Safranal prevents rotenone-induced oxidative stress and apoptosis in an in vitro model of Parkinson's disease through regulating Keap1/Nrf2 signaling pathway

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Abstract: Safranal, a major constituent of saffron, possesses antioxidant and anti-apoptotic properties showing considerable neuroprotective effects. However, whether safranal shows therapeutic effect on Parkinson’s disease (PD) remains unknown. In this study, we aimed to investigate the potential effect of safranal on PD using an in vitro model of PD induced by rotenone. We found that safranal significantly inhibited rotenone-induced cell death in a dose-dependent manner. Moreover, safranal also markedly suppressed the reactive oxygen species (ROS) generation and cell apoptosis induced by rotenone. Further investigation showed that safranal inhibited the expression of kelch-like ECH-associated protein 1 (Keap1) and promoted the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) in rotenone-induced dopaminergic neurons. Meanwhile, the downstream antioxidant enzyme genes of Nrf2 including glutathione S transferase (GST), glutamate-cysteine ligase catalytic subunit (GCLc), NADPH-quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) were also induced by safranal in rotenone-induced dopaminergic neurons. However, the knockdown of Nrf2 significantly abrogated the protective effect of safranal on rotenone-induced neurotoxicity. Taken together, our study suggests that safranal protects against rotenone-induced neurotoxicity associated with Nrf2 signaling pathway implying that safranal may serve as a potent and promising therapeutic drug for the treatment of PD.

Key words: Safranal, Parkinson’s disease, ROS, cell apoptosis, Nrf2.

Introduction

Parkinson’s disease (PD) is a severe type of neurodegenerative disorder, the hallmark of which is continuous loss of dopaminergic neurons in the substantia nigra pars compacta leading to dopamine depletion in the striatum (1). PD is an age-related disease with approximately 1% incidence rate in the older population with age greater than 60 in the industrialized countries (2). Despite the advance in the treatments, the current drugs can only temporarily remit the symptoms that improve patients’ life quality to a limited degree. Therefore, it is of great importance to develop potential and effective therapeutic drugs for treating PD.

Saffron, with a scientific name of *Crocus sativus* L. (*C. sativus*), has been used as a traditional medicine for many years (3-4). Saffron and its constituents have a wide range of anti-inflammatory, antioxidant, anti-apoptotic and immunomodulatory effects that have been employed as an alternative treatment for many diseases including coronary artery diseases, neurodegenerative disorders, diabetes, bronchitis, asthma and so on (3). Safranal, a monoterpenic aldehyde occupying 70% of the volatile fraction, is the main constituent of saffron (5). In recent years, increasing evidence has demonstrated that safranal has a variety of pharmacological properties including anti-inflammatory, antioxidant, anti-apoptotic, anti-ischemic, antiinflammatory, anti-depressant, anti-inflammatory, and neuroprotective effects (6-9). However, whether safranal has a treatment effect on PD remains unknown.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor that regulates a variety of antioxidant and cytoprotective genes to protect against oxidative stress (10). The transcription activity of Nrf2 is controlled by kelch-like ECH-associated protein 1 (Keap1), which binds to Nrf2, promoting Nrf2 degradation; conversely, Nrf2 releases from Keap1 and translocates to the nucleus, binding to antioxidant response elements and activating the transcription of antioxidant enzymes including glutathione S transferase (GST), glutamate-cysteine ligase catalytic subunit (GCLc), NADPH-quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) (11). A recent study has suggested that the Keap1/Nrf2 signaling plays an important role in neurodegenerative diseases including PD (12). The activation of Nrf2 signaling shows a protective effect against PD (13).

