure 2 (*Continued*)

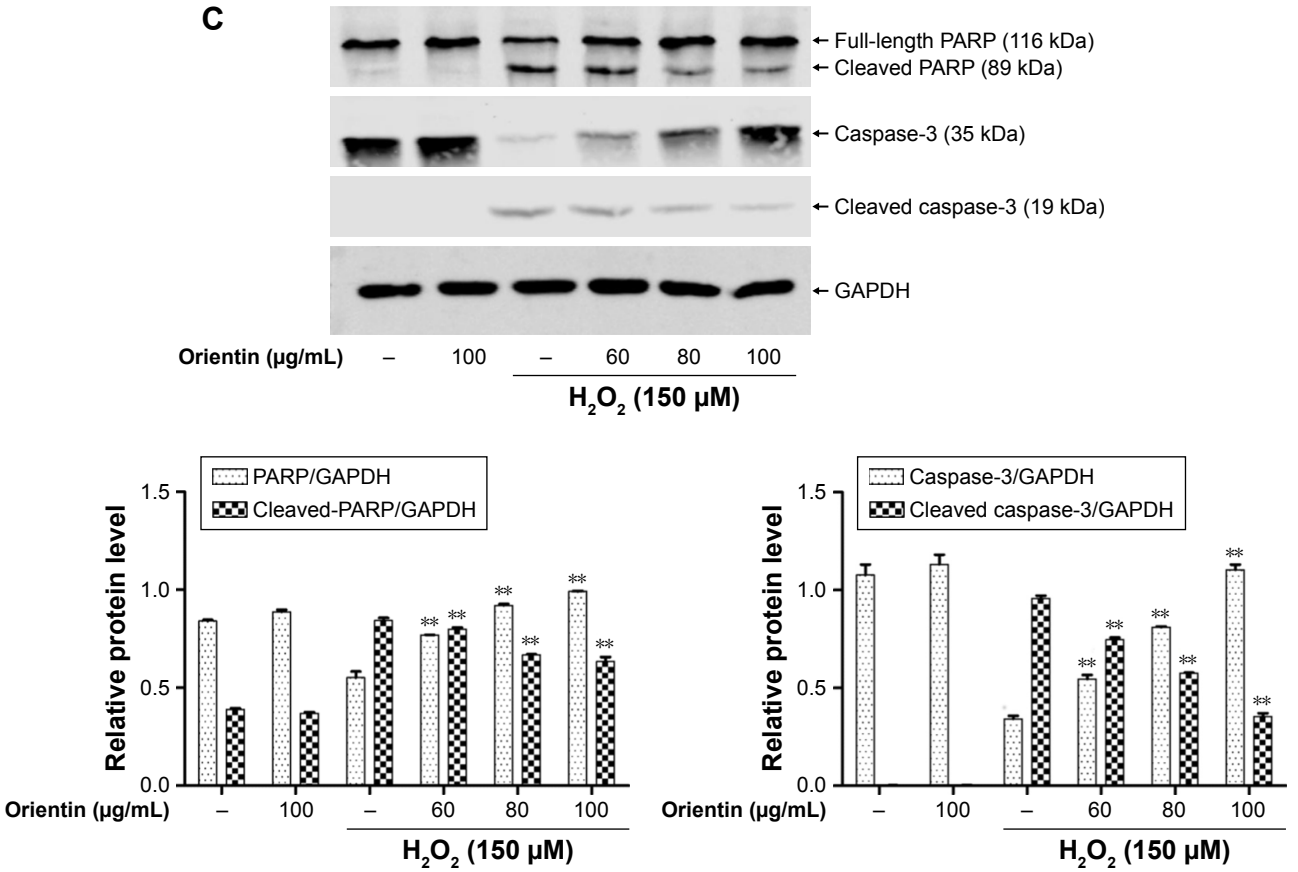


Figure 2 Orientin alleviated H_2O_2-induced apoptosis in PC12 cells.
Notes: Orientin alleviated H_2O_2-induced nuclear morphological changes (**A**), apoptosis rate (**B**), and cleavage of PARP and caspase-3 (**C**) in PC12 cells. Data are presented as mean±SD (n=3). Scale bars: 100 μm. Significant differences are indicated with asterisks (**$P<0.01$). Compared with single H_2O_2 group (**C**).

