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Original article

Protective and therapeutic activity of honokiol in reversing motor deficits and neuronal degeneration in the mouse model of Parkinson's disease

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ABSTRACT

Background: Parkinson's disease (PD) is a progressive and profound movement disorder resulting from neurodegeneration in the nigrostriatal dopaminergic system, but current treatment neither cures nor stops PD from advancing. Based on the ability to suppress oxidative stress, excitotoxicity, and neuroinflammation, the potential of honokiol as a novel neuroprotective agent for PD treatment was determined.

Methods: The hemi-parkinsonian model was used to investigate the protective and therapeutic effects of honokiol on motor dysfunctions and dopaminergic neurodegeneration in mice, with a single unilateral striatal injection of 6-hydroxydopamine (6-OHDA).

Results: One day after 6-OHDA-induced lesion, the mice exhibited spontaneous ipsilateral turning, motor imbalance, and incoordination which were mild with a single administration of honokiol prior to 6-OHDA injection. Thereafter, honokiol was continually applied daily for 14 days, which ameliorated apomorphine-induced contralateral rotation and reduced the loss of tyrosine hydroxylase-immunoreactive (TH-ir) fibers in the lesioned striatum. In addition, honokiol posttreatment, beginning on day 8 after 6-OHDA lesion, for 14 days efficiently rescued motor deficits and recovered the TH-ir neuronal loss in both the lesioned striatum and the ipsilateral substantia nigra. The 6-OHDA-induced increases in nigrostriatal expression of inducible nitric oxide synthase (iNOS) and decreases in that of nNOS were also reversed by honokiol posttreatment.

Conclusions: These findings revealed that honokiol has both protective and therapeutic effects on motor impairments and dopaminergic progressive damage, at least in part through modulation of NOS signaling, in 6-OHDA-lesioned mice. Honokiol may represent a potential therapeutic candidate for the management of motor symptoms and neurodegeneration in PD.

Introduction

Parkinson's disease (PD), the most common adult-onset movement disorder, is characterized by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta, resulting in the consequent loss of their projection fibers in striatum. The hallmark features of PD exhibit motor impairments, including bradykinesia, tremor, rigidity, and postural instability. Although the etiology and pathogenesis of PD remain unclear, various environmental and genetic factors have been widely considered to influence the susceptibility to PD [1]. Multiple epidemiological and experimental studies have demonstrated that environmental toxicants such as herbicides, pesticides, solvents, metals, and neurotoxicants may elicit the progressive loss of specific neuronal cells, thereby leading to neurodegeneration and PD [1,2].

Current PD medications rely heavily on dopamine (DA) replacement agents [3]. Although such therapies are efficacious in improving the motor symptoms and quality of life for patients during the early stages of PD, long-term use of these agents cannot halt disease progression and often causes side effects, including unwanted dyskinesia, psychotic symptoms, and mood

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disturbances [4]. Most current therapies could only partially alleviate the pathological symptoms, but fail to inhibit the neurodegenerative progression.

Traditional medicine has been prescribed centuries to treat movement disorders, such as head shaking or hand trembling, that mimic the symptoms of PD. Some herbal medicines are indicated to improve the motor or non-motor symptoms in PD and delay PD progression [5,6]. A herbal medicine Anchanling, mainly containing bioactive compound magnolol, has been observed to attenuate PD pathology [7]. Magnolol and its isomer honokiol are the main active biphenol ingredients in various *Magnolia* species which are widely used in traditional medicine to treat various neurological disorders, *e.g.* anxiety and nervous tension [8]. Our previous studies have shown that honokiol and magnolol could prevent neuronal cell death through attenuation of oxidative stress, excitotoxicity, neuronal inflammation, and glial activation in cell culture and *in vivo* [9,10]. Moreover, subchronic magnolol treatment protected neuronal damage and improved behavioral deficits in PD mouse model [11]. In fact, honokiol exhibits neuroprotective activity against neurotoxin- and oxidant-induced cell death and is more effective than magnolol on neuroprotection against neurotoxin-induced neurotoxicity [8,12]. Thus, honokiol may have high neuroprotective activity and be considered as a promising candidate for PD therapy. The present study determined whether honokiol exhibits the preventive and therapeutic effects on PD in hemi-parkinsonian mouse model with unilateral 6-OHDA-induced lesions of striatal DA neurons. After the beneficial effects of subchronic honokiol treatment (starting 30 min prior to the unilateral striatal 6-OHDA injection for 14 days) on motor deficits and neurotoxicity was observed, a posttreatment paradigm was used to assess if honokiol could promote motor and neuronal recovery. As high levels of inducible/neuronal nitric oxide synthase (i/nNOS) were found in substantia nigra (SN) of patients and animal models of PD [13,14], the effects of honokiol on NOS expression were also delineated.

