

Polydatin protects neuronal cells from hydrogen peroxide damage by activating CREB/Ngb signaling

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Abstract. Oxidative stress-induced neuronal cell death contributes significantly to the physiological processes of a number of neurological disorders. Polydatin (PD) has been reported to protect against Alzheimer's disease (AD), ischemic stroke and traumatic brain injury. However, the underlying neuroprotective mechanisms remain to be elucidated. The current study suggested that PD activates AKT/cAMP response element-binding protein (CREB) signaling and induces neuroglobin (Ngb) to protect neuronal cells from hydrogen peroxide (H₂O₂) *in vitro*. PD inhibited the H₂O₂-induced neuronal cell death of primary mouse cortical neurons and N2a cells. Functional studies showed that PD attenuated H₂O₂-induced mitochondrial dysfunction and mitochondrial reactive oxygen species production. Mechanistically, PD was verified to induce the phosphorylation of AKT and CREB and increase the protein level of Ngb. The luciferase assay results showed that Ngb transcriptional activity was activated by CREB, especially after PD treatment. It was further indicated that PD increased the transcription of Ngb by enhancing the

binding of CREB to the promoter region of Ngb. Finally, Ngb knockdown largely attenuated the neuroprotective role of PD against H₂O₂. The results indicated that PD protected neuronal cells from H₂O₂ by activating CREB/Ngb signaling in neuronal cells, indicating that PD has a neuroprotective effect against neurodegenerative diseases.

Introduction

The brain is prone to oxidative stress-induced cell damage due to its high oxygen demand, the abundance of redox-active metals and relatively high levels of oxidizable polyunsaturated fatty acids (1). Neuronal cells are especially vulnerable to oxidative stress (1). Excessive oxidative stress leads to neuronal cell death, a key physiological process in a number of neurological disorders, including neurodegenerative disease (2,3), stroke (4) and traumatic brain injury (5). Therefore, neuroprotective antioxidants have long been considered promising for the treatment of neuronal disorders (6). As a crucial mediator of oxidative stress, hydrogen peroxide (H₂O₂) leads to lipid peroxidation, mitochondrial dysfunction and DNA damage, which ultimately causes neuronal cell death (7). Indeed, H₂O₂ treatment of cultured neuronal cells can be used to mimic oxidative stress *in vitro* (8).

Polydatin (PD) is a single compound isolated from the Chinese medicine *Polygonum cuspidatum* (Japanese knotweed) (9). This compound has a number of biological properties, including anti-inflammatory (10) and antioxidative (11) properties. It has also been reported that PD serves a neuroprotective role against neurodegenerative diseases, including Alzheimer's disease (12), Parkinson's disease (13), cerebral ischemia (14-16), traumatic brain injury (17) and spinal cord injury (18,19). In the brain, PD can upregulate the antioxidant Nrf2 to reduce oxidative stress (16,20) and alleviate Parkinsonism in MPTP-model mice by enhancing glycolysis in dopaminergic neurons (21). In cultured cells, PD has been reported to protect SH-SY5Y neuronal cells by promoting Atg5-mediated autophagy (22) and prevent

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A β -induced neuronal cytotoxicity by enhancing autophagy and decreasing oxidative stress (23). Although PD has been reported to protect neuronal cells and decrease oxidative stress, the neuroprotective roles of PD in primary neurons and the underlying neuroprotective mechanisms remain to be elucidated.

The present study further investigated the Nrf2-independent mechanisms underlying the role of PD in serving anti-oxidative and neuroprotective functions. Through a cell-based Ngb gene promoter-reporter assay, it was identified that PD could significantly induce Ngb expression (24). Ngb was originally found as a vertebrate globin that is predominantly expressed in the brain (25). Accumulating data has suggested that Ngb is a crucial endogenous neuroprotectant for a number of neurological disorders, including stroke, traumatic brain injury and neurodegenerative diseases (26). Ngb can directly interact with mitochondria and maintain mitochondrial function, as Ngb suppresses mitochondrial permeability transition pore opening by binding to voltage-dependent anion-selective channel 1 (27) and reduces mitochondrial reactive oxygen species (ROS) production by inhibiting mitochondrial complex III (28,29). In addition, Ngb has been reported to protect against oxidative stress-induced neuronal cell death (30). Notably, Ngb is reported to be regulated by cAMP response element-binding protein (CREB) (31). However, whether CREB/Ngb signaling axis mediates the neuroprotective effects of PD has not been investigated. The present study, therefore, hypothesized that PD might protect against H₂O₂-induced mitochondrial dysfunction and neurotoxicity by upregulating CREB/Ngb signaling.

The present study examined the effects of PD on H₂O₂-induced mitochondrial dysfunction and neurotoxicity and explored whether CREB/Ngb signaling is involved in the beneficial effects of PD on mitochondrial function and neuronal survival. The results showed that PD protects neuronal cells from H₂O₂-induced mitochondrial dysfunction and neurotoxicity via activation of CREB/Ngb signaling.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), Neurobasal medium, L-Glutamine, B-27 Supplement, geneticin, penicillin, streptomycin, Lipofectamine[®] 2000, Lipofectamine[®] 3000 and MitoSOX Red Mitochondrial Superoxide Indicator were purchased from Thermo Fisher Scientific, Inc. Fetal bovine serum (FBS) and 0.05% trypsin-EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc.). The N2a cell line was purchased from the American Type Culture Collection. The chicken anti-Ngb primary antibody was purchased from BioVendor. AKT and phosphorylated CREB (Ser133; p-CREB) antibodies were purchased from Cell Signaling Technology, Inc. The MAP2 antibody was purchased from Novus Biologicals, LLC. β -actin antibody was purchased from Sigma-Aldrich (Merck KGaA). Mouse Ngb short interfering (si)RNA1, siRNA2 and siRNA3 were purchased from OriGene Technologies, Inc. Negative control (NC) siRNA was purchased from Santa Cruz Biotechnology, Inc. The pCMV-HA vector and CREB Dominant-Negative Vector Set were purchased from Takara Bio USA, Inc. The Dual-Luciferase Reporter Assay System

was purchased from Promega Corporation. The pGL3-Basic vector, the pGEM-T Easy Vector System II and the Dual Luciferase Reporter Assay System were purchased from Promega Corporation. Reporter plasmids including the P2033(-2027/+6) and P2033(CRE2del) plasmids (mutated Ngb promoter plasmid with deletion of the specific CRE site at -854) were constructed as described previously (31). PD, with a purity of 98.95%, was purchased from MedChemExpress, LLC and DMSO was used as the solvent.

