Neuroscience 285 (2015) 70–80

PAEONIFLORIN INHIBITION OF 6-HYDROXYDOPAMINE-INDUCED APOPTOSIS IN PC12 CELLS VIA SUPPRESSING REACTIVE OXYGEN SPECIES-MEDIATED PKCδ/NF-κB PATHWAY

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Abstract—Parkinson's disease (PD) is second only to Alzheimer's disease as the most common devastating human neurodegenerative disorder. Despite intense investigation, no curative therapy is available for PD. Paeoniflorin, a monoterpene glucoside isolated from the *Paeonia lactiflora* Pall., possesses wide pharmacological effects in the nervous system. This study aims at evaluating the effect of paeoniflorin on 6-hydroxydopamine (6-OHDA)-induced apoptosis and to characterize involved signal transduction pathways in PC12 cells. Our results showed that paeoniflorin suppresses mitochondria-mediated apoptosis of PC12 cells induced by 6-OHDA, and anti-apoptotic effects of paeoniflorin on PC12 cells might mainly result from its antioxidant capability by increasing glutathione (GSH). Moreover, we also found that paeoniflorin can dramatically attenuate the 6-OHDA-induced nuclear factor κB (NF-κB) translocation without affecting phosphorylation of Akt, JNK, p38, and ERK1/2. 6-OHDA-induced protein kinase Cδ (PKCδ) upregulation was blocked by paeoniflorin treatment in PC12 cells. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium or NF-κB inhibitor BAY 11-7082 could partially attenuate 6-OHDA-induced cell death. Together, our results indicate that the inhibition of PC12 cell apoptosis by paeoniflorin might be mediated, at least in part, by inhibiting reactive oxygen species (ROS)/PKCδ/NF-κB signaling pathway. This evidence supports the pharmacological potential of paeoniflorin in the management of neurodegenerative disorders associated with oxidative stress, including PD.

Key words: oxidative stress, apoptosis, Parkinson's disease, *Paeonia lactiflora* Pall.

INTRODUCTION

Parkinson's disease (PD) is second only to Alzheimer's disease as the most common devastating human neurodegenerative disorder characterized by a profound loss of midbrain dopamine neurons in the substantia nigra pars compacta (Vieira et al., 2013; Darvas et al., 2014). The brain is particularly vulnerable to oxidative stress because of its high oxygen consumption (for a review, see Lau et al., 2007). Earlier studies have demonstrated a significant increase in markers for oxidative stress in the brains of PD patients as compared to controls (Ikawa et al., 2011). Reactive oxygen species (ROS) are necessary for normal cellular functions such as signaling and synaptic plasticity, but can become detrimental to neuronal function when they accumulate excessively in the brain (Wypijewska et al., 2010). Therefore, treatment with antioxidants might theoretically act to retard spreading of neuronal damage and to improve neurological outcome (Koppula et al., 2012).

Although dopamine replacement therapy is remarkably effective in the treatment of idiopathic PD by masking or reducing disease symptoms (Lobb, 2014), there is no disease-modifying neuroprotective or neurorestorative therapy available (for a review, see Stocchi and Olanow, 2013). The limitations associated with dopaminergic agents prompted us to investigate new disease modifying therapies to aim at neuroprotection. As oxidative stress contribute to the pathogenesis and progression of PD (for a review, see Subramaniam and Chesselet, 2013), it is reasonable to assume that antioxidants would prevent the progression of PD.

Natural products have established a strong position as leads for drug discovery (for a review, see Campos et al., 2011; Brown et al., 2014). For many natural products, the mechanism of action is unknown, hampering drug development. Based on a retrospective review of the historical role of a number of Chinese herbal medicine used for the treatment of PD, it was shown that the *Paeonia lactiflora* Pall. as a component of traditional Chinese medicine prescriptions might potentially provide natural treatment for PD. Paeoniflorin, a monoterpene glucoside, is known to be one of the principal active components of *P. lactiflora* Pall. (Sun et al., 2012). Paeoniflorin has been reported to possess various pharmacological effects on central

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Abbreviations: 6-OHDA, 6-hydroxydopamine; DPI, diphenyleneiodonium; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; LDH, lactate dehydrogenase; LPO, lipid hydroperoxides; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor κB; PC, pheochromocytoma; PCR, polymerase chain reaction; PD, Parkinson's disease; PKCδ, protein kinase Cδ; ROS, reactive oxygen species.

http://dx.doi.org/10.1016/j.neuroscience.2014.11.008
0306-4522/

nervous system such as anti-oxidative, anti-depressant, anti-apoptotic, anti-inflammatory, and neuroprotective effects (Nam et al., 2013; Qiu et al., 2013; Wang et al., 2013; Wu et al., 2013). Moreover, paeoniflorin is unique among antioxidants because it can cross the blood–brain barrier representing a promising opportunity for the treatment for PD (Cao et al., 2006). However, the precise mode of paeoniflorin-neuroprotective action in the treatment and management of PD remains elusive.