Materials and methods

Materials and chemicals

Tyrosine hydroxylase (TH), iNOS, and nNOS antibodies were purchased from Novus Biologicals, Novus Biologicals, Inc. (Littleton, Colo., USA) Sodium chloride, sucrose, and Tris Base were purchased from J.T. Baker (Mallinckrodt, Kent., USA). Paraformaldehyde and hydrogen peroxide were purchased from Riedel-de Haen. Other chemicals were obtained from Sigma. Honokiol was synthesized by our research team as previously described [12].

Animal surgical procedure

Male NMRI mice (10–11 weeks, 35–45 g) were supplied from the Laboratory Animal Center of Tzu Chi University (Hualien, Taiwan), housed 4–5 animals per cage with a light/dark cycle of 12/12 h, and had free access to food and water in their home cages at room temperature (25 ± 2 °C). All experiments were carried out in accordance with Guide for the Care and Use of Laboratory Animals and with approval from the Review Committee of the Tzu Chi University for the use of animal (No. 99039).

The surgical procedure was adopted with some modifications as previously described [11]. Mice were initially anesthetized with a mixture of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively, *ip*) and then received a single injection of 6-OHDA (15 μg/3 μl, containing 0.2% ascorbic acid) into the right striatum to achieve unilateral striatal lesions. The sham mice were injected with an equivalent volume of saline with 0.2% ascorbic acid. The lesion was performed using a Hamilton syringe at the following coordinates: AP: −0.9 mm; ML: −1.9 mm; DV: −2.2 mm with respect to bregma.

Treatment protocols

Experiment I: subchronic honokiol treatment, starting 30 min prior to the unilateral striatal 6-OHDA injection, for 14 days.

To detect the protective activity of honokiol on neurotoxicity, mice were pretreated with honokiol (5 mg/kg, *ip*) or corn oil (1 ml/kg) 30 min prior to surgical incision and unilateral striatal 6-OHDA injection. Twenty-four hours after lesions, the cylinder turning test and beam walking test were conducted. The mice were then consecutively received honokiol or corn oil daily for 14 days. The changes in motor behaviors and neuronal activity in nigrostriatal DA system of the hemi-parkinsonian mice were determined.

Experiment II: honokiol posttreatment, starting on the 8th days after unilateral striatal 6-OHDA lesion, for 14 days.

The eventual loss of nigrostriatal DA neurons became significant one week after intrastriatal 6-OHDA lesion in mice [15]. In order to clarify the possible therapeutic effect of honokiol on progressive neurodegeneration, honokiol (0, 1, and 5 mg/kg, *ip*) were consecutively administrated to the mice with severe motor deficits at day 7 daily for 14 days, starting on 8th days after the unilateral striatal 6-OHDA lesion. The changes in motor behaviors, neuronal activity in nigrostriatal DA system and NOS expression were determined.

Behavioral studies

The following behavioral tests were conducted before and/or at different time points after 6-OHDA lesion over a period of 3 weeks. An investigator who was blinded to the group and the results of other behavioral tests analyzed all video recordings.

The cylinder turning test

The measurement of spontaneous turning behavior in hemi-parkinsonian mice was modified based on previously established protocol [16]. In cylinder turning test, mice were placed individually into the center of a transparent plastic column (20 cm diameter: 32 cm height) and recorded with a video camera to evaluate asymmetry locomotion. The spontaneous turning preference in mice with unilateral striatal damage induced by 6-OHDA was monitored. The direction for animal movement included ipsilateral turning (toward the lesion side) and contralateral turning (away from the lesion side).

