Neuroprotective effect of paeoniflorin on H_2O_2-induced apoptosis in PC12 cells by modulation of reactive oxygen species and the inflammatory response

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Abstract. Paeoniflorin (PF) is a product derived from *Paeoniae Radix* and is commonly prescribed in traditional Chinese medicine. PF has been reported to exhibit neuroprotective, anti-ischemic, antioxidant, anti-inflammatory and anticancer effects. The neuroprotective properties of PF have been demonstrated in animal models of various neuropathologies. The present study investigated the effects of PF on hydrogen peroxide (H_2O_2)-induced apoptosis in PC12 cells, to improve the understanding of the mechanisms underlying its neuroprotective properties. The H_2O_2-induced apoptosis of PC12 cells resulted in a reduction in the B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein ratio and the activation of caspase-3. PF treatment was observed to reverse the apoptotic process and to modulate the expression levels of a number of apoptosis-associated proteins. Furthermore, PF significantly mitigated the H_2O_2-induced reduction in cell viability, in addition to scavenging reactive oxygen species and preventing the release of lactate dehydrogenase from the PC12 cells. In addition, the apoptosis-associated activation of nuclear factor (NF)-κB was inhibited in the PF-treated cells, and the expression levels of tumor necrosis factor α and interleukin (IL)-1β were reduced. In conclusion, the present study demonstrated that PF was able to reduce H_2O_2-induced toxicity by blocking the activation of the neuroinflammatory factor NF-κB. These results suggest that PF may be a valuable neuroprotective agent for the treatment of neurological disease and injury.

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Introduction

Paeoniae Radix is a well-known herb and is used widely as a component of traditional Chinese prescriptions to treat certain types of dementia, traumatic injury and inflammation. Paeoniflorin (PF), a product derived from *Paeoniae Radix*, has been reported to exhibit neuroprotective, anti-ischemic, antioxidative, anti-inflammatory and anticancer effects. The neuroprotective potential of PF has been demonstrated in animal models of various neuropathologies (1-4).

Reactive oxygen species (ROS) are produced by various enzymatic reactions and chemical processes, which are essential for numerous physiological functions, in addition to serving as secondary messengers in the human body (5). A number of neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's, are characterized by severe and/or prolonged oxidative stress (6). The primary outcome of oxidative stress is the irreversible damage of macromolecules by ROS (7). The association between oxidative stress and inflammation is due to the activation of nuclear factor (NF)-κB and activator protein-1, and the inhibition of nuclear factor (erythroid-derived 2)-like 2, peroxynitrite-mediated endothelial dysfunction, altered nitric oxide levels and macrophage migration (8). Previous studies have indicated that PF protects neurons against ischemia-reperfusion injury by reducing the expression levels of intracellular adhesion molecule 1 and tumor necrosis factor α (TNF-α), resulting in reduced inflammation in infarcted brain regions, and PF prevents chronic cognitive damage by downregulating the expression of NF-κB in hippocampal astrocytes (4,9). The present study investigated the neuroprotective effect of PF following H_2O_2-induced injury in PC12 cells and the possible signaling pathways involved.

Materials and methods

Reagents and cell line. PF (purity, 98.5%) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). The PC12 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA).

MTT cell proliferation assay. Cell viability was measured using an MTT assay as described in a previous study (9).

The PC12 cells received different treatments, including no treatment (control), 200 μM H_2O_2 alone or 200 μM H_2O_2 in combination with 20, 40 or 80 μM PF. Briefly, the cells were seeded into 96-well plates ($3.0x10^3$/well) and cultured for 6 h. MTT solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h. Next, 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan precipitate. Absorbance was then measured at 570 nm using a ThermoMax microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). Cell viability is expressed as a percentage relative to the untreated control.

Lactate dehydrogenase (LDH) release assay. The rate of cell death was further assessed by measuring the leakage of LDH into the surrounding medium, as described in a previous study (6). Briefly, following treatment of the PC12 cells, the supernatants of each group were collected. The quantity of LDH released was determined using a Neutral Red LDH Cytotoxicity Assay Kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Wuhan, China). Optical absorbance was measured at 440 nm using the ThermoMax microplate reader.

Measurement of intracellular ROS levels. Intracellular H_2O_2 and low-molecular weight peroxides are able to oxidize 2',7'-dichlorofluorescin diacetate (DCFH-DA) to dichlorofluorescein (DCF), which is highly fluorescent under absorption analysis. A DCFH-DA fluorescent probe from a Reactive Oxygen Species Assay kit (Beyotime Institute of Biotechnology) was used to measure ROS generation, as previously reported (6). Following treatment, cells were incubated with 10 mM DCFH-DA for 30 min at 37˚C and washed twice with phosphate-buffered saline. Subsequently, the DCF fluorescence was measured using the ThermoMax microplate spectrofluorometer at excitation and emission wavelengths of 485 and 530 nm, respectively.