cleavage and PARP degradation were also decreased after PP2 treatment. The negative inhibitor PP3 had no effect on the activation of AKT/MAPK and cutting of apoptotic proteins (Figure 4D). These results indicate that inhibition of H_2O_2-induced cell apoptosis by orientin was achieved through the regulation of Src-mediated activation of AKT/MAPK signaling.

Orientin cleared ROS to decrease Src-MAPK/AKT-dependent cell apoptosis

Excessive exogenous H_2O_2 stimulates an antioxidant system disorder in cells and reduces the ability to clear endogenous ROS, thus causing excessive production of ROS and consequently OS damage and cell death.[18] To determine whether orientin could clear the ROS produced in response to H_2O_2, PC12 cells were preincubated with 60, 80, and 100 μg/mL orientin for 2 hours and then stimulated by H_2O_2 for 30 minutes. Analysis of intracellular ROS production using the ROS probe CM-H2DCFDA showed that orientin significantly decreased H_2O_2-induced ROS production in PC12 cells (Figure 5A). To determine whether H_2O_2-induced cell apoptosis was due to the increase in the level of intracellular ROS, PC12 cells were incubated with the ROS inhibitor NAC for 2 hours and then stimulated by H_2O_2 for 24 hours. Measurement of the cleavage conditions of caspase-3 and PARP showed that NAC inhibited caspase-3 activation and PARP degradation (Figure 5C). We further detected the phosphorylation of Src, MAPKs, and AKT after 2 hours NAC pretreatment, followed by H_2O_2 stimulation for 45 minutes, 1.5 hour, and 4 hours, respectively. Figure 5B and D shows that NAC restrained the activation of Src, MAPKs, and AKT. Overall, these data suggest that orientin alleviates H_2O_2-induced PC12 cell apoptosis through the inhibition of ROS-mediated activation of Src-MAPK/AKT signaling pathways.

Discussion

NDDs, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis, have a long disease course, are difficult to cure, can shorten life span, and cause disability.[19] There are many pathological factors in NDDs, including autophagy system disorder,[20] unfolded protein response (UPR),[21] inflammatory injury,[22] apoptosis,[23] OS,[24] and mitochondrial dysfunction.[25] Among these factors, OS and its role in the pathological