Rotenone, a naturally lipophilic compound with insecticide-like properties, suppresses mitochondrial electron transfer chain complex I and induces the formation of reactive oxygen species (ROS), leading to cell apoptosis (14-15). Rotenone has been used as a common neurotoxic agent to develop PD models in vitro and in vivo (16-19). To date, there is still a lack of effective pharmacological therapies for PD. The development of neuroprotective drugs for PD from natural origins has drawn considerable interest in recent years (20). Consi...
dering the antioxidant and anti-apoptotic effect of safranal, we hypothesized that safranal may prevent the oxidative stress and inhibit the loss of dopaminergic neurons in PD. To test this hypothesis, we used an in vitro model of PD induced by rotenone to detect the effect of safranal on oxidative stress and cell apoptosis. Interestingly, we found that safranal effectively inhibited rotenone-induced ROS generation and cell apoptosis in dopaminergic neurons associated with Keap1/Nrf2 signaling pathway. Our results suggest that safranal may serve as a potent pharmacological therapy for PD.

Materials and Methods

Primary dopaminergic cell culture

Primary dopaminergic cells were isolated from rat embryos as described previously (21). Briefly, E17 embryos were dissected from pregnant Sprague-Dawley rats (Laboratory Animal Center of College of Medicine of Xi’an Jiaotong University, Xi’an, China). The mesencephalic tissues were removed from the embryonic rat brains and incubated with Ca²⁺ and Mg²⁺-free Hank’s balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) at 4°C. Then, the tissues were digested in phosphate-buffered saline (PBS) containing 0.02% EDTA and 0.1% trypsin (Sigma, St. Louis, MO, USA) at 20°C followed by dissociation in HBSS containing 10% fetal calf serum (FCS; Gibco, Rockville, MD, USA). After filtration through 50-μm pore-sized nylon mesh filter, the dissociated cells were centrifuged at 200 g for 5 min. The cell pellets were collected and resuspended in neurobasal medium supplemented with 10% FCS, 5 μg/ml gentamicin, 1 mM Glutamax, 0.1 × B27 supplement and 1% penicillin and streptomycin mix (Gibco). Cells were seeded in plates pre-coated with 0.1 mg/ml poly-L-ornithine (Sigma) and cultured in a humidified incubator containing 5% CO₂ at 37°C. After 7 days, the cells were used for the experiments in vitro. The experimental procedures of animal use were approved by the Institutional Animal Care And Use Committee of Ankang Hospital of Traditional Chinese Medicine.

Cell treatments

Cells were incubated with rotenone (Sigma) at different concentrations (0.5, 5, 50, 100 and 200 nm) for 24 h to choose the effective dose of rotenone. Cells were incubated with different concentrations of safranal (Sigma; 10, 15, 20, and 50 μg/ml) for 4 h and then incubated with the effective rotenone dose for 24 h. For gene silencing, Nrf2 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was transiently transfected into cells according to the manufacturer’s instructions.

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoi-
lum bromide (MTT) assay

Cell viability was detected by MTT assay. Cells were seeded in 96-well plates at a density of 4 × 10⁴ cells/well after pretreatment with safranal for 4 h with indicated concentrations following treatment with 100 nM rotenone for 24 h. Then, MTT (Sigma; 0.5 mg/ml) was added to the cultures (20 μl/well) and incubated for 4 h. Dimethyl sulfoxide (Sigma) was added to the cultures (200 μl/well) to dissolve the formazan crystals for 15 min. The absorbance at 490 nm was detected using a microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

ROS determination

The generation of ROS was detected using the 2',7'-dichlorofluoresceindiacetate (DCFH-DA) method. Briefly, a 1-ml aliquot of cells (8×10⁶ cells/ml) was incubated with 50 μM DCFH-DA (Sigma) for 30 min at 37°C in a dark place. After washes with PBS, the fluorescence intensity was measured with an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence spectrophotometer (Bio-Tek Instruments).

Caspase-3 activity assay

Cell apoptosis was detected by caspase-3 activity assay using a commercial kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s instructions. Briefly, cells were lysed by lysis buffer, and the protein concentration was assessed using a BCA kit (Beyotime, Haimen, China). A total of 100 μg protein in 50 μl lysis buffer were incubated with 5 μl DEVD-p-nitroanilide (4 mM) supplemented with 50 μl reaction buffer for 2 h at 37°C. The absorbance at 405 nm was detected using a microplate spectrophotometer (Bio-Tek Instruments).