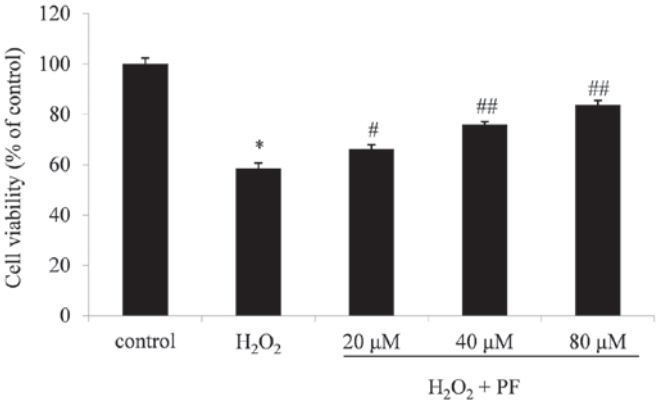

Figure 1. Protective effect of PF on the H_2O_2-induced reduction of cell viability in PC12 cells. *P<0.01 vs. control group, #P<0.05 and ##P<0.01 vs. the H_2O_2 group. H_2O_2, hydrogen peroxide; PF, paeoniflorin.

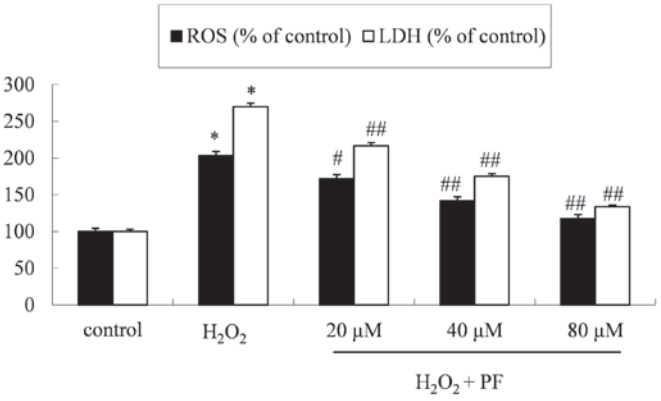

Figure 2. Effects of PF on H_2O_2-induced increase in ROS accumulation and LDH release in PC12 cells. *P<0.01 vs. the control group, #P<0.05 and ##P<0.01 vs. the H_2O_2 group. H_2O_2, hydrogen peroxide; PF, paeoniflorin; ROS, reactive oxygen species; LDH, lactate dehydrogenase.

Hoechst 33258 staining. PC12 cells at the logarithmic-growth phase were seeded into 96-well plates ($1x10^4$/well). The cells were cultured in H_2O_2 alone or with 80 μM PF. A third group of cells received no treatment and was used as a control group. Next, the cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature, then washed and stained with Hoechst 33258 (Sigma-Aldrich) for 30 min at 37˚C. PC12 cells were observed under a Nikon 80i fluorescence microscope equipped with a UV filter (Nikon Corporation, Tokyo, Japan).

Western blot analysis. PC12 cells were seeded in 6-well plates ($3.0x10^5$/well) and pretreated with 200 μM H_2O_2 alone or 200 μM H_2O_2 + 80 μM PF for 6 h. A third group of cells received no treatment and was used as a control group. After incubation the culture medium was collected for detection of the levels of TNF-α and interleukin (IL)-1β. Cells were collected and lysed in a buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF and protease inhibitor mixture (1:500; Sigma-Aldrich) for cell lysates. Cell lysates were subjected to 10% SDS-polyacrylamide gel (Invitrogen, Thermo Fisher Scientific, USA) electrophoresis, then transferred onto polyvinylidine fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were subsequently probed with antibodies, including rabbit polyclonal caspase-3 (#9662), cleaved poly(ADP-ribose) polymerase (PARP; #9541), B-cell lymphoma 2 (Bcl-2; #2872) and Bcl-2-associated X (Bax; #2772) antibodies purchased from Cell Signaling Technology, Inc. (1:1,000; Danvers, MA, USA). Mouse monoclonal NF-κB-p65RelA (1:800; sc-8008) and rabbit polyclonal p-NF-κB Ser536 (1:500; sc-33020) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and mouse monoclonal β-actin antibody (1:10,000; #ab6276) from Abcam (Cambridge, MA, USA). Immunoblots were developed using horseradish peroxidase (HRP)-conjugated secondary antibodies. Bound antibodies were visualized using Immobilon Western Chemiluminescent HRP substrate (EMD Millipore) and quantified by densitometry using a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Berkeley, CA, USA). Densitometric analyses of bands were adjusted against β-actin, which functioned as a loading control. The percentage increase or reduction in protein