Cell culture and transfection. The animal protocol (approval number 2016#0101) was approved by the ethical committee of Hunan Normal University. The 10 week-old male and female C57BL/6 mice (weight, 25-30 g) were purchased from Hunan SJA Laboratory Animal Co., LTD, and bred in animal cages in the controlled environment with 12/12-h light/dark cycle, room temperature of 21-23°C and humidity of 55±10%. A total of four pregnant female mice (E16) were used for the present study. They were anesthetized with isoflurane (5%) for ~3 min. Anesthetic adequacy was checked continuously by observing the respiration rate and checking tailpinches. Then they were sacrificed via immediate decapitation. Once it was confirmed that the mouse was dead, the fetuses were surgically remove. The fetal mouse pups were immediately decapitated, as immediate decapitation is the fastest, most humane and standard procedure to sacrifice fetal mice for purposes of extracting brains and growing primary neuron culture. Animals were removed from the study and sacrificed if any of the following clinical signs/conditions were found: Persistent recumbence; inability to rise; loss of righting reflex; pain or distress that cannot be alleviated by analgesics; difficulty with ambulation (paralysis, fractures and trauma); severe central nervous system signs (including circling, rolling, persistent seizures or convulsions); abnormal breathing (dyspnea) and cyanosis; body condition score (32) of ≥ 2 (out of 5); excessive weight loss; vomiting/diarrhea resulting in severe dehydration; and tumor production specific endpoints (maximum tumor diameter, ≤ 20 mm) (33). As described previously (29), primary mouse cortical neuronal cultures were prepared from the cortices of E16 C57/BL6 female mouse embryos and plated onto 12-, 24-, or 48-well plates precoated with poly-D-lysine (Sigma-Aldrich; Merck KGaA) at a density of 3.5×10^5 cells/ml in neurobasal medium (NBM) plus 2% B27, 0.8 mM L-glutamine and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin (Thermo Fisher Scientific, Inc.). Half of the medium was changed every 3 days. The purity of primary mouse cortical neurons on day 8 was determined by calculating the ratio of MAP2-positive cells to the total number of viable cells (Fig. S1). These data indicated that >95% of the cells in the cultures were neurons.

Mouse N2a neuronal cells were cultured in complete DMEM (DMEM containing 10% FBS and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin) and maintained in a humidified (5% CO₂, 37°C) incubator. For transfection, N2a cells at ~80% confluence in 12 wells plates were transfected with 30 pmol NC siRNA or Ngb siRNAs (siNgb1 Target sequence: GCCAGG CTCTTCGCCCTGGAA, siNgb2 Target sequence: GCCAAG CTGTTTACTTTCCAA, siNgb3 Target sequence: GGTGAT GCTAGTGATTGAT) with 3 μl Lipofectamine[®] 3000 or 3 μg plasmids [mouse Ngb promoter plasmid [P2033(-2027/+6)], pCMV-CREB or pCMV-CREBs133a or pCMV-KCREB] with

3 μ l Lipofectamine[®] 2000 at 37°C according to the manufacturer's instructions for 6 h, followed by replacement of medium with fresh complete DMEM and culture for another 18 or 42 h. The transfected cells were treated with 200 μ M H₂O₂ in the presence or absence of 10 μ M PD at 37°C for 16 h for MTT assays, or for 4 h for JC-1 mitochondrial membrane potential and ROS production assay.

3-(4,5-Dimethylthiazol-2-yl)-2,5-dimethyltetrazoliumbromide (MTT) assay. An MTT assay was carried out as described previously (29). After different treatments, N2a cells were incubated with fresh DMEM containing 0.5 mg/ml MTT for 4 h in a 37°C cell culture incubator. Next, the medium was aspirated and DMSO was added to each well to dissolve the MTT formazan crystals. The absorbance of each sample at 570 nm was measured with a Molecular Device Spectramax M5 plate reader. Percentages of live cell counts were used for assay normalization.

Lactate dehydrogenase (LDH) assay. LDH assays were used to measure neurotoxicity as previously described (27). Following treatment, the medium was removed and the cells were lysed with PBS containing 0.1% Triton at room temperature for 5 min. Subsequently, the lysate was collected and centrifuged at 380 x g for 10 min at 4°C. The supernatant was used for LDH activity measurement using a cytotoxicity detection kit (LDH) following the manufacturer's instructions (Roche Applied Science).

Mitochondrial membrane potential ($\Delta\Psi_m$) measurements. $\Delta\Psi_m$ measurements were performed as described previously (29). Following treatment, primary mouse cortical neurons or N2a cells were stained with 2.0 μ g/ml 5',6',6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) at 37°C for 20 min, followed by washing with Hanks' Balanced Salt Solution (HBSS) three times. Then, the cellular fluorescence was detected with a microplate reader (green fluorescence, excitation 489 nm and emission 529 nm; red fluorescence, excitation 570 nm and emission 600 nm) and imaged with a fluorescence microscope with 200x magnifications. Four random microscopic fields in each group were selected. The $\Delta\Psi_m$ was calculated as the fluorescence ratio of red (JC-1 aggregates) to green (JC-1 monomers).

Mitochondrial ROS measurements. Mitochondrial ROS production was assessed with MitoSOX Red Mitochondrial Superoxide Indicator according to the manufacturer's instructions. Briefly, following treatment, the medium was aspirated and HBSS was added to primary mouse cortical neurons or N2a cells, which were diluted with MitoSOX Red dye (10 μ M) and incubated in a cell incubator at 37°C in the dark for 30 min. After loading the dye, the cells were washed twice with HBSS and the cellular fluorescence was imaged with a fluorescence microscope with 200x magnifications. Four random fields in each group were selected. The mitochondrial ROS was also measured at 510 nm excitation and 580 nm emission with a microplate reader.

Luciferase assay. N2a cells were seeded into 24-well plates and transfected with the indicated plasmids, including the

mouse Ngb promoter plasmid P2033(-2027/+6) or mutated Ngb promoter plasmid P2033(CRE2del) plasmid (200 ng/well) and pRL-TK (10 ng/well). The wild-type and mutated mouse Ngb promoter were cloned into the pGL3-Basic vector (Promega Corporation) as previously described (31). After 24 h, cells were treated with H₂O₂ or PD as described. Reporter assays were performed with Dual Luciferase Reporter Assay System (Promega Corporation). All assays were carried out in a Veritas Microplate Luminometer (Turner BioSystems). The activity was normalized and defined as the Firefly/*Renilla* luciferase ratio. Each experiment was performed at least three times.