Beam walking test

The assessment of motor balance and coordination in hemi-parkinsonia mice was modified based on previously established protocol [16]. Before surgery and drug treatment, the mice were trained to traverse a metallic round beam with 12 mm diameter elevated 50 cm above the bench in three consecutive trials each day for three days. The total length of the beam was 60 cm and placed on a table and ended directly into the animal's goal cage. Prior to testing, mice were habituated in the room for 30 min. The home cage for each mouse was used as the escape box to increase the motivation of mouse to traverse the beam. Then the mice that successfully walked along the beam were randomly separated into different experimental groups. Under testing, the distance as mouse walked along the beam was recorded, with a maximum time (cut-off time) of 20 s allowed.

Apomorphine-induced rotational behavior

One to three weeks after the surgery, apomorphine-induced animal rotational behaviors were performed to identify the motor function in 6-OHDA-induced unilateral striatal lesions as

previously described [11]. Initially, mice were allowed to habituate to their environment for 30 min before rotation test. Then mice were injected with apomorphine (0.5 mg/kg, *ip*) and placed in individual glass bowls attached to an automatic rotameter system (TSE, Bad Homburg, Germany). Each circular movement exceeding 30^0 was registered. The contralateral movements and ipsilateral were counted for 60 min. Results were expressed as the total numbers of ipsilateral net turns minus contralateral net turns for 60 min.

Immunohistochemistry

The mouse brain sections were stained to visualize the cells that were positive for TH, and iNOS/nNOS as previously described [11]. After completion of the apomorphine-induced rotational tests, the mice from the sham (corn oil + vehicle), 6-OHDA-lesioned, and honokiol treated groups were anaesthetized with ketamine/xylazine and transcardially perfused with heparin solution (0.05% heparin in 0.1 M phosphate buffered saline, PBS), and subsequently followed by ice cold fixative (4% paraformaldehyde). The brains were removed, postfixed, and then dehydrated in 30% sucrose. Coronal sections (25 micrometer thickness) were cut on a freezing microtome, transferred to slides, and immersed in 0.1 M PBS. After a preincubation with 0.5% normal goat serum/0.2% Triton in PBS, the sections were incubated with the primary antibody (1:500 for TH; 1:100 for iNOS/nNOS) for 16–18 h at 4 °C. To develop the immunoperoxidase color interaction, the sections were incubated with a biotinylated secondary antibody to goat anti-rabbit IgG for 1 h, rinsed again three times, and stained with 0.05% diaminobenzidine/0.03% H_2O_2 in PBS for 5 min. A negative control without primary antibody was performed in each experiment.

For quantitative examination of TH positive fibers or iNOS/nNOS positive cells, coronal sections in the striatum and SN were analyzed and photographed. The images in the region of interest (ROI) were captured using a CCD camera mounting on Nikon Eclipse E800 microscope. To determine the DA fibers in striatum and SN of both hemisphere, the mean optical density (OD) was measured and quantified from the TH-ir stained sections. The damage was calculated as the ratio of the TH-ir positive area in the lesioned side divided by the TH-ir positive area of the contralateral intact side. The number of iNOS/nNOS positive cells was counted in the ipsilateral hemispheres of the stereotaxic ROI in each striatal and SN sections. For each animal, three consecutive sections were counted and the results were pooled to obtain a total mean cell count. Four to five animals/group were used, and cells in striatum and SN on lesioned side were counted in a 0.5 mm^2 counting grid. To correct for variability in lighting conditions, all images were photographed under identical conditions. All DA fiber density and iNOS/nNOS positive cells were quantified using Image-J software (National Institute of Health).

Data analysis

The statistical test and number of animals used in each experimental are indicated in the Results and each figure legend. Most data are expressed as mean ± SEM. Statistical significance of difference between groups was determined by two-way ANOVA followed by Bonferroni *post-hoc* analysis. A p value of less than 0.05 was considered statistically significant.