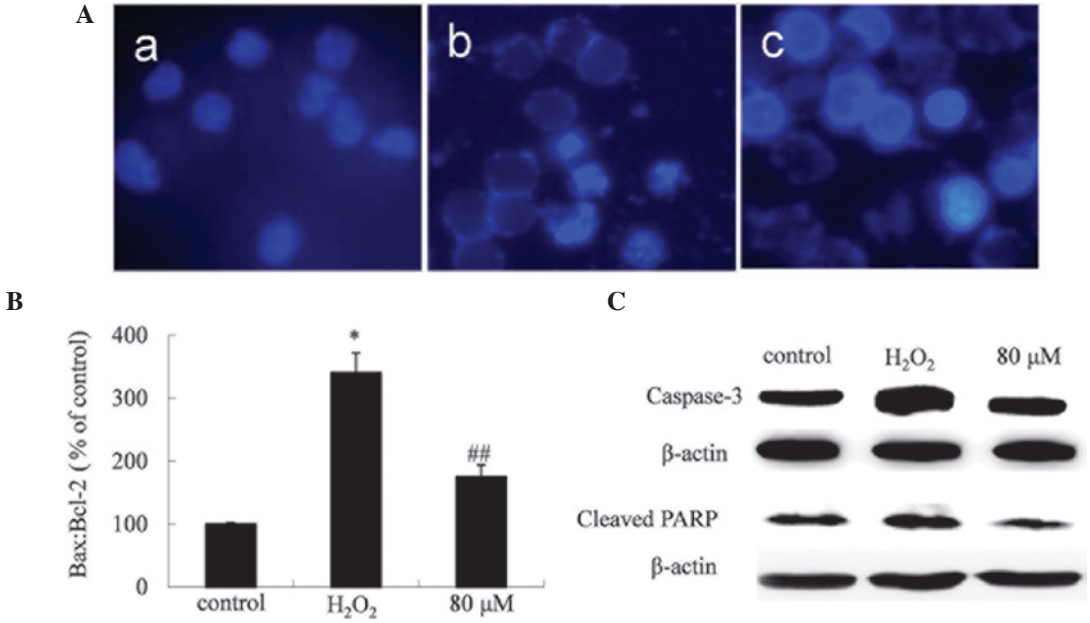

Figure 3. PF protected PC12 cells against H_2O_2-induced apoptosis. (A) Morphological analysis of nuclear chromatin in a) control, b) H_2O_2 and c) 80 μM PF treated cells with Hoechst 33258 staining. Cells displayed condensed chromatin and apoptotic nuclei following H_2O_2 treatment, while PF treatment markedly reversed these effects. (B) Bax:Bcl-2 ratio in the control, H_2O_2 and 80 μM PF groups. (C) PF reversed the H_2O_2-induced increase in the expression levels caspase-3 and cleaved PARP in PC12 cells. *P<0.01 vs. the control group and $^{\#\#}$P<0.01 vs. the H_2O_2 group. H_2O_2, hydrogen peroxide; PF, paeoniflorin; PARP, poly(ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

expression levels was estimated by comparison to a vehicle control. Experiments were performed in triplicate, separately.

TNF-α and IL-1β assays. The culture medium was collected in microcentrifuge tubes and subjected to centrifugation for 10 min. The supernatants were separated out and the expression levels of TNF-α and IL-1β were detected using Human TNF-alpha Quantikine (DTA00C) and Human IL-1 beta/IL-1F2 Quantikine ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS software for Windows, version 18.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation and were analyzed by one-way analysis of variance. Multiple comparisons between groups were performed using the Student-Newman-Keuls method and P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of PF on cell viability. The viability of cells incubated with 200 μM H_2O_2 was 58.6±2.4% of the control value (P<0.01; Fig. 1). The viabilities of cells treated with 200 μM H_2O_2 + 20, 40 or 80 μM PF were increased in a dose-dependent manner to 66.3±1.6 (P<0.05 vs. H_2O_2), 75.9±1.1 and 83.4±1.7% (P<0.01 vs. H_2O_2) of the control values, respectively (n=3; Fig. 1). These results clearly indicate that PF attenuated the H_2O_2-induced cytotoxicity in the PC12 cells.