Western blotting analysis. Cells were lysed in RIPA buffer and centrifuged at 13,500 g for 10 min at 4°C. Supernatants were measured with a Bradford assay (Bio-Rad Laboratories, Inc.) and 30 μ g of total protein were separated using a 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat dried milk in TBST (TBS containing 0.1% Tween-20) for 1 h at room temperature, followed by immunoblotting and incubation overnight at 4°C with the primary antibodies as follows: CREB (1:1,000; Cell Signaling Technology, cat. no. #4820), phospho-CREB antibody (1:1,000; Cell Signaling Technology, #9198), Ngb (1:500; Abnova, 2B5-A7), β -actin antibody (1:3,000; Sigma, A5441). Subsequently, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (both 1:5,000; cat. nos. #31430 and #31460; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. After washing with TBST, immunoreactive proteins were determined by an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Images were captured with ChemiDoc Imaging Systems (Bio-Rad Laboratories). Quantification of protein band intensity obtained by Western blotting analysis was analyzed with ImageJ 1.52a (<http://rsb.info.nih.gov/ij/>) and normalized to the actin band intensity.

Electrophoretic mobility shift assay (EMSA). An EMSA was performed as described previously (29,34). N2a cell nuclear extracts were prepared with a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Inc.). Oligonucleotides containing wild-type CRE2 (5'-TGGGGGCTGAGGTGATCC AAC-3') were synthesized and labeled with biotin using a biotin 3-end DNA-labeling kit (Thermo Fisher Scientific, Inc.) and annealed to obtain double-stranded DNA probes. DNA/nuclear protein/antibody binding reactions for the supershift assay were performed using the LightShift EMSA Optimization and Control kit (Thermo Fisher Scientific, Inc.). The reaction mixtures were loaded onto a 6% nondenaturing polyacrylamide gel and electrophoresed in 0.5x TBE buffer at 100 V at 4°C. Following electrophoresis, the DNA/nuclear protein/antibody complexes were transferred to a nylon membrane (Ambion; Thermo Fisher Scientific, Inc.), followed by cross-linking of the membrane at 120 mJ/cm² for 60 sec using a UV cross-linker (Stratagene; Agilent Sumitomo Dainippon Pharma Co., Ltd.). Biotin-labeled DNA was further detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Inc.) and exposed to film (X-Omat; Kodak).

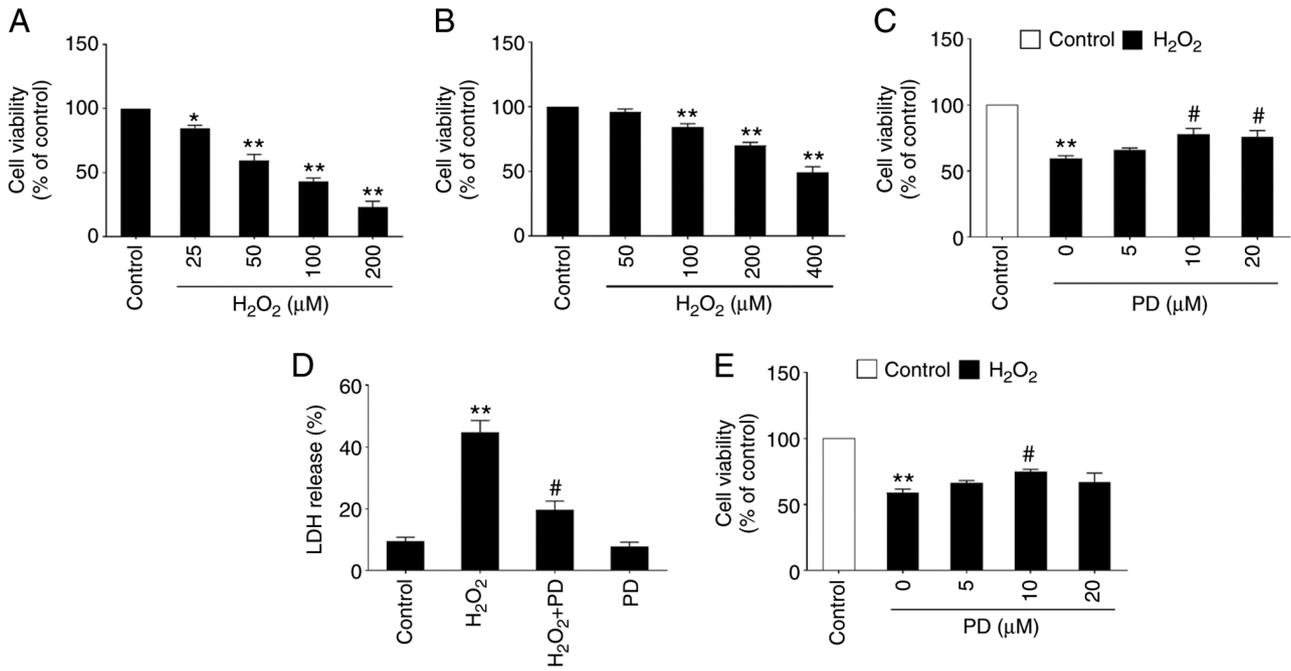


Figure 1. PD protects primary mouse cortical neurons and N2a neuronal cells against H₂O₂-induced neurotoxicity. (A) Cell viability was measured by MTT assay in primary mouse cortical neurons cultured in 48-well plates treated with the indicated dose of H₂O₂ for 16 h. n=4, data are expressed as the mean ± SD, *P<0.05, **P<0.01 compared with the control (0 μM H₂O₂). (B) Cell viability was measured by MTT assay in N2a cells cultured in 48-well plates treated with the indicated dose of H₂O₂ (0, 50, 100, 200 and 400 μM) for 16 h. Data are expressed as the mean ± SD, *P<0.05, **P<0.01 compared with the control (0 μM H₂O₂). (C) Cell viability was measured by MTT assay in primary mouse cortical neurons cultured in 48-well plates treated with 50 μM H₂O₂ and the indicated dose of PD for 16 h. Data are expressed as the mean ± SD, **P<0.01 compared with the control (DMSO); n=4, #P<0.05 compared with PD (0 μM). (D) Ratio of LDH release was measured by LDH assay in primary mouse cortical neurons cultured in 48-well plates treated with DMSO (control) or 50 μM H₂O₂ with or without 10 μM PD. **P<0.01 compared with the control (DMSO); #P<0.05 compared with H₂O₂ (50 μM). (E) Cell viability was measured by MTT assay in N2a cells cultured in 48-well plates treated with 400 μM H₂O₂ and the indicated dose of PD (0, 5, 10 and 20 μM) for 16 h. n=5, data are expressed as the mean ± SD, **P<0.01 compared with the control (DMSO); #P<0.05 compared with H₂O₂ (50 μM). PD, polydatin; LDH, lactate dehydrogenase.