Results

Experiment I

A single dose pretreatment of honokiol prevented motor deficit in the cylinder turning test and beam walking test in 6-OHDA-lesioned mice

The cylinder turning test and beam walking test were conducted to identify the motor deficits in unilateral striatal lesions induced by 6-OHDA. In acute pretreatment condition,

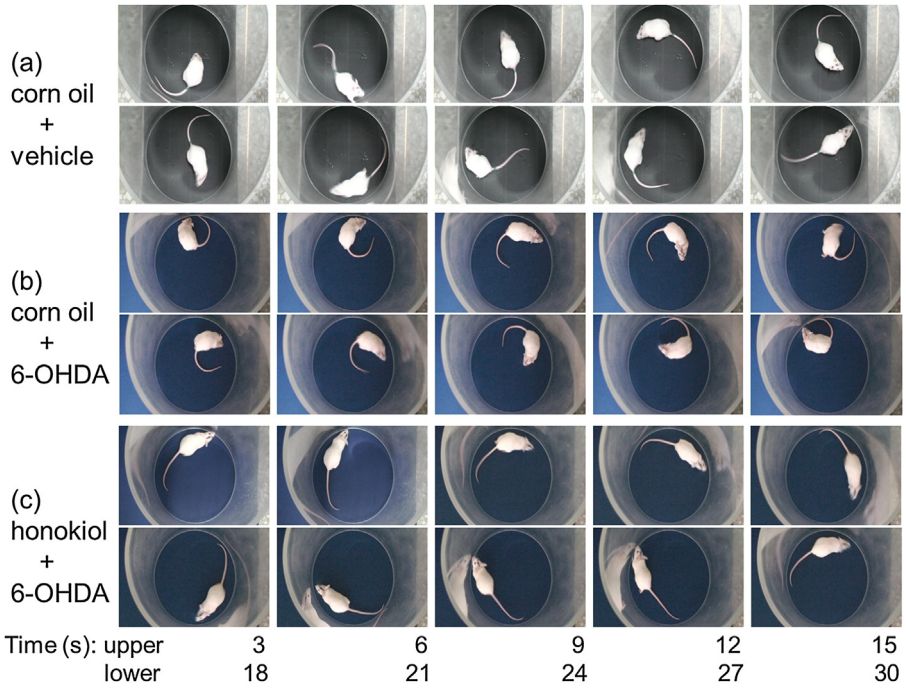

Fig. 1. Pretreatment with honokiol protected the rotatory behaviors at 24 h after unilateral striatal 6-OHDA lesion in mice. The locomotor pattern was recorded in an open-field arena every 3 s for 30 s. The sham-operated mice treated with corn oil + vehicle executed a random walk in a cylinder turning test (a). The behavior of unilateral 6-OHDA-lesioned mice showed only ipsilateral circling turning in a diameter of 6 ± 2 cm (b). The behavior of mice pretreated with honokiol (5 mg/kg) 30 min prior to 6-OHDA lesion showed ipsilateral circling turning in a diameter of 18 ± 2 cm (c). Data are expressed as mean ± SEM from 4 animals (n = 4).

mouse was given a single administration of honokiol (5 mg/kg) or corn oil at 30 min before intrastriatal 6-OHDA injection into the right striatum. On the next day the cylinder turning test was performed. Results showed that mice in corn oil + vehicle group randomly walked around in open field without direction preference (Fig. 1a). The unilateral 6-OHDA-injected mice exhibited the spontaneous ipsilateral rotatory behavior (towards the lesioned side), with ipsilateral circling in a diameter of 6 ± 2 cm (Fig. 1b). The mice lesioned by 6-OHDA with honokiol pretreatment had little disturbance of motor function and the diameter of rotatory behavior with ipsilateral circling was 18 ± 2 cm (Fig. 1c). The mice in honokiol + vehicle group walked and moved normally (data not shown).

The beam walking assay was performed after the cylinder turning test. This task intended the mouse to stay upright and travel across an elevated narrow beam to a safe platform. As shown in Fig. 2A, the mice in corn oil + vehicle group were able to travel the full length (60 cm) of the beam in less than 5 s. The 6-OHDA-lesioned mice, with significant bradykinesia and hind-limb fault, fell off the beam. However, the mice pretreated with honokiol 30 min prior to 6-OHDA lesion could tardily travel ~60% of the beam length.

One day after 6-OHDA-lesion, mice exhibited total walking distance of 3 ± 2 cm, while the mice in corn oil + vehicle group completely traversed the tested distance of 60 cm (Fig. 2B). The walking distance of mice with honokiol pretreatment was 30 ± 3 cm after 1 day 6-OHDA-lesion. Honokiol itself did not influence the walking ability of mice. These data indicated that the 6-OHDA-lesioned mice exhibited the pronounced asymmetry locomotion and a reduced capacity to remain on the narrow beam and travel across. Acute honokiol pretreatment significantly reduced the motor dysfunction of 6-OHDA-lesioned mice.