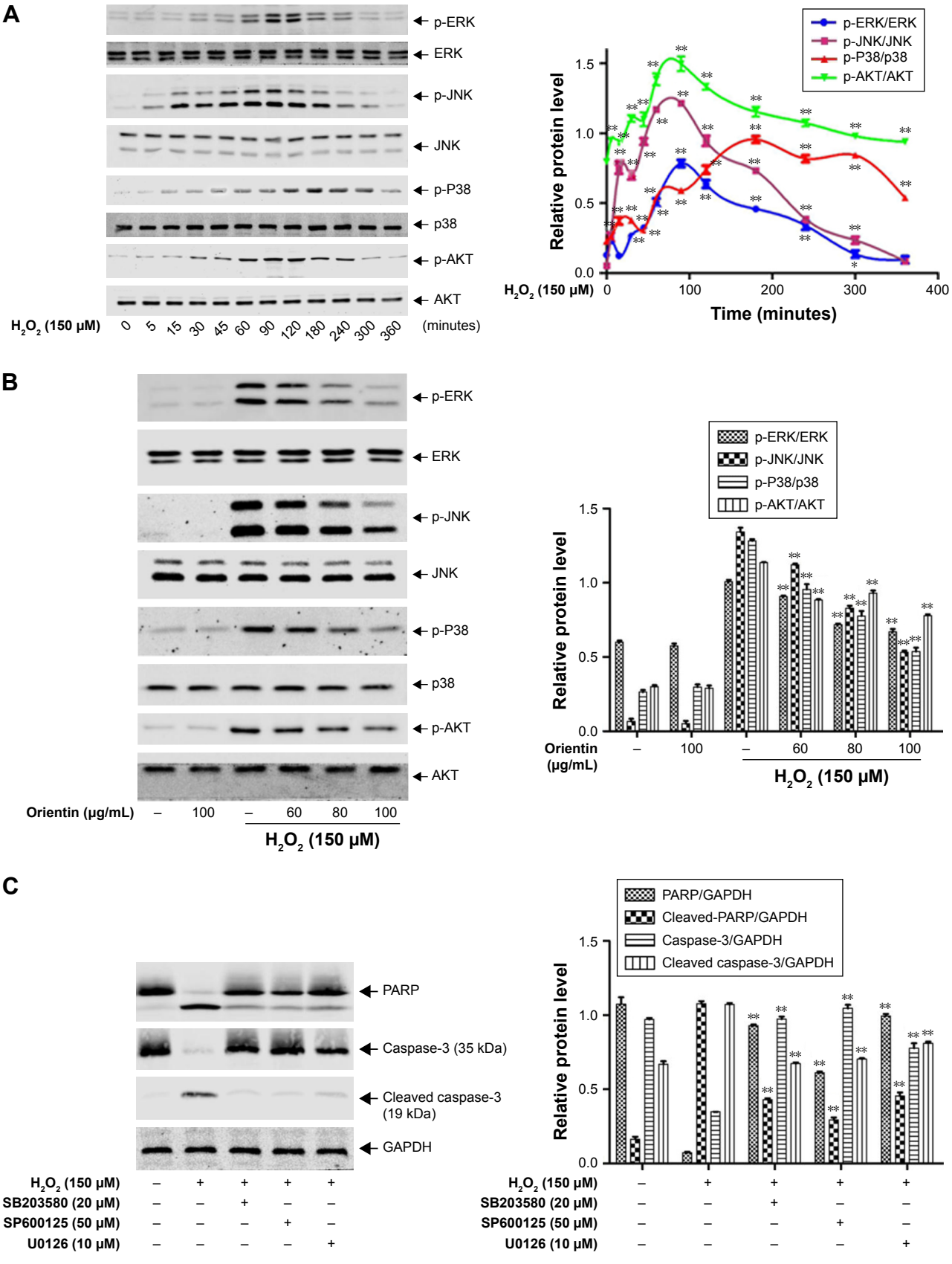


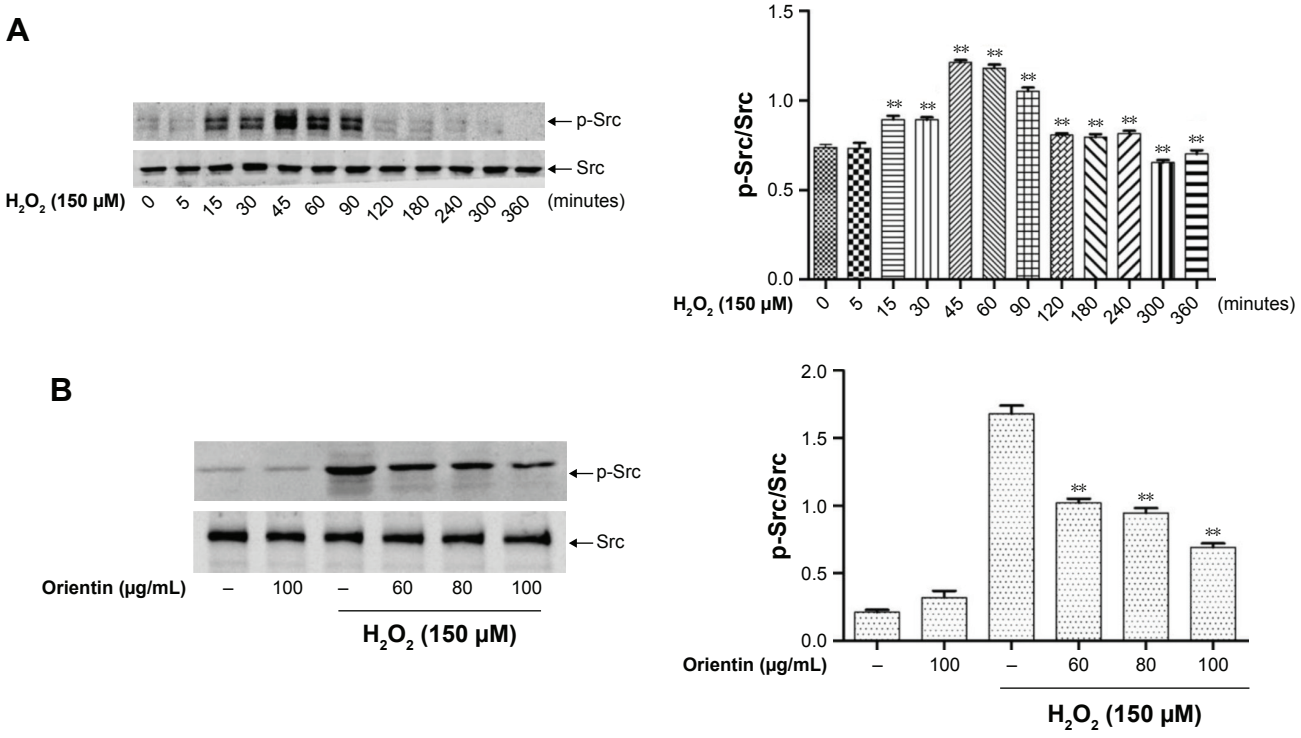
Figure 3 Orientin decreased MAPK/AKT signaling-dependent cell apoptosis induced by H_2O_2.

Notes: (**A**) Activation of ERK, JNK, p38, and AKT by H_2O_2 in a time-dependent manner. (**B**) Inhibition of H_2O_2-induced ERK, JNK, p38, and AKT phosphorylation by orientin in a dose-dependent manner. (**C**) Reversal of PARP and caspase-3 cleavage by the inhibitors of ERK, JNK, and p38 (U0126, SP600125, and SB203580, respectively). Data are presented as mean±SD (n=3). Significant differences are indicated with asterisks (*$P<0.05$, **$P<0.01$). Compared with H_2O_2 (0 μM) group (**A**); compared with single H_2O_2 group (**B** and **C**).

process of NDDs have received significant attention. Generally, excessive production of free radicals, ROS, and reactive nitrogen species or the deregulation of detoxifying and/or repairing systems causes OS, either individually or together. Therefore, it would be very practical to develop drugs for the clearance of oxidative free radicals or activation of the antioxidant defense system for NDD treatment. This study, for the first time, provides in vitro results showing that through the clearance of H_2O_2-induced ROS and reduced activation of ROS-dependent Src-MAPK/AKT signaling pathways, orientin protected PC12 cells against H_2O_2-induced cell apoptosis and oxidative damage.

Many studies have confirmed that H_2O_2 induces apoptosis in a variety of neuronal cells. The most likely mechanism underlying this observation is that H_2O_2 excessively consumes anti-oxidases in neuronal cells, resulting in redox balance disorders, and ultimately cell death.[26,27] Our results showed that orientin alleviated H_2O_2-induced apoptosis in PC12 cells via the restoration of cell viability, reduction of cell apoptosis rates, and improvement of nuclear morphology. Caspase-3 is the most crucial end-cleaving enzyme during the process of cell apoptosis; it cleaves and inactivates the DNA repair enzyme PARP, which plays key roles in DNA repair and stimulates the cell apoptosis program.[28] Western blotting results showed that orientin blocked caspase-3 activation and PARP degradation. Together, these data suggest that orientin significantly alleviates H_2O_2-induced apoptosis in PC12 cells.