Statistical analysis. All data are expressed as the mean ± standard deviation of ≥3 independently repeated experiments. Differences between groups were analyzed by one-way ANOVA followed by Tukey-Kramer's test using GraphPad Prism 7 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

PD protects primary mouse cortical neurons and N2a neuronal cells against H₂O₂-induced neurotoxicity. Primary cultured mouse cortical neurons and mouse N2a cells were treated with H₂O₂ at different doses and neuronal cell viability was measured by MTT assay. The data showed that treatment with H₂O₂ for 16 h significantly reduced cell viability of primary mouse cortical neurons and N2a cells in a dose-dependent manner (Fig. 1A and B). It was found the two cells have a different sensitivity to PD. Primary mouse cortical neurons are more sensitive to PD than N2a cells. It was found that ~30-40% neuronal cell death was induced by 50 and 200 μM H₂O₂ in primary mouse cortical neurons and N2a cells, respectively (Fig. 1A and B) and these H₂O₂ concentrations were selected for further experiments. To investigate whether PD can protect neuronal cells from oxidative stress, MTT assay and LDH assay were used for measurement of cytotoxicity. The primary mouse cortical neurons are non-dividing cells, but N2a cells are proliferated cells. If PD has a promoting effect on the proliferation of N2a cells, there would be more

intracellular LDH in N2a cells, which may be accompanied by more LDH release. The present study thus performed an LDH assay with primary mouse cortical neurons and MTT assay for N2a cells, respectively. Primary mouse cortical neurons were treated with H₂O₂ (50 μM) plus PD (0, 5, 10 and 20 μM) for 16 h, followed by the MTT assay. The results showed that PD dose-dependently inhibited H₂O₂ (50 μM)-induced neurotoxicity in primary mouse cortical neurons. Compared with the control, 10 and 20 μM PD could inhibit H₂O₂-induced neurotoxicity. However, the cell viability of these two concentrations was significantly different compared with the control. Considering that the primary mouse neurons are more sensitive to PD treatment, there may be more reverse effects with a high dose of PD in primary mouse neurons; 10 μM PD exhibited the maximal protective effects among the tested doses (Fig. 1C). Moreover, LDH assays showed that 10 μM PD significantly prevented H₂O₂ (50 μM)-induced LDH release in primary mouse cortical neurons (Fig. 1D). Similarly, treatment with PD (10 μM) significantly attenuated neurotoxicity in N2a cells, which was induced by H₂O₂ at a dose of ≤400 μM (Fig. 1E). These results indicated the potent neuroprotective effects of PD.

PD attenuates H₂O₂-induced Δψ_m and oxidative stress in neurons and N2a cells. Mitochondrial dysfunction has been proposed as a mediator of neurodegeneration (35,36). To investigate whether PD attenuates H₂O₂-induced Δψ_m, a JC-1 assay was performed to determine the Δψ_m in primary mouse