Effect of subchronic honokiol treatment with one single dose pretreatment on motor deficit and neuronal loss induced by 6-OHDA

Mice were treated daily with honokiol for two weeks, starting at 30 min before 6-OHDA injection. The beam walking test was examined again 7 days after 6-OHDA injection. The performance of individuals in each group was similar to that observed 1 day after 6-OHDA injection (Fig. 2C).

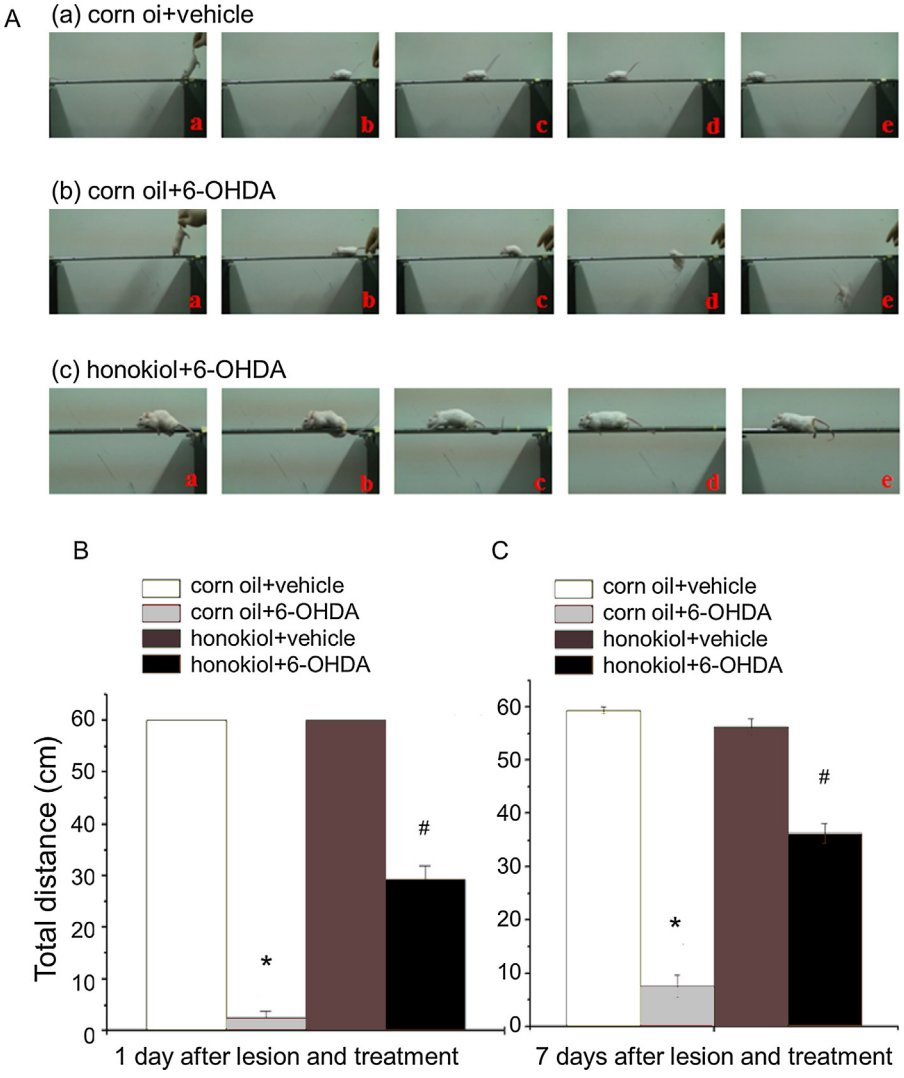

Fig. 2. Effects of honokiol pretreatment on beam walking test in unilateral striatal 6-OHDA lesioned mice. A single dose of honokiol (5 mg/kg, *ip*) was administrated of 30 min prior to unilateral 6-OHDA injection into the right striatum of mice. (A) Photographs showing the beam walking test 24 h after mice under unilateral striatal lesion and treatment with corn oil + vehicle (a), corn oil + 6-OHDA (b), and honokiol + 6-OHDA (c). Analysis of the total distance that mice traveled in a narrow beam (B) 1 day and (C) 7 days after unilateral striatal lesion. Data are expressed as mean ± SEM from 8 animals (n = 8). *$p < 0.05$ compared with corn oil + vehicle group; #$p < 0.05$ compared with corn oil + 6-OHDA group (two-way ANOVA followed by Bonferroni *post-hoc* analysis).