Many signaling pathways, including the MAPK,[29] PI3K/AKT,[30] and NF-κB,[31] play important roles in the neuronal apoptosis induced by OS. Our experimental results showed that H_2O_2 stimulated the activation of MAPKs (ERK, JNK, and p38) and AKT signaling pathways in a time-dependent manner, and orientin decreased H_2O_2-induced phosphorylation of MAPKs and AKT signaling proteins. When cells were incubated with specific inhibitors of ERK, JNK, p38, and AKT, H_2O_2-induced apoptosis in PC12 cells was inhibited to different degrees. These results indicate that alleviation of H_2O_2-induced apoptosis by orientin is mediated by the suppression of MAPK/AKT signaling pathways. The Src family proteins usually play a housekeeping role in cell proliferation, differentiation, stress, and apoptosis. Src is usually used as an early indicator of the activation of downstream signaling.[32] In this study, H_2O_2 stimulated the activation of Src in PC12 cells, and Src was activated earlier than MAPKs and AKT. However, orientin decreased H_2O_2-induced phosphorylation of the Src signaling protein. When PC12 cells were incubated with the Src-specific inhibitor PP2, H_2O_2-induced activation of MAPKs and AKT signaling was inhibited. More importantly, MAPK/AKT-mediated cell apoptosis was also inhibited by PP2. The above results indicate that alleviation



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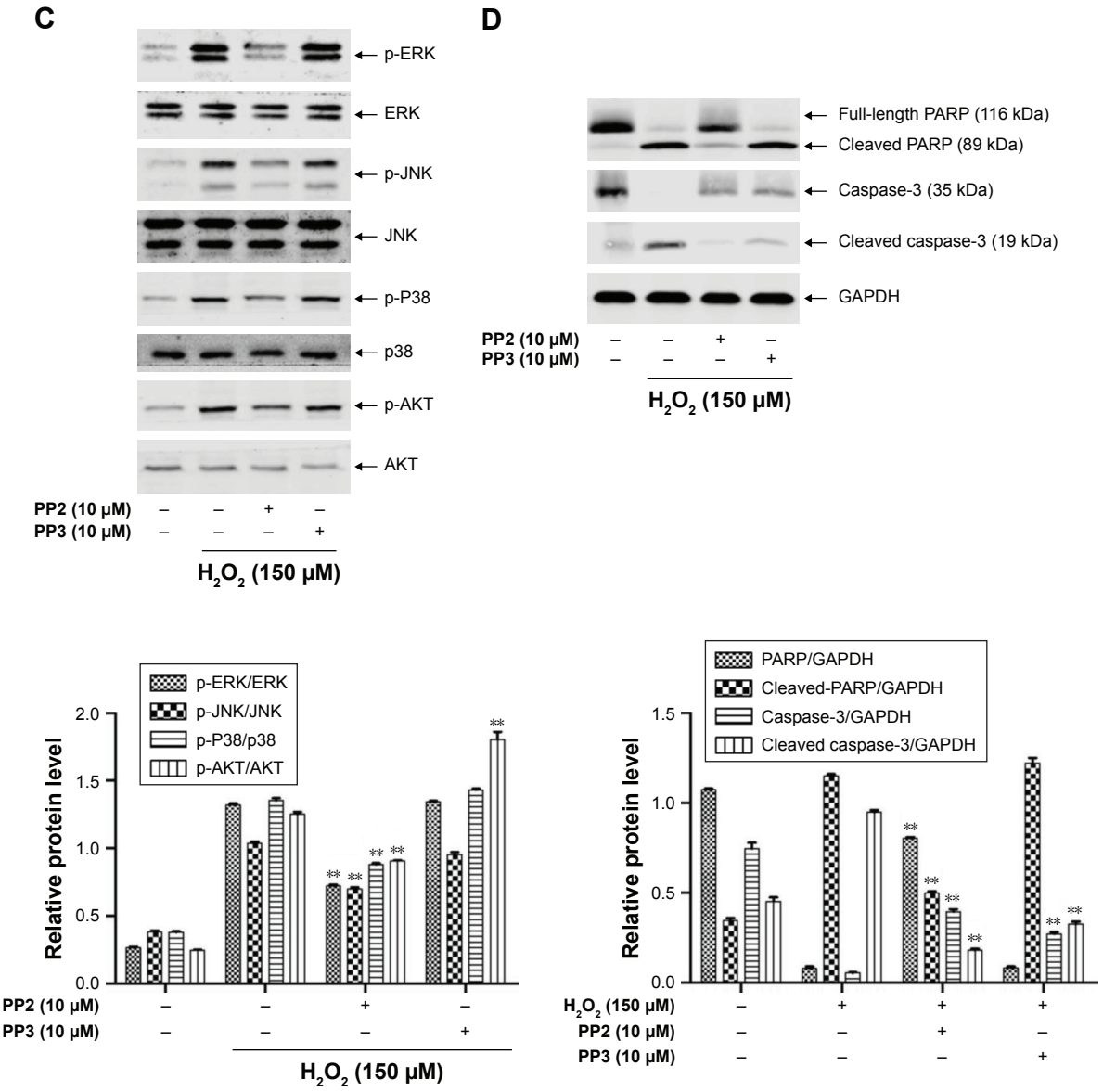