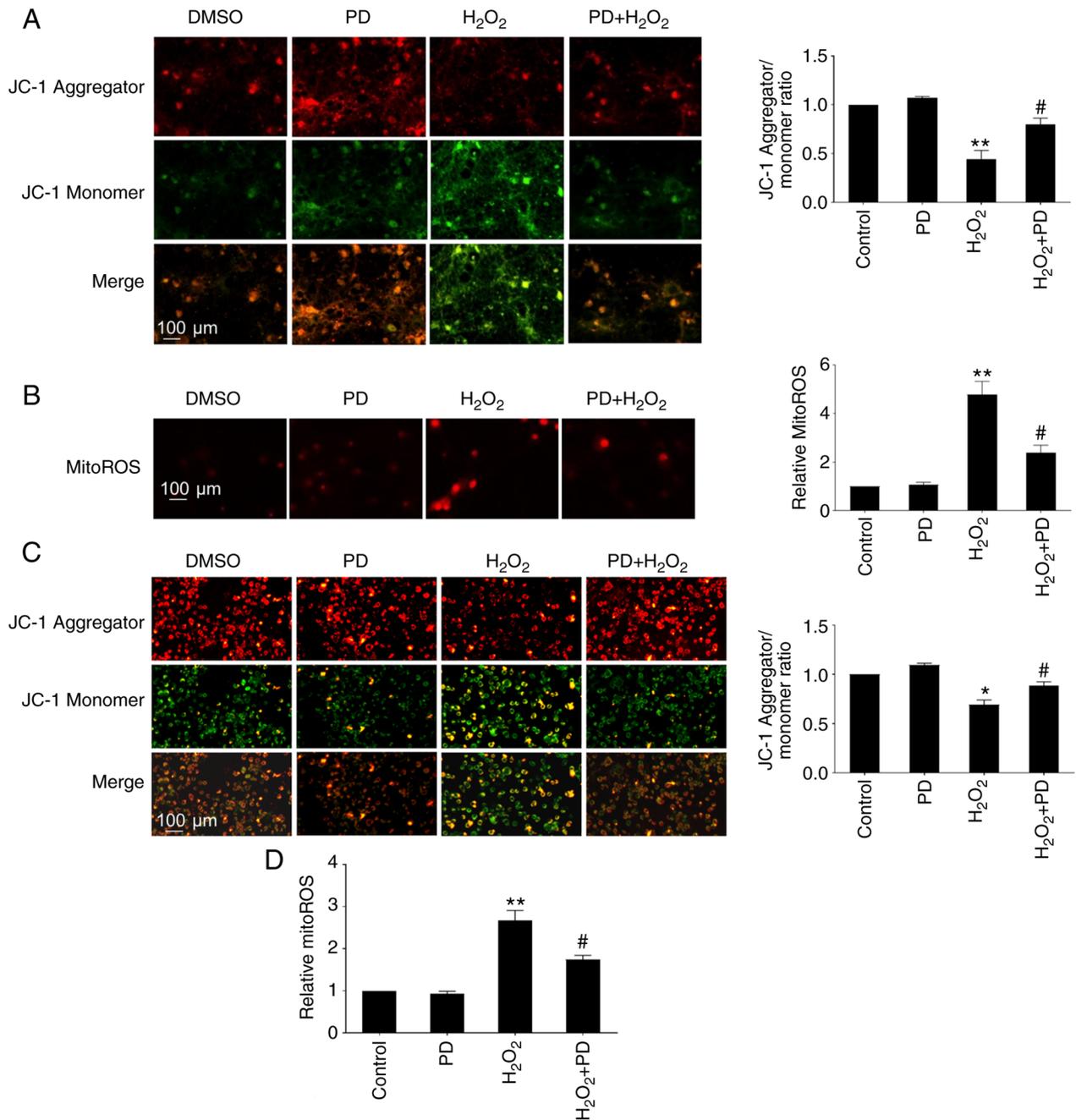


Figure 2. PD attenuates H₂O₂-induced mitochondrial dysfunction ($\Delta\psi_m$) and oxidative stress in neurons and N2a cells. (A) $\Delta\psi_m$ was measured by JC-1 dye in primary mouse cortical neurons cultured in 24-well plates treated with DMSO, 10 μ M PD, 50 μ M H₂O₂, or 10 μ M PD + 50 μ M H₂O₂ for 4 h. Fluorescence of the JC-1 aggregates (red) and monomers (green) in primary mouse cortical neurons was measured using fluorescence microscopy. The ratio of JC-1 aggregates/monomers was plotted. (B) Mitochondrial ROS were measured in primary mouse cortical neurons cultured in 24-well plates treated with DMSO, 10 μ M PD, 50 μ M H₂O₂, or 10 μ M PD + 50 μ M H₂O₂ for 4 h. Fluorescence was measured using fluorescence microscopy. The fluorescence intensity was plotted. (C) $\Delta\psi_m$ was measured by JC-1 dye in N2a cells cultured in 24-well plates treated with DMSO, 10 μ M PD, 200 μ M H₂O₂, or 10 μ M PD + 200 μ M H₂O₂ for 4 h. Fluorescence of the JC-1 aggregates (red) and monomers (green) in N2a cells was measured using fluorescence microscopy. The ratio of JC-1 aggregates/monomers was plotted. (D) Mitochondrial ROS contents were measured in N2a cells cultured in 24-well plates treated with DMSO, 10 μ M PD, 200 μ M H₂O₂, or 10 μ M PD + 200 μ M H₂O₂ for 4 h. n=4, data are expressed as the mean \pm SD, *P<0.05 vs. control, **P<0.01 vs. control (DMSO); #P<0.05 vs. H₂O₂. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; PD, polydatin; ROS, reactive oxygen species.

cortical neurons treated with H₂O₂ (50 μ M) or H₂O₂ (50 μ M) plus PD (10 μ M). It has been reported that the mitochondrial dysfunction that occurs as early as 4 h can affect neuronal cell viability (35). $\Delta\psi_m$ was thus detected at the early time point 4 h. The results showed that the JC-1 red/green fluorescence ratio in neurons significantly decreased after H₂O₂ (50 μ M) treatment compared with the untreated control, while this

decrease was significantly attenuated by cotreatment with PD (10 μ M; Fig. 2A). Similar observations were also found in N2a cells, as PD (10 μ M) treatment significantly preserved the $\Delta\psi_m$, which was dramatically reduced by H₂O₂ (200 μ M) treatment (Fig. 2C). To further investigate whether PD inhibits H₂O₂-induced mitochondrial oxidative stress, MitoSOX dye was used. The results showed that PD treatment significantly

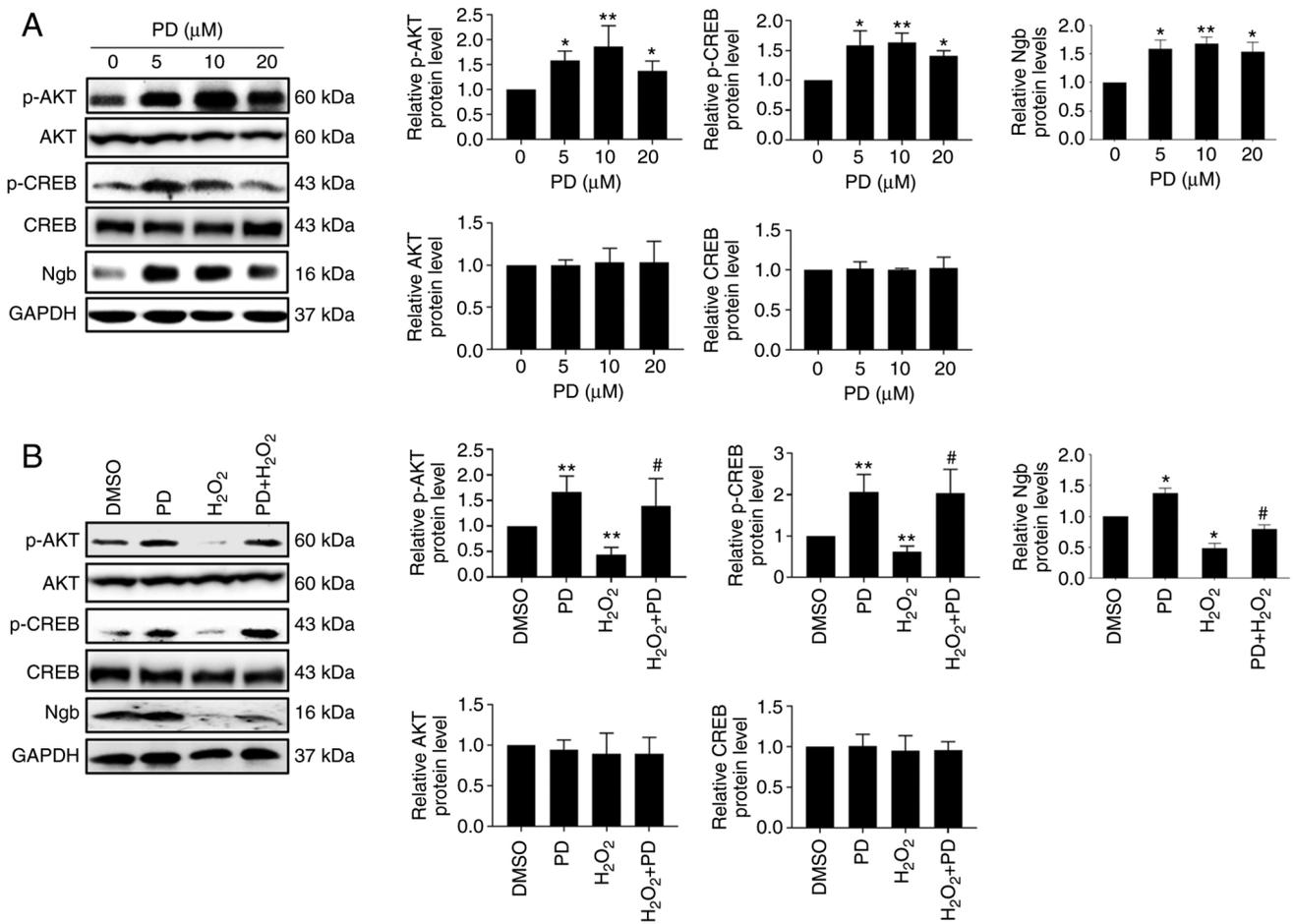


Figure 3. PD attenuates H₂O₂-reduced p-AKT, p-CREB and Ngb expression in N2a cells. (A) Protein levels of p-AKT, AKT, p-CREB, CREB and Ngb were measured by western blotting in N2a cells cultured in 60 mm dishes treated with the indicated dose of PD, n=3. Band intensities of p-AKT and p-CREB were normalized to AKT and CREB, respectively. Band intensities of AKT, CREB and Ngb were normalized to GAPDH. n=3, data are expressed as the mean \pm SD, *P<0.05, **P<0.01 compared with the control (DMSO). (B) Protein levels of p-AKT, AKT, p-CREB, CREB and Ngb were measured by western blotting in N2a cells cultured in 6-well plates treated with DMSO, 10 μ M PD, 200 μ M H₂O₂, or 10 μ M PD + 200 μ M H₂O₂, n=3. Band intensities of p-AKT and p-CREB were normalized to AKT and CREB, respectively. Band intensities of AKT, CREB and Ngb were normalized to GAPDH. Data are expressed as the mean \pm SD, *P<0.05, **P<0.01 compared with control (DMSO); #P<0.05 compared with H₂O₂. PD, polydatin; p-, phosphorylated; CREB, cAMP response element-binding protein; Ngb, neuroglobin.