Two weeks after 6-OHDA-lesion, the apomorphine-induced rotational behavior was monitored, followed by the pathological investigation to assess the extent of neuronal damage. Results demonstrated that apomorphine induced a marked and significant contralateral rotation in 6-OHDA-injected mice at day 15 (Fig. 3A). Importantly, when mice received honokiol treatment prior to 6-OHDA lesion and continued daily for 14 consecutive days, the number of contralateral turns induced by apomorphine was significantly reduced (Fig. 3A).

To verify lesions and evaluate whether the extent of apomorphine-induced contralateral rotation was predictive of

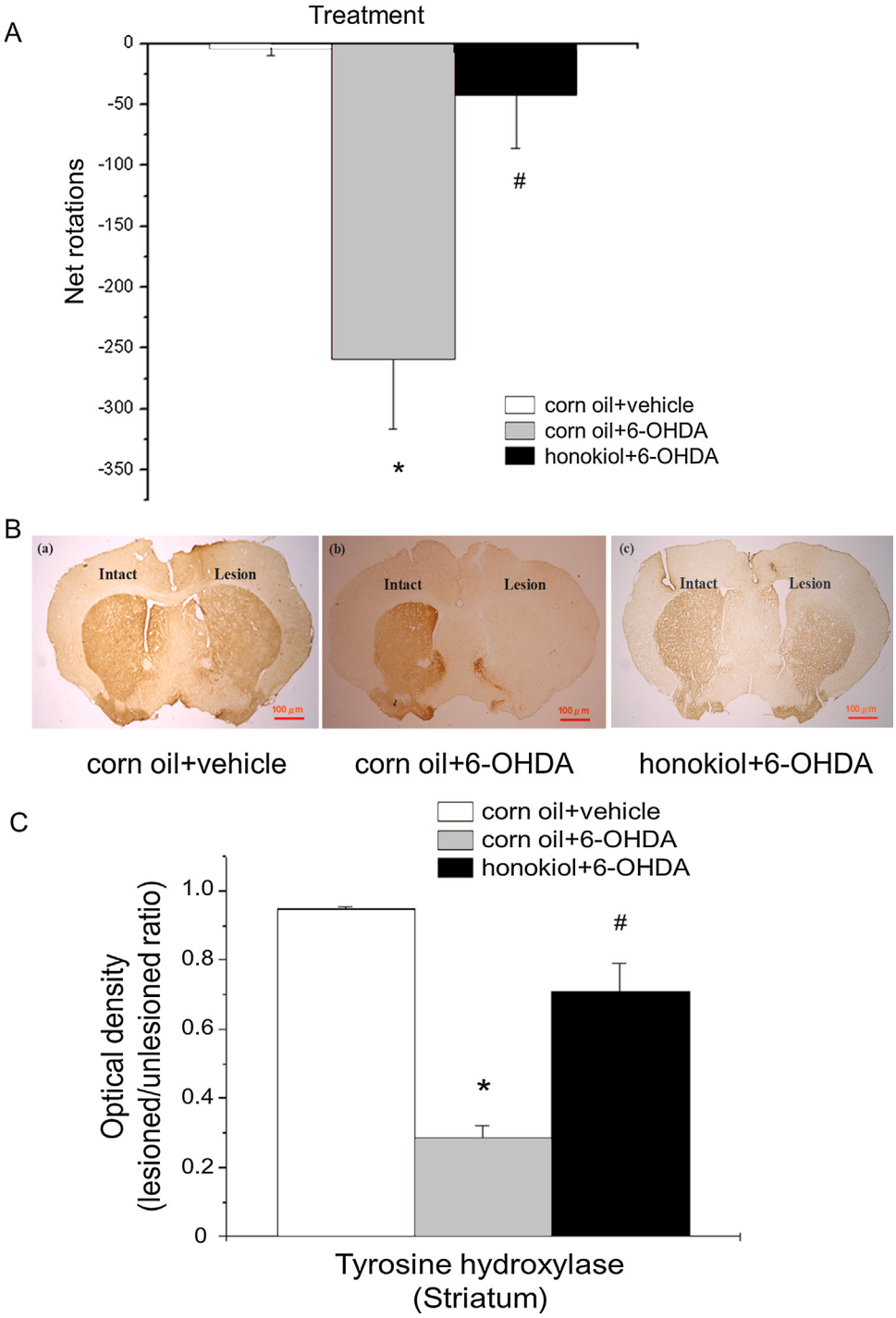

Fig. 3. Effects of subchronic honokiol treatment with a single pretreatment on rotational behavior and striatal TH-ir expression in unilateral striatal 6-OHDA lesioned mice. The mice were administrated with honokiol (5 mg/kg, *ip*) 30 min before 6-OHDA-induced unilateral striatal lesion and subsequently applied daily for 14 days. (A) Histogram showing the number of contralateral rotations in mice treated with corn oil + vehicle, corn oil + 6-OHDA, and honokiol + 6-OHDA. The mice were challenged with apomorphine to induce the rotational behaviors which were counted for 60 min. Data are expressed as mean ± SEM from 8 animals (n = 8). $^{*}p < 0.05$ compared with corn oil + vehicle group; $\#p < 0.05$ compared with corn oil + 6-OHDA group (two-way ANOVA followed by Bonferroni *post-hoc* analysis). (B) Representative photomicrographs illustrate the striatal TH-ir fibers in mice treated with vehicle (a), corn oil + 6-OHDA (b), and honokiol + 6-OHDA (c). Scale bar represents a length of 0.1 mm. (C) Summary data are expressed as mean ± SEM from 4 animals (n = 4). $^{*}p < 0.05$ compared with corn oil + vehicle group; $\#p < 0.05$ compared with corn oil + 6-OHDA group (two-way ANOVA followed by Bonferroni *post-hoc* analysis).