Figure 4 Orientin decreased Src-mediated MAPK/AKT signaling-dependent cell apoptosis induced by H_2O_2.

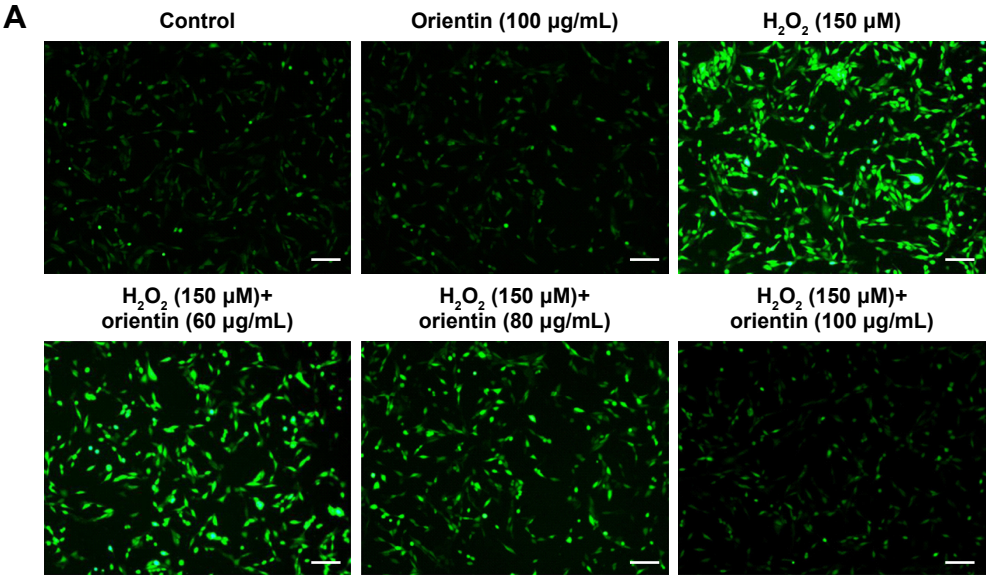
Notes: (**A**) H_2O_2 activated Src in a time-dependent manner. (**B**) Orientin inhibited H_2O_2-induced Src activation. (**C**) PP2 (Src inhibitor) decreased the level of MAPK and AKT phosphorylation activated by H_2O_2; however, PP3 (negative inhibitor) had no effect. (**D**) PP2 (Src inhibitor) completely reversed H_2O_2-induced cleavage of PARP and caspase-3, whereas PP3 (negative inhibitor) had no significant effect. Data are presented as mean±SD (n=3). Significant differences are indicated with asterisks (**$P<0.01$). Compared with H_2O_2 (0 μM) group (**A**); compared with single H_2O_2 group (**B**–**D**).