attenuated H₂O₂-induced mitochondrial ROS production in primary mouse cortical neurons (Fig. 2B) and N2a cells (Fig. 2D). Taken together, these results indicated that PD attenuates H₂O₂-induced mitochondrial dysfunction and oxidative stress in primary mouse cortical neurons and N2a cells.

PD attenuates H₂O₂-reduced p-AKT, p-CREB and Ngb expression in N2a cells. To investigate the molecular mechanism underlying the neuroprotective effects of PD, western blotting analysis was performed. The results showed that PD significantly induced the phosphorylation of AKT and CREB and Ngb protein expression in a dose-dependent manner and that there was no effect on the AKT and CREB protein expression (Fig. 3A). H₂O₂ induces neuronal cell death by reducing the levels of CREB and p-CREB (37). In agreement with this study, the results of the present study showed that H₂O₂ (200 μ M) treatment led to a significant reduction in p-AKT, p-CREB and Ngb expression in N2a cells and that there was no effect on the AKT and CREB protein expression. Nevertheless, this decrease was rescued by cotreatment with PD (10 μ M) (Fig. 3B). Taken together, these results suggested

that PD attenuates H₂O₂-reduced p-AKT, p-CREB and Ngb expression in N2a cells.

PD upregulates Ngb via CREB in N2a cells. To investigate whether CREB is required for PD-induced Ngb upregulation, a mouse Ngb promoter plasmid [P2033(-2027/+6)] and wild-type CREB or mutant CREB (CREBs133a or KCREB) were cotransfected into N2a cells for 24 h, followed by PD (10 μ M) treatment for another 16 h. The results showed that mutant CREB significantly attenuated PD-induced Ngb promoter activity (Fig. 4A). Moreover, it was observed that KG501, a potent inhibitor of CREB, significantly attenuated PD-induced Ngb promoter activity (Fig. 4B). These results indicated that CREB is responsible for PD-induced Ngb promoter activity. To determine whether PD promotes the binding of CREB to CRE in the mouse Ngb promoter, EMSA was performed. The results showed that compared with DMSO-treated N2a cell nuclear extracts, PD-treated N2a cell nuclear extracts produced a stronger super-shift band after incubation with p-CREB antibody and specific Ngb CRE oligos, indicating that PD significantly induces the binding of p-CREB to the specific CRE site located at -854 in