DA degeneration, TH-ir protein expression was analyzed in both intact and lesioned striatum by immunohistochemistry staining. A conspicuous reduction in TH-ir fiber density was observed in the lesioned striatum compared with the intact site and corn oil + vehicle group (Fig. 3B). There was no difference in TH-ir staining in the contralateral striatum between lesioned and sham operated mice. The reduced TH-ir protein expression in lesioned striatum was significantly recovered by honokiol treatment (Fig. 3B and C). These data demonstrated that mice with more severe DA loss tended to engage in more motor dysfunction. Subchronic honokiol treatment, starting 30 min prior to the unilateral striatal 6-OHDA injection, for 14 days reduced the extent of striatal DA loss and attenuated the elevation of rotational behaviors induced by apomorphine in unilateral striatal 6-OHDA-lesioned mice.

Experiment II

Honokiol posttreatment improved motor deficit and rescued neuronal loss in 6-OHDA lesioned mice

To determine the therapeutic potential of honokiol on motor impairment and neurotoxicity after severe unilateral DA denervation, only mice exhibiting vigorous motor deficits with more than 120 contralateral rotations induced by apomorphine when tested 7 days after 6-OHDA lesion were applied in the following tests. Mice meeting this criterion tended to maintain a strong contralateral preference, suggesting sufficient lesions (Fig. 4A).

These lesioned mice were administrated daily with honokiol (1 and 5 mg/kg) for 14 consecutive days (subchronic posttreatment), starting on day 8 after unilateral 6-OHDA injection into striatum. The apomorphine-induced rotational test was performed again