Western blot analysis

The total, cytosolic and nuclear protein fractions were extracted using a commercial protein extraction kit (Beyotime) as per the recommended protocols by the manufacturer. Equivalent amounts of 50 μg proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then electrohoresically transferred onto a polyvinylidene difluoride membrane (Millipore, Boston, MA, USA) using semidy transfer (Bio-Rad, Hercules, CA, USA). After blocking with 3% non-fat milk in Tris-buffered saline (TBS) at 37°C for 1 h, the membrane was incubated with primary antibodies including anti-Bax (1:500), anti-Bcl-2 (1:500), anti-Keap1 (1:300), anti-Nrf2 (1:600), anti-β-actin (1:800) and anti-Lamin B (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with primary antibodies at 4°C overnight, the membrane was washed with TBS-0.05%Tween-20 (TBST) and probed with horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with primary antibodies at 4°C overnight, the membrane was washed with TBS-0.05%Tween-20 (TBST) and probed with horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology) for 1 h at 37°C. After washes with TBST, the protein bands were developed by a Pierce ECL Western blotting kit (Pierce, Rockford, IL, USA). Densitometry was quantified by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA were extracted using TRIZOL reagent (Invitrogen). The cDNA was synthesized by reverse transcription using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). The PCR amplification was conducted using SYBR Green PCR Master Mix (Applied Biosystem, Carlsbad, CA, USA). β-actin was used as the internal control. Relative gene expression was analyzed by 2⁻ΔΔCt method. The primers used were as follows: Nrf2 forward, 5’-ATG GCCACACTTTTCTGGAC-3’ and
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The results showed that safranal had no cytotoxicity on dopaminergic neurons (Fig. 1B) but significantly attenuated rotenone-induced cell death in a dose-dependent manner (Fig. 1C). The data suggest that safranal has a protective effect on rotenone-induced cell death in dopaminergic neurons.

Safranal inhibits rotenone-induced ROS and apoptosis

To further investigate the effect of safranal on rotenone-induced neurotoxicity, we detected the ROS generation and cell apoptosis changes. The results showed that the intracellular ROS level was significantly induced by rotenone treatment, whereas pretreatment with safranal to rotenone remarkably decreased the level of ROS generation (Fig. 2A). The cell apoptosis induced by rotenone was also significantly abrogated by pretreatment with safranal (Fig. 2B). Furthermore, rotenone treatment significantly upregulated the protein expression of pro-apoptotic Bax (Fig. 2C) and downregulated the protein expression of anti-apoptotic Bcl-2 (Fig. 2D). As expected, pretreatment with safranal markedly inhibited the rotenone-induced increase of Bax (Fig. 2C) and reduction in Bcl-2 (Fig. 2D). No significant changes were detected in cells treated with safranal alone. These results indicate that safranal can protect dopaminergic neurons from rotenone-induced neurotoxicity through inhibiting ROS generation and apoptosis.

Safranal promotes Nrf2 nuclear accumulation in rotenone-induced dopaminergic neurons

Keap1/Nrf2 signaling is involved in the protection of PD (12). To investigate whether Keap1/Nrf2 signaling reverse, 5’-AGATGTCAAGCGGCTACCTT-3’; HO-1 forward, 5’-CGTGCAGAGAATTCTGAGTTC-3’ and reverse, 5’-CGGCTGGAATTCTGAGTTC-3’; NQO1 forward, 5’-CGGTACTTGCCTGCCTTTG-3’ and reverse, 5’-AGACGCTTTACGTAGTGCTG-3’; GST forward, 5’-CTCTGCCCCGTGACCGTGAC-3’ and reverse, 5’-ATTTGTTTTGCATCCACCGG-3’; GCLC forward, 5’-CTCTGCCCCGTGACCGTGAC-3’ and reverse, 5’-ATTTGTTTTGCATCCACCGG-3’; and β-actin forward, 5’-CCCATCTATGAGGTTACGC-3’ and reverse, 5’-TTTAATGTCACGCACGATTTC-3’.