Effect of PF on LDH and ROS levels. The neuroprotective effect of PF was further investigated by measuring ROS accumulation and levels of LDH release following treatment. Pretreatment with PF attenuated the H_2O_2-induced increase in levels of ROS and LDH release (Fig. 2).

PF protects PC12 cells against H_2O_2-induced apoptosis. Alterations of cellular morphology were assessed using Hoechst 33258 staining in order to characterize the degree of H_2O_2-induced PC12 cell death (Fig. 3A). The nuclei of the PC12 cells treated with H_2O_2 appeared fragmented, indicating that apoptosis affected the morphology of the cells. However, treatment with 80 μM PF for 6 h clearly reduced the percentage of necrotic and apoptotic cells.

Expression levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax were measured using western blot analysis. H_2O_2 was observed to increase the Bax:Bcl-2 ratio in the PC12 cells, while the PF treatment produced an opposite effect (Fig. 3B). Furthermore, the H_2O_2-induced elevation of the expression levels of caspase-3 and cleaved PARP appeared significantly reduced in cells treated with PF (Fig. 3).

PF suppresses the expression levels of NF-κB and its associated inflammatory factors. Western blot analysis indicated that the expression levels and activity of NF-κB were elevated in cells treated with H_2O_2 alone. Treatment with PF appeared to significantly reduce this H_2O_2-induced NF-κB activity (Fig. 4A). The levels of total NF-κB protein displayed a marked reduction in cells treated with PF (P<0.01). Further analysis indicated that the levels of p-NF-κB (Ser536), the active form of NF-κB, were significantly reduced by the PF treatment (P<0.01).

Inhibitory effect of PF on the expression levels of TNF-α and IL-1β. Western blot analysis indicated that treatment with PF reversed the H_2O_2-induced elevation of the expression levels

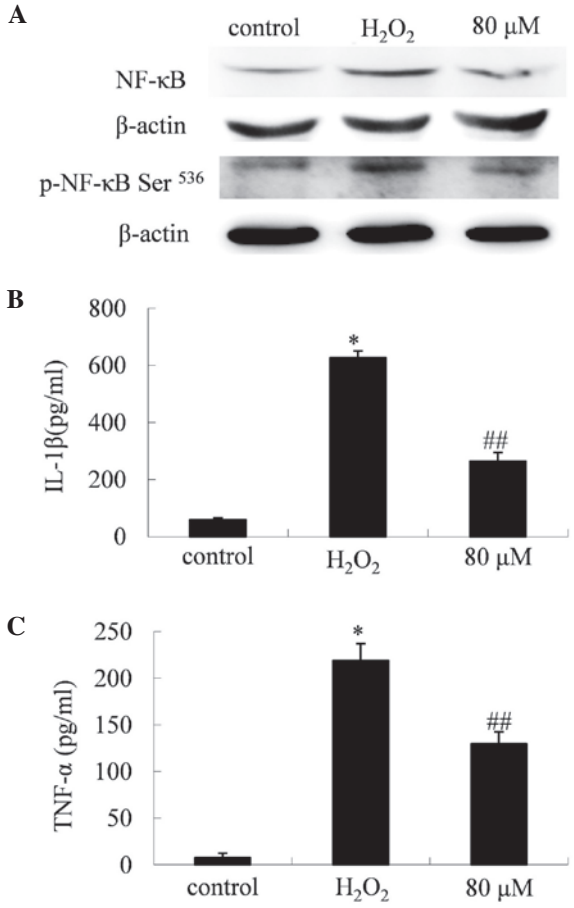

Figure 4. PF suppressed the expression of NF-κB and its associated inflammatory factors. (A) PF reduced the H_2O_2-induced increase in the expression levels of (A) NF-κB and its active form p-NF-κB Ser536, (B) IL-1β and (C) TNF-α. *P<0.01 vs. the control group and ##P<0.01 vs. the H_2O_2 group. H_2O_2, hydrogen peroxide; PF, paeoniflorin; NF-κB, nuclear factor-κB; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α.

of IL-1β (P<0.01; Fig. 4C) and TNF-α (P<0.01; Fig. 4B) in the PC12 cells.