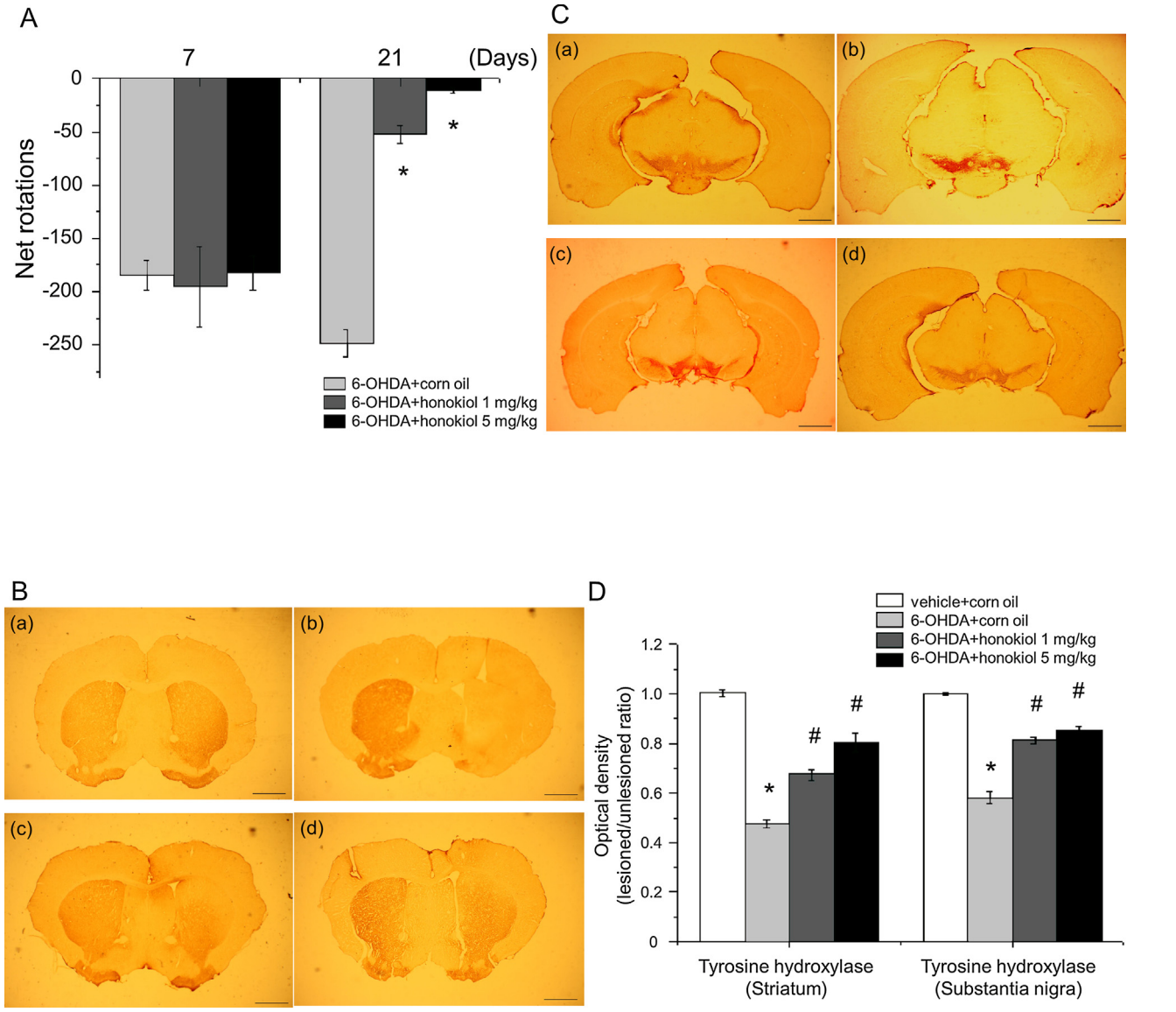

Fig. 4. Effects of subchronic honokiol post-treatment on rotational behavior and nigrostriatal TH-ir expression in 6-OHDA lesioned mice. Honokiol was administrated on day 8 after 6-OHDA-induced unilateral lesion in striatum and continually applied daily for 14 days. (A) Seven and 21 days after 6-OHDA injection, the contralateral rotational behaviors induced by apomorphine challenge were detected. Values are mean ± SEM from 8 animals (n = 8). *$p < 0.05$ compared with 6-OHDA + corn oil groups. Photomicrographs illustrate the experimental groups from the (B) striatum and (C) SN: vehicle + corn oil (a), 6-OHDA + corn oil (b), 6-OHDA + honokiol (1 mg/kg, c), and 6-OHDA + honokiol (5 mg/kg, d). (D) Quantification of immunohistochemical staining TH expression from the striatum and SN in 6-OHDA lesioned mice. Values are mean ± SEM from 4 to 5 animals (n = 4-5). *$p < 0.05$ compared with vehicle + corn oil groups; #$p < 0.05$ compared with 6-OHDA + corn oil groups (two-way ANOVA followed by Bonferroni *post-hoc* analysis).