The aim of this study was to test the hypothesis that inhibition of 6-OHDA-induced apoptosis in pheochromocytoma (PC)-12 cells by paeoniflorin is mediated through a ROS-mediated protein kinase Cδ (PKCδ)/nuclear factor κB (NF-κB) signaling pathway. Indeed, Guo et al. give the first evidence that this hypothesis holds true (Guo et al., 2012). Results in the current report support our hypothesis and provide novel insights into the mechanisms of paeoniflorin in the inhibition of PC12 cells apoptosis.

EXPERIMENTAL PROCEDURES

Cell culture and treatments

The PC12 cell line derived from rat pheochromocytoma can differentiate and exhibit features of sympathetic neurons under the influence of nerve growth factor. We used differentiated PC12 cells as a model of sympathetic neurons, a vulnerable population in PD. PC12 cells were obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China), and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. To initiate neurodifferentiation, the medium was changed to include 100 ng/mL of nerve growth factor (Promega Corporation, Madison, WI, USA) for 9 days. Cells were seeded at a density of 2×10^5 cells/mL. Cultured cells were pretreated for 2 h with paeoniflorin at various concentrations followed by exposure to 6-OHDA at indicated times. In some experiments, cells were treated with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI) (10 μM) (Sigma, St Louis, MO, USA), caspase-3 inhibitor Z-DEVD-FMK (50 μM) (Sigma), or NF-κB inhibitor BAY 11-7082 (20 μM) (Sigma) in the presence and absence of paeoniflorin or/and 6-OHDA for 24 h. The final concentrations of all solvents for treatment of the cells, including control cultures, were maintained at 0.25%.

MTS cytotoxicity assay

The cell viability was determined using CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, USA) according to the manufacturer's instructions. Absorbance was measured using an automatic microplate reader at a wavelength of 490 nm to calculate the cell survival percentages.

Lactate dehydrogenase (LDH) leakage assays

LDH activity was measured using a cytotoxicity detection kit (Genmed, Westbury, NY, USA) and a spectrophotometric microplate reader ((ELx800; BioTek, Winooski, VT, USA) and compared as a percentage of LDH activity from cells lysed with 0.1% Triton X-100 for 20 min at room temperature.

Flow cytometry

Cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (BD Biosciences, San Jose, CA, USA), according to manufacturer's protocol. The population of Annexin V-positive cells was evaluated by flow cytometry (BD FACScan; Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Nuclear and cytoplasmic extracts from PC12 cells were prepared using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China). Cytosolic cytochrome *c* was determined by the Quantikine® rat/mouse cytochrome *c* assay kit (R&D systems, Minneapolis, MN, USA). DNA fragmentation was measured using Cell Death Detection ELISA Plus Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). Activation of NF-κB in the nuclear extracts was estimated using ELISA-based TransAM™ NF-κB p65 Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol.

Caspase assay

Cellular caspase-3 and caspase-9 activities were measured with commercially available colorimetric assay kit (Beyotime Biotechnology, Haimen, China). PC12 cells were lysed with lysis buffer supplied with the kit. The soluble fraction of the cell lysate was then assayed for caspase-3 and caspase-9 activities using Ac-DEVD-pNA, a colorimetric substrate for caspase-3, and Ac-LEHD-pNA, a colorimetric substrate for caspase-9, as described in the manufacturer's protocol, respectively.

GSH assays

The levels of GSH were determined by using the components provided in Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The concentration of total GSH was calculated according to the equation in the protocol, and was then normalized to protein concentration.

ROS assays

The fluorescent probe, 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), provided in OxiSelect™ Intracellular ROS Assay Kit obtained from Cell Biolabs Inc. (San Diego, CA, USA) was used to measure intracellular ROS levels. Fluorescence was measured in