Discussion

PF is the main component of *Paeoniae Radix* used in traditional Chinese medicine, and has been reported to exhibit numerous pharmacological effects. The results of the present study indicated that PF may protect PC12 cells from H_2O_2-induced oxidative injury. PF was observed to regulate H_2O_2-induced oxidative stress, indicated by the modulation of LDH and ROS levels in PC12 cells. PF was also demonstrated to reduce H_2O_2-induced apoptosis and promote overall cell survival. Furthermore, the results indicated that PF downregulated H_2O_2-induced neuroinflammation by regulating NF-κB-associated inflammatory signals.

Oxidative stress has been extensively implicated in the pathophysiology of cerebral ischemia and stroke (10). Hypoxia is a crucial initiator of the loss of neurocytes and apoptosis is considered to be a pivotal source of damage to neurocytes during this process (11). The accumulation of ROS may lead to various forms of oxidative modification of proteins, lipids and DNA, resulting in cellular damage (12). The present study demonstrated that 200 μM H_2O_2 was able to significantly stimulate the accumulation of ROS and the release of LDH (203.1 and 270.1% of control values, respectively) in PC12 cells. Furthermore, the cell survival rate in the H_2O_2 group was 58.6±2.4% of the control value.

PF treatment appeared to markedly improve these oxidative conditions. The ROS levels in the 20, 40 and 80 μM PF treatment groups were 171.8, 141.6 and 117.4% of the control group, respectively. The LDH expression levels of the 20, 40 and 80 μM PF treatment groups were 217.0, 175.1 and 133.8% of the control group, respectively. These results indicated that the PF treatment produced a significant reduction in H_2O_2-induced toxicity and oxidative stress in the PC12 cells.

ROS are widely recognized to be key mediators of cell survival, proliferation, differentiation and apoptosis (5,13,14). Previous studies have demonstrated that proteins of the Bcl-2 family, including Bax and Bcl-2, are associated with apoptosis induced by ROS-generating agents (Ji BS,Renaud,Pan). In addition, ROS may activate caspase-3, which results in the cleavage of PARP, a 116-kDa nuclear poly (ADP-ribose) polymerase, which appears to be involved in DNA repair in response to environmental stress. PARP may be cleaved by numerous caspase-1-like caspases *in vitro* and is one of the primary cleavage targets of caspase-3 *in vivo*. Furthermore, ROS may activate caspase-3, which results in the cleavage of PARP into an 89-kDa fragment (6,15-17,20). In the present study, a Hoechst 33258 staining assay indicated that treatment with 200 μM H_2O_2 alone induced notable cell apoptosis in PC12 cells, while 80 μM PF produced a reduction in the extent of apoptosis-associated nuclear fragmentation (Fig. 3A). Furthermore, H_2O_2-induced apoptosis was associated with an increase in the Bax:Bcl-2 ratio and with the activation of caspase-3. Treatment with PF was observed to downregulate the expression of the pro-apoptotic protein Bax, and to upregulate the anti-apoptotic protein Bcl-2. The results of the present study also demonstrated that caspase-3 and cleaved PARP were modulated by PF treatment.

Oxidative stress-induced neuroinflammation has been reported to be a vital factor in nerve injury and associated diseases (5,8,21). Numerous studies have suggested that chronic inflammation is implicated in neurodegenerative disease and injury (1,4,21-23). A number of well-established inflammatory target proteins, including matrix metalloproteinase-9, cyclooxygenase-2, inducible nitric oxide synthase and certain adhesion molecules have been associated with ROS generation, which is also induced by proinflammatory cytokines, peptides, peroxidants and infection (5,13,24,25). Increasing inflammatory stress has been reported to correlate with oxidative stress during the progression of neurodegenerative disease (5,19,26). NF-κB, a proinflammatory transcription factor, functions as the 'first responder' to various generators of cellular stress, including free radicals and pro-inflammatory cytokines (e.g. TNF-α) and bacterial biomolecules) (27). In the present study, H_2O_2 was observed to induce an inflammatory response involving NF-κB and its associated signals. Following H_2O_2 treatment, the levels of NF-κB and its active form, p-NF-κB (Ser536), were elevated, as were the levels of TNF-α and IL-1β. However, cells cocultured with 80 μM PF exhibited reduced levels of these inflammatory factors, indicating that PF modified the apoptotic process, in addition to correcting the abnormal inflammatory signals induced by H_2O_2.

In conclusion, PF treatment significantly reduced H_2O_2-induced apoptosis and ROS accumulation, promoted cell survival and downregulated neuroinflammation in PC12 cells. Thus, PF may serve as a protective agent against oxidative stress and scavenger of intracellular ROS, and may offer a novel pharmacological preventative or palliative treatment for ischemic cerebral injury.

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