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TANSHINONE IIA PROTECTS DOPAMINERGIC NEURONS AGAINST 6-HYDROXYDOPAMINE-INDUCED NEUROTOXICITY THROUGH miR-153/NF-E2-RELATED FACTOR 2/ANTIOXIDANT RESPONSE ELEMENT SIGNALING PATHWAY

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Abstract—Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder with increased oxidative stress, the underlying vital process contributing to cell death. Tanshinone IIA (Tan IIA), a major bioactive diterpene quinone of Salva miltiorrhiza, had been proved effective in the MPTP model through its anti-inflammatory activity. Here in this research, we found that Tan IIA prevented the loss of nigrostriatal dopaminergic neurons by activating the NF-E2-related factor 2 (Nrf2)–antioxidant response element (ARE) pathway. The cytotoxicity of 6-hydroxydopamine (6-OHDA) was attenuated by the treatment of Tan IIA in SH-SY5Y cells, which significantly reduced 6-OHDA-induced lactic dehydrogenase release and reactive oxygen species production. Further study indicated that Tan IIA contributed to the nuclear accumulation of Nrf2, which bound to the ARE sequence, and activated ARE-regulated genes, including heme oxygenase-1, glutamate cysteine ligase catalytic subunit (GCLC) and glutamate cysteine ligase modifier subunit (GCLM). Tan IIA also protected against damage to mitochondrial membrane potential, reduced the translocation of cytochrome c from the mitochondria to the cytoplasm and the activation of Caspase-9 and Caspase-3. Moreover, we demonstrated the above effects were performed in Nrf2-dependent manner. Further studies revealed that Tan IIA reduced the enhancement of miR-153 by 6-OHDA, which targeted the 3′-UTR of Nrf2, and suppressed its expression and activation. Additionally, neurodegeneration caused by *in vivo* stereotaxic injection of 6-OHDA could also be ameliorated by the administration of Tan IIA. Taken together, our results strongly suggest that Tan IIA may be beneficial for the treatment of PD, and also confirm that targeting the Nrf2/ARE pathway is a promising strategy for therapeutic intervention in PD.

Key words: Parkinson's disease, 6-OHDA, tanshinone IIA, Nrf2, ARE, oxidative stress.

INTRODUCTION

Parkinson's disease (PD) is emerging as the second most common progressive neurodegenerative disorder, which is pathologically characterized by selective loss of dopaminergic neurons in the substantia nigra. Although the exact mechanisms underlying PD are still largely unknown, oxidative stress and inflammatory effects are thought to be the critical factors that induce the onset of both familial and sporadic PD. High oxygen consumption and high lipid content make neural tissues more sensitive to oxidative stress than other organs. Unregulated reactive oxygen species (ROS) exert deleterious effects through disrupting cellular redox homeostasis and causing extensive damage to macromolecules of the cell, resulting in oxidative stress. Reduced glutathione peroxidase (Kish et al., 1985), peroxidase and catalase activity (Ambani et al., 1975), as well as increased basal lipid peroxidation (Dexter et al., 1989) and DNA oxidative damage (Jenner and Olanow, 1998) in the dopaminergic system of PD patients demonstrated that dopaminergeic neurons of PD patients are exposed to heightened oxidative stress.

6-Hydroxydopamine (6-OHDA), a hydroxylated dopamine (DA) metabolite (Cohen and Heikkila, 1974), produces hydrogen peroxide, superoxide radical, and hydroxyl radical during its autoxidation. 6-OHDA damages the catecholaminergic neurons since it has high affinity for the catecholamine transporters due to its chemical similarity with catecholamines. Intrastriatal injection of 6-OHDA induces a parkinsonian pattern of dopaminergic neuronal loss in rodents. 6-OHDA injury mimics many pivotal features of degenerating neurons observed in human PD samples, which include oxidation and nitration of proteins, proteasome inhibition, increased protein ubiquitination, glutathione depletion, and the accumulation of activated signaling proteins (Deumens et al., 2002). Notably, 6-OHDA has been

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Abbreviations: 6-OHDA, 6-hydroxydopamine; ARE, antioxidant response element; BSA, bovine serum albumin; Cyt C, cytochrome c; DA, dopamine; DAPI, 4′,6-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; DOPAC, dihydroxyphenylacetic acid; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; GSH, Glutathione; HO-1, heme oxygenase-1; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; HUVECs, human umbilical vein endothelial cells; HVA, homovanillic acid; LDH, lactic dehydrogenase; MTT, momotetrazolium; NQO-1, NADPH quinone oxidoreductase-1; Nrf2, NF-E2-related factor 2; PD, Parkinson's disease; ROS, reactive oxygen species; siRNA, Small interference RNA; SM, Salva miltiorrhiza; Tan IIA, tanshinone IIA; TH, tyrosine hydroxylase.

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found in the caudate nucleus of human brain biopsies (Curtius et al., 1974) and the urine of L-dopa-treated PD patients (Andrew et al., 1993), which indicates that 6-OHDA may participate in the pathogenesis of PD as an endogenous hydroxylated metabolite of DA. The above traits render 6-OHDA as an ideal model to elucidate the pathogenic mechanism of neurodegeneration in PD.