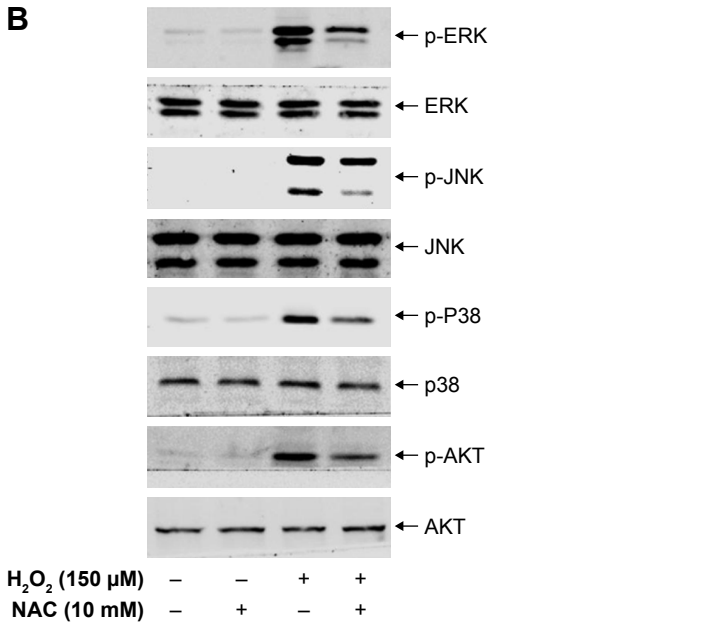
of H_2O_2-induced apoptosis by orientin is dependent on the Src-MAPK/AKT signaling pathways.

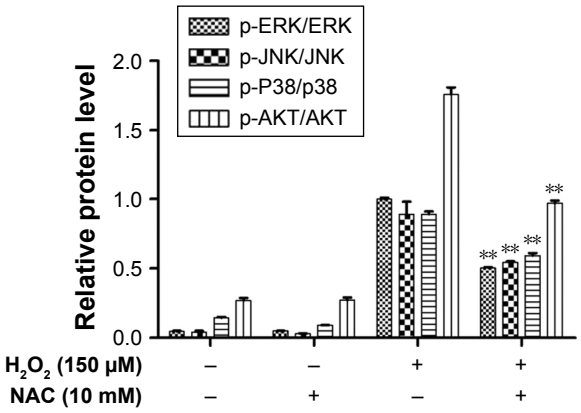
Many studies have previously confirmed that OS induces neuronal apoptosis, which can be mediated by many signaling pathways.[33–36] As expected, H_2O_2 induced PC12 cells to produce a large amount of ROS, and pre-incubation with orientin significantly inhibited H_2O_2-induced ROS production in PC12 cells. To determine whether Src-MAPK/AKT signaling-dependent cell apoptosis was mediated by ROS in our experimental system, we preincubated cells with ROS-specific scavenger NAC. The results showed that NAC inhibited H_2O_2-induced activation of Src. Additionally, MAPKs and AKT signaling molecules located downstream of Src were also inhibited. More importantly, H_2O_2-induced cell apoptosis in PC12 cells was also inhibited by NAC. Overall, orientin alleviated H_2O_2-induced apoptosis in PC12 cells by inhibiting ROS-mediated activation of Src-MAPK/AKT signaling.