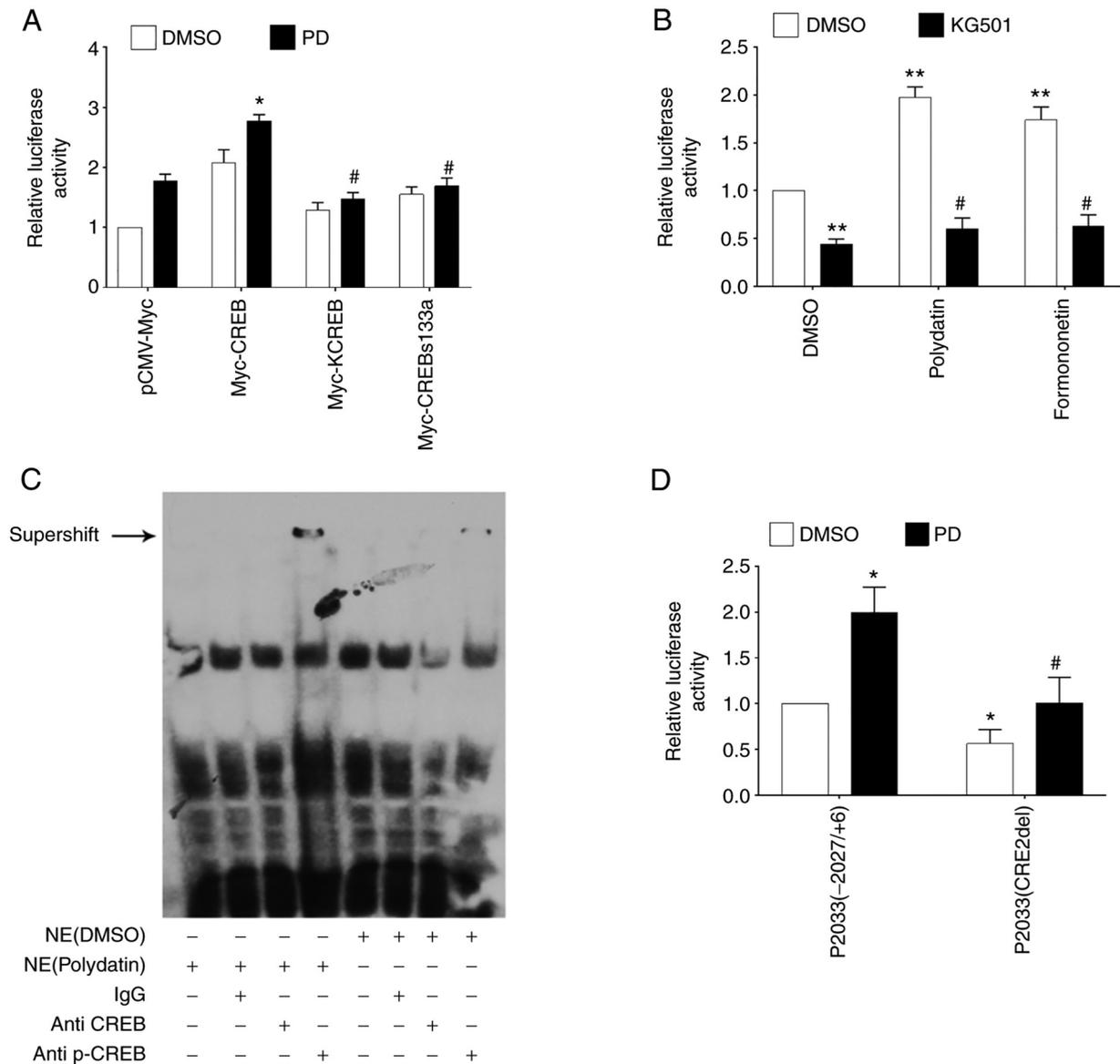


Figure 4. PD upregulates Ngβ via CREB in N2a cells. (A) Mouse Ngβ promoter plasmid P2033(-2027/+6) and wild-type CREB or mutant CREB (KCREB or CREBs133a) were cotransfected into N2a cells for 24 h, followed by PD treatment for another 16 h. Relative Ngβ promoter activity was measured with a luciferase assay. * $P < 0.05$ compared with N2a cells transfected with pCMV and treated with PD (10 μM); # $P < 0.05$ compared with N2a cells transfected with Myc-CREB and treated with PD (10 μM). (B) The mouse Ngβ promoter plasmid P2033(-2027/+6) was transfected into N2a cells for 24 h. Cells were treated with or without KG501, PD and formononetin as described for another 16 h. Relative Ngβ promoter activity was measured with a luciferase assay. ** $P < 0.01$ compared with N2a cells treated with DMSO; # $P < 0.05$ compared with N2a cells treated with PD or formononetin. (C) Supershift assay of p-CREB binding to the CRE in the Ngβ promoter. Ngβ CRE biotin-labeled oligos were incubated with the nuclear extract of N2a cells treated with DMSO or polydatin, with or without the addition of antibodies against CREB and p-CREB. (D) The mouse Ngβ promoter plasmid P2033(-2027/+6) or mutated Ngβ promoter plasmid P2033(CRE2del) plasmid was transfected into N2a cells for 24 h. Then, the cells were treated with PD for another 16 h. Relative activities were measured with a luciferase assay. * $P < 0.05$ compared with N2a cells treated with DMSO; # $P < 0.05$ compared with N2a cells treated with PD. PD, polydatin; Ngβ, neuroglobin; CREB, cAMP response element-binding protein; p-, phosphorylated; NE, nuclear extract.

the promoter region of the mouse Ngβ gene (Fig. 4C). To further investigate whether the specific CRE site in the Ngβ promoter region is responsible for PD-induced Ngβ promoter activity, P2033(-2027/+6) or P2033(CRE2del), mutated Ngβ promoter plasmid with deletion of the specific CRE site at -854) plasmids were transfected into N2a cells for 24 h followed by PD treatment for another 16 h. The results showed that deletion of the specific CRE site significantly attenuated basal Ngβ promoter activity and PD-induced Ngβ promoter activity (Fig. 4D). Taken together, these results suggested that PD upregulates Ngβ via CREB in N2a cells.

PD inhibits neuronal cell death, maintains mitochondrial function and reduces ROS production via Ngβ. Next, the role of Ngβ in mediating PD protection against H_2O_2 (200 μM) was determined. Transfection of Ngβ-specific siRNA (siNgβ-2 and siNgβ-3) into N2a cells for 48 h successfully led to the knockdown of endogenous Ngβ (Fig. 5A) and siNgβ-2 was chosen for further experiments. It was found that knockdown of Ngβ significantly attenuated the protection of PD against H_2O_2 via an MTT assay (Fig. 5B). Next, the role of endogenous Ngβ in mediating PD-preserved mitochondrial membrane potential in N2a cells was determined. It was found that the JC-1 ratio