Statistical analysis

The data were reported as mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance followed by Bonferroni post-hoc test using Statistical Package for the Social Science (SPSS) software package version 11.5 software (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered to be statistically significant.

Results

Safranal protects rotenone-induced cell death

Treatment with different concentrations of rotenone dose-dependently induced cell death of dopaminergic neurons, with approximately 50% reduction observed at 100 nM (Fig. 1A). Next, we investigated the effect of safranal on 100 nM rotenone-induced apoptosis. The results showed that safranal had no cytotoxicity on dopaminergic neurons (Fig. 1B) but significantly attenuated rotenone-induced cell death in a dose-dependent manner (Fig. 1C). The data suggest that safranal has a protective effect on rotenone-induced cell death in dopaminergic neurons.
is involved in the protective effect of safranal against rotenone-induced neurotoxicity, we detected the expression of Keap1 and Nrf2 in rotenone-induced dopaminergic neurons. The results showed that rotenone treatment significantly upregulated Keap1 expression (Fig. 3A) and decreased the nuclear Nrf2 expression (Fig. 3B). However, the pretreatment with safranal to rotenone markedly decreased the Keap1 expression (Fig. 3A) and upregulated the nuclear Nrf2 expression (Fig. 3B). The results implied that Keap1/Nrf2 signaling may be involved in the protective effect of safranal against rotenone-induced neurotoxicity.

Safranal increases the expression of antioxidant genes downstream of Nrf2 in rotenone-induced dopaminergic neurons

To further confirm the effect of safranal on Keap1/Nrf2 signaling in rotenone-induced dopaminergic neurons, we detected the expression of antioxidant genes downstream of Nrf2. The results showed that the expression of GST (Fig. 4A), GCLc (Fig. 4B), NQO1 (Fig. 4C) and HO-1 (Fig. 4D) decreased by rotenone treatment was significantly reversed by pretreatment with safranal. The results further verify that safranal promotes the antioxidant capability of dopaminergic neurons induced by rotenone.

Knockdown of Nrf2 abrogates the protective effect of safranal

To further investigate whether Keap1/Nrf2 signaling is involved in the protective effect of safranal, we blocked the Keap1/Nrf2 signaling by knockdown of Nrf2 and then detected the protective effect of safranal against rotenone-induced neurotoxicity. The results showed that transfection of Nrf2 siRNA significantly decreased the mRNA (Fig. 5A) and protein (Fig. 5B) expression of Nrf2. The knockdown of Nrf2 significantly abolished the protective effect on safranal against rotenone-induced cell death (Fig. 6A), cell apoptosis (Fig. 6B) and ROS generation (Fig. 6C). These results suggest that safranal protects dopaminergic neurons against rotenone-induced neurotoxicity at least in part through Keap1/Nrf2 signaling.

Discussion

In the present study, we have demonstrated that safranal inhibited rotenone-induced cytotoxicity in dopa-
minergic neurons, suggesting a direct neuroprotective role of safranal against PD. The protective effect of safranal is associated with the activation of Nrf2 signaling that inhibits the oxidative stress and cell apoptosis induced by rotenone. It has been reported that safranal is a safe compound with a very low toxicity in vivo (22). Therefore, safranal may be potentially useful for therapeutic applications of PD.