Salva miltiorrhiza (SM) is widely used in traditional Chinese medicine, and has been widely used in the treatment of cardiovascular diseases, hepatocirrhosis, hepatitis, and chronic kidney disease. Tanshinone IIA (Tan IIA), a major bioactive diterpene quinone of SM, is used in the treatment of coronary heart disease, cerebrovascular disease, hepatitis and hepatocirrhosis due to its multiple pharmacological activities including anti-oxidant, anti-inflammatory, and anti-neoplastic effects (Sung et al., 1999; Ji et al., 2008; Hong et al., 2010; Qin et al., 2010). Lin et al. have reported that damage to the human umbilical vein endothelial cells (HUVECs, ECV-304) induced by hydrogen peroxide was attenuated by Tan IIA through its anti-oxidant and anti-inflammatory approach (Lin et al., 2006). Recently, it has been reported that Tan IIA has neuroprotective effects against cerebral ischemia/reperfusion injury and traumatic injury of the spinal cord in rats. Tan IIA could scavenge free radicals (Ren et al., 2015) and prevent the loss of nigrostriatal dopaminergic neurons by inhibiting NADPH oxidase and iNOS in the MPTP model of PD (Ren et al., 2015). Whether Tan IIA has any neuroprotective effect in 6-OHDA-induced PD model is still largely unknown.

The redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2) plays a key role in orchestrating cellular antioxidant defenses and maintaining redox homeostasis. With the stimulation of oxidative stress, Nrf2 binds to and regulates the antioxidant response element (ARE) genes such as heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase-1 (NQO-1), and glutathione-synthesizing enzymes to rescue the cells from oxidative stress and neuroinflammation. Tan IIA was reported to protect against liver injury and suppress cholesterol accumulation in human macrophages via the activation of Nrf2/ARE. Whether antioxidation of Tan IIA in dopaminergic cells is related to the activation of Nrf2/ARE needs further exploration. In this study, we tested the hypothesis that Tan IIA has therapeutic effects in the 6-OHDA-induced cellular and animal model of PD via the regulation of Nrf2/ARE.

EXPERIMENTAL PROCEDURE

Reagents

Tan IIA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The chemical structure of Tan IIA is shown in Fig. 1A, and its purity was greater than 98%. RPMI-1640's medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). 6-OHDA, ascorbic acid, momotetrazolium (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). The test kits for measurement of lactic dehydrogenase (LDH), superoxide dismutase (SOD) were purchased from the Nanjing Institute of Jiancheng Bioengineering (Nanjing, China). All other chemicals and solvents used were of analytical grade unless stated specified.

Primary antibodies to Nrf2, HO-1, glutamate cysteine ligase catalytic subunit (GCLC), glutamate cysteine ligase modifier subunit (GCLM), NQO-1, Lamin A and β-actin were purchased from Santa Cruz Biotechnology. Primary antibodies to cleaved Caspase-9 and cleaved Caspase-3 were purchased from Cell signaling technology (Beverly, MA, USA). Primary antibody to tyrosine hydroxylase (TH) was purchased from Chemicon (Bill Card City, MA, USA). Alexa Fluor 594 goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA, USA).

Neuroblastoma SH-SY5Y cell culture and 6-OHDA exposure

Human neuroblastoma SH-SY5Y cells were obtained from ATCC and maintained in DMEM-F12 supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2. The neurotoxin 6-OHDA·HBr was dissolved in 0.02% ascorbic acid and prepared fresh for each experiment. Cultures were exposed to 100 μM 6-OHDA for 24 h before being harvested for various assays.

Cell viability/death assessment

Cell viability was measured by the MTT assay. Briefly, the cells were seeded in 96-well plates (5×10^3 cells/well). Twenty-four hours later, the cells were co-treated with 100 μM 6-OHDA and vehicle alone (PBS including 1% DMSO) or Tan IIA (5, 10, 20, 40 and 80 μg/ml, dissolved in PBS including 1% DMSO) for 24 h in the continued presence of vehicle or Tan IIA. After incubation, cells were incubated at 37 °C for 4 h in 0.5 mg/ml MTT solution, and the media were carefully removed. 100 μl of DMSO was added to each well to dissolve the violet formazan crystals. The absorbance at 570 nm was measured. All values were normalized to the control group.

6-OHDA-induced cell death was quantified by measuring LDH release from damaged cells into the culture medium according to the manufacture's instruction (Roche). In brief, 150 μl of LDH reaction reagent was added with 50 μl medium taken from the cell culture wells. The absorbance was measured at 490 nm using a spectrophotometer plate reader. The absorbance was proportional to the amount of LDH in the medium. Data were expressed as the percentage of cell death that was calculated according to the manufacturer's instruction.

ROS determination

Levels of oxidative stress were determined by measuring intracellular ROS generation as detected using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay. Briefly, cells were seeded in 96-well plates at the density of 7×10^3 per well for overnight incubation. After treatment with various concentrations of test samples, cells were incubated in serum-free medium containing 25 μM DCFH-DA at 37 °C for 30 min. ROS generation was measured by the