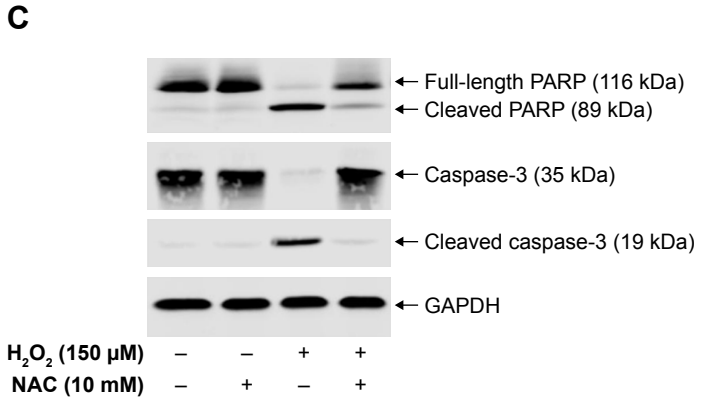
Conclusion

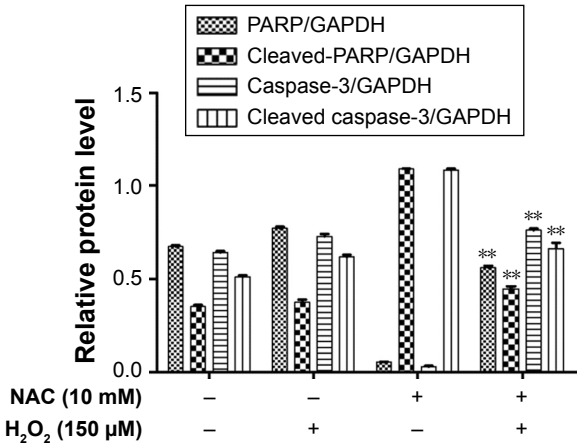
This is the first study showing that orientin alleviates H_2O_2-induced apoptosis in PC12 cells in vitro. This is probably











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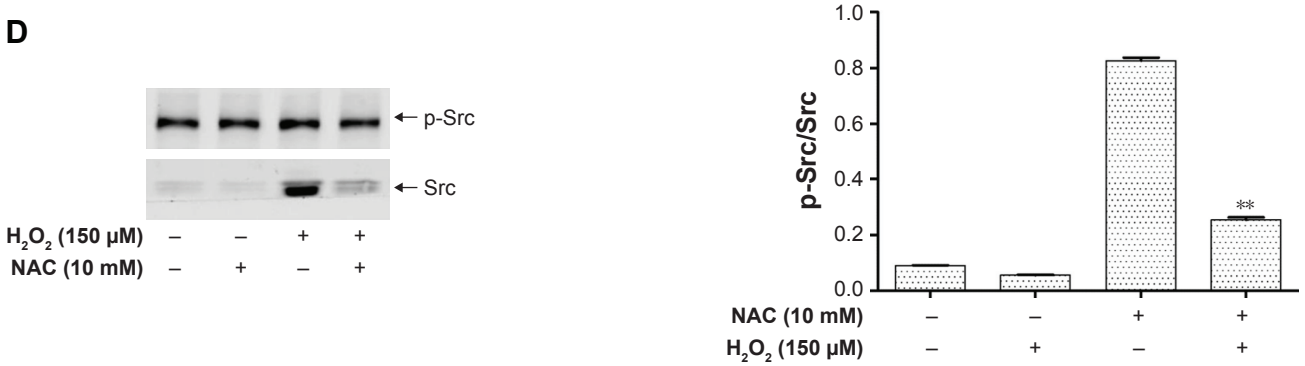

Figure 5 ROS mediated the neuroprotective effect of orientin.
Notes: (**A**) Orientin cleared H_2O_2-induced intracellular ROS. (**B–D**) NAC (ROS scavenger) inhibited H_2O_2-induced MAPKs and Src activation. (**C**) NAC reversed H_2O_2-induced cleavage of PARP and caspase-3. Data are presented as mean±SD (n=3). Scale bars: 100 μm. Significant differences are indicated with asterisks (**$P<0.01$). Compared with single H_2O_2 group (**B–D**).

because by inhibiting H_2O_2-induced ROS production, orientin further inhibited the activation of Src-MAPK/AKT signaling to alleviate apoptosis induced by OS damage. Thus, we suggest orientin as a potential antioxidant drug for the treatment of NDDs induced by neural OS.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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