Saffron has been used as an alternative treatment for many diseases due to its widely pharmacological activities such as antioxidant and anti-apoptotic effects (3). Saffron extract can inhibit chronic stress induced oxidative damage in rat liver, kidneys and brain (23). The extract of saffron inhibited cell viability in lung cancer cell (24), reduced serum inflammatory markers in sensitized guinea pigs (25-26), and decreased human lymphocytes' cytokines (27). Saffron extract also reduces oxidative myocardial damage and attenuates cardiac cell apoptosis, providing a protective effect against ischemia-reperfusion injury (7, 28). The renal ischemia-reperfusion-induced oxidative damage is also alleviated by aqueous saffron extract (29). Smooth muscle relaxant activity of Crocus sativus (saffron) and its constituents were also reported (30). As the main constituent of saffron, safranal also exhibits considerable antioxidant and anti-apoptotic effects. Hosseinzadeh et al. reported that safranal inhibited cerebral ischemia-induced oxidative damage in rat hippocampus (31). Saffran exhibits strong antioxidant and anti-apoptotic potential in cardiac cells suffering ischemia-reperfusion injury (32). In isoproterenol-induced myocardial infarction, treatment with safranal significantly decreased oxidative stress and preserved myocardial function (33). The age-related oxidative stress such as lipid peroxidation and nitric oxide formation are suppressed by safranal in rat liver and brain (8, 34). In diabetic nephropathy, safranal reduces renal dysfunction and damage through its antioxidant and anti-inflammatory activities (35-36). The glucose/serum deprivation-induced cell death can be also prevented by safranal (37). These reports demonstrate that safranal possesses strong antioxidant and anti-apoptotic activities. Considering the oxidative stress associated with neuron apoptosis in PD, safranal may exhibit a protective effect against PD. As expected, we found that safranal significantly inhibited ROS generation and apoptosis in dopaminergic neurons induced by PD, an in vitro model of PD. Saffron has been suggested as an antidote or a protective agent against chemical or natural toxicities (38) in which safranal may play an important role. Our findings as well as these above reports support a useful treatment effect in oxidative stress-related diseases.

The neuroprotective effect of safranal has also been widely studied. Saffron retards retinal degeneration through inhibiting photoreceptor cell degeneration (39-40). The quinolinic acid-induced oxidative damage in rat hippocampus is alleviated by safranal treatment (41). Saffran also has improving effects on crushed-injured sciatic nerve function and attenuates neuropathic pain induced by spinal nerve transaction (42-43). The spinal cord injury induced neuron apoptosis in a rat model of traumatic injury is also prevented by safranal, and safranal inhibited cell apoptosis through inhibiting pro-apoptotic protein Bax and promoting anti-apoptotic protein Bcl-2 (44). In line with these findings, we also found that safranal inhibited dopaminergic neuron apoptosis through regulating Bax and Bcl-2 expression.

The Keap1/Nrf2 signaling pathway plays an important role in age-associated neurodegenerative diseases (12). Nrf2 knockout promotes the nigral degeneration induced by the mitochondrial complex I inhibitor (45-48). The knockout of Nrf2 also increases the susceptibility to rotenone (49). Overexpression of Nrf2 alleviates the PD symptoms in a mouse model (50-51). Additionally, knockdown of Keap1 increases the resistance to rotenone in cortical cultures (52). A recent study demonstrated that the activation of Nrf2 signaling by a novel compound ITC-3 inhibits oxidative stress and abolishes PD-associated motor deficits in an animal model of PD (13). In this study, we found that safranal could inhibit Keap1 expression induced by rotenone and promoted Nrf2 nuclear accumulation that led to transcription of antioxidant gene expression, by which safranal alleviated rotenone-induced neurotoxicity in dopaminergic neurons. Saffranal has been reported to have the potential to inhibit Keap1 and activate Nrf2 signaling against oxidative stress (53).

Taken together, our study for the first time demonstrated a protective effect of safranal that suppressed oxidative stress and apoptosis in dopaminergic neurons induced by rotenone. Our data indicate that safranal may serve as a potent therapeutic drug for the treatment of PD. However, these results were obtained using an in vitro cellular model of PD. Further investigation is warranted to verify the effect of safranal in animal and clinical models of PD.
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