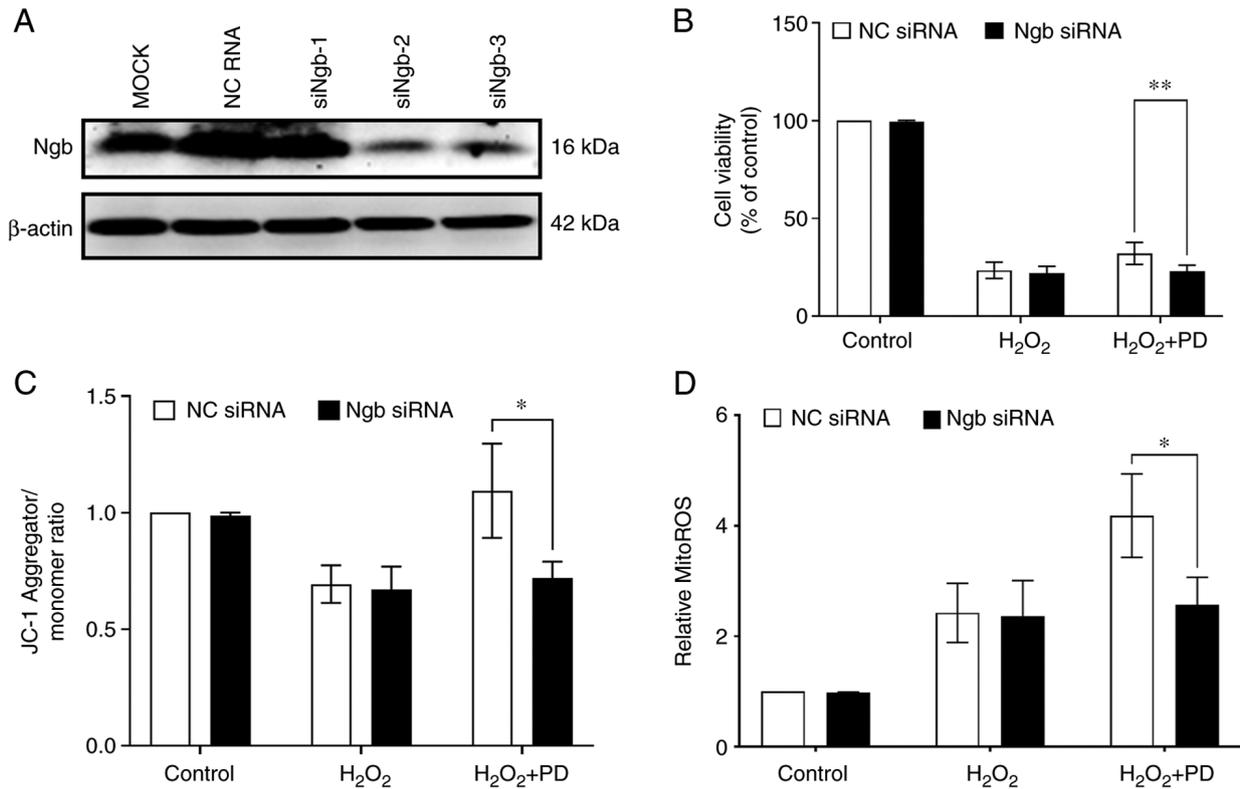


Figure 5. PD inhibits neuronal cell death, maintains mitochondrial function and reduces ROS production by upregulating Ngb. (A) Ngb protein levels were measured by western blotting in N2a cells transfected with Ngb-specific siRNA (siNgb-1, siNgb-2 and siNgb-3) for 48 h. (B) N2a cells were transfected or not with Ngb-siRNA2 for 24 h and then treated with 200 μ M H₂O₂ or 200 μ M H₂O₂ plus 10 μ M PD for 16 h. Cell viability was measured by MTT assay. n=3, data are expressed as the mean \pm SD, **P<0.01. DMSO was used as a vehicle (control) and the final concentration of DMSO in cells was 0.1%. (C) N2a cells were transfected or not with Ngb-siRNA2 for 24 h and then treated with 200 μ M H₂O₂ or with 200 μ M H₂O₂ plus 10 μ M PD for 4 h. The mitochondrial membrane potential was measured by a JC-1 mitochondrial membrane potential assay kit. n=4, data are expressed as the mean \pm SD, *P<0.05. DMSO was used as a vehicle (control) and the final concentration of DMSO in cells was 0.1%. (D) N2a cells were transfected or not with Ngb-siRNA2 for 24 h and then treated with 200 μ M H₂O₂ or with 200 μ M H₂O₂ and 10 μ M PD for 4 h. Mitochondrial ROS production was measured with a mitoROS assay kit. n=4, data are expressed as the mean \pm SD, *P<0.05. DMSO was used as a vehicle (control) and the final concentration of DMSO in cells was 0.1%. Ngb, neuroglobin; ROS, reactive oxygen species; si, short interfering; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide.

was significantly reduced in Ngb siRNA-transfected N2a cells compared with NC siRNA-transfected N2a cells under PD plus H₂O₂ treatment conditions (Fig. 5C). Furthermore, it was observed that there was a significant increase in ROS production in N2a cells treated with siNgb-2 transfection plus PD and H₂O₂ compared with NC siRNA transfection plus PD and H₂O₂ (Fig. 5D). These results indicate that PD inhibits neuronal cell death, maintains mitochondrial function and reduces ROS production via Ngb.

Discussion

Oxidative stress-induced neuronal cell death contributes significantly to the physiological processes of a number of neurological disorders, including neurodegenerative disease (2), stroke (4) and traumatic brain injury (5). The present study found that PD potently attenuated H₂O₂-induced mitochondrial dysfunction and mitochondrial ROS production. As a result, PD significantly attenuated H₂O₂-induced neuronal cell death and apoptosis. Moreover, it was found that activated AKT/CREB signaling and increased Ngb expression was crucial for mediating the neuroprotective role of PD against H₂O₂-induced neurotoxicity. These results suggested that PD protects neuronal cells against oxidative stress by

upregulating AKT/CREB/Ngb signaling. Notably, PD can cross the blood-brain barrier (38). As oxidative stress participates in a number of physiological functions and pathologies of neuronal disorders, the present study further highlights the possible therapeutic potential of PD to treat various neuronal disorders. Future studies will be focused on testing the pharmacokinetics and efficacy of PD in animal models of Alzheimer's disease.

The present study found that PD attenuates H₂O₂-induced mitochondrial dysfunction and mitochondrial ROS production, which can be ascribed to the activation of CREB signaling, at least in part. CREB, which belongs to the CREB/ATF family of transcription factors, is a critical component of the neuroprotective transcriptional network (39). In response to stimuli, CREB can be phosphorylated at the Ser133 site, enter cell nuclei and bind to CREB responsive elements to promote the transcription and expression of multiple antioxidant and detoxifying genes, thereby inhibiting neuronal oxidative injury (40). Among these genes, Ngb is a key candidate (31). In the present study, PD significantly elevated Ngb promoter activity and mRNA and protein levels. Notably, treatment with the CREB inhibitor KG-501 and ectopic overexpression of CREB s133a and the KCREB mutant in N2a cells largely attenuated PD-induced Ngb and neuroprotection. In

addition, Ngb knockdown by specific Ngb siRNA largely attenuated PD-induced neuronal cell protection against H₂O₂. In agreement with the present study, it has been reported that Ngb is able to protect neuronal cells against oxidative stress (30,41). Notably, the neuroprotective roles of Ngb are associated with its ability to modulate mitochondria. Our previous data suggested that Ngb can directly interact with mitochondria and maintain mitochondrial function by binding to and suppressing the mitochondrial permeability transition pore (27) and reduce mitochondrial ROS production by inhibiting mitochondrial complex III (28,29). In agreement with our studies, accumulating studies suggest that PD protects the mitochondria of neuronal cells, myocardial cells, hepatocytes and arterial smooth muscle cells after acute severe hemorrhagic shock (42-45). Therefore, these studies indicate that PD protects against H₂O₂-induced mitochondrial dysfunction and neurotoxicity, in which Ngb might serve a key role.

Although the antioxidative and neuroprotective role of PD is mediated by the activation of CREB/Ngb signaling, the possibility that Nrf2 may also be involved cannot be ruled out. The antioxidative role of PD is mediated by the activation of Nrf2 (10,46,47). However, the direct physical interaction of PD with Nrf2 has not been demonstrated. As the members of the bZip family, CREB and Nrf2 may form a heterodimer (48). Notably, Nrf2 is a downstream target of CREB, as PKA-CREB signaling contributes to Exendin-4-induced Nrf2/ARE activation (49) and CREB is required for Nrf2 mediated induction of antioxidant genes heme oxygenase-1 (48). These studies suggest that CREB is upstream of Nrf2 and serves a central role in controlling Nrf2 signaling. Based on the studies of the authors and other studies, it is possible that PD activates CREB/Ngb and CREB/Nrf2 signaling pathways in parallel. This will be further investigated by employing Nrf2-deficient neurons in future studies. Considering that a number of Nrf2 activators are strong radical scavengers but also have some severe adverse effects and poor bioavailability (8), it is possible that compounds with multiple antioxidative effects may be more effective and have fewer adverse effects.

In conclusion, the results of the present study suggested that PD significantly induced AKT/CREB signaling and Ngb upregulation, which mediated the antioxidative effect of PD, at least in part, thereby protecting neuronal cells from H₂O₂-induced neurotoxicity. These results might provide novel molecular insights to explain the potent neuroprotective effects of PD in a variety of oxidative stress-associated neurological disorders.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL and SX designed the experiments; HZ, YL, HL, CW, YX, HW and NL performed the experiments; and NL, YL, SY, XY and SX helped with data analysis and manuscript writing. HZ and YL confirm the authenticity of all the raw data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The animal protocol (approval number 2016#0101) was approved by the ethical committee of Hunan Normal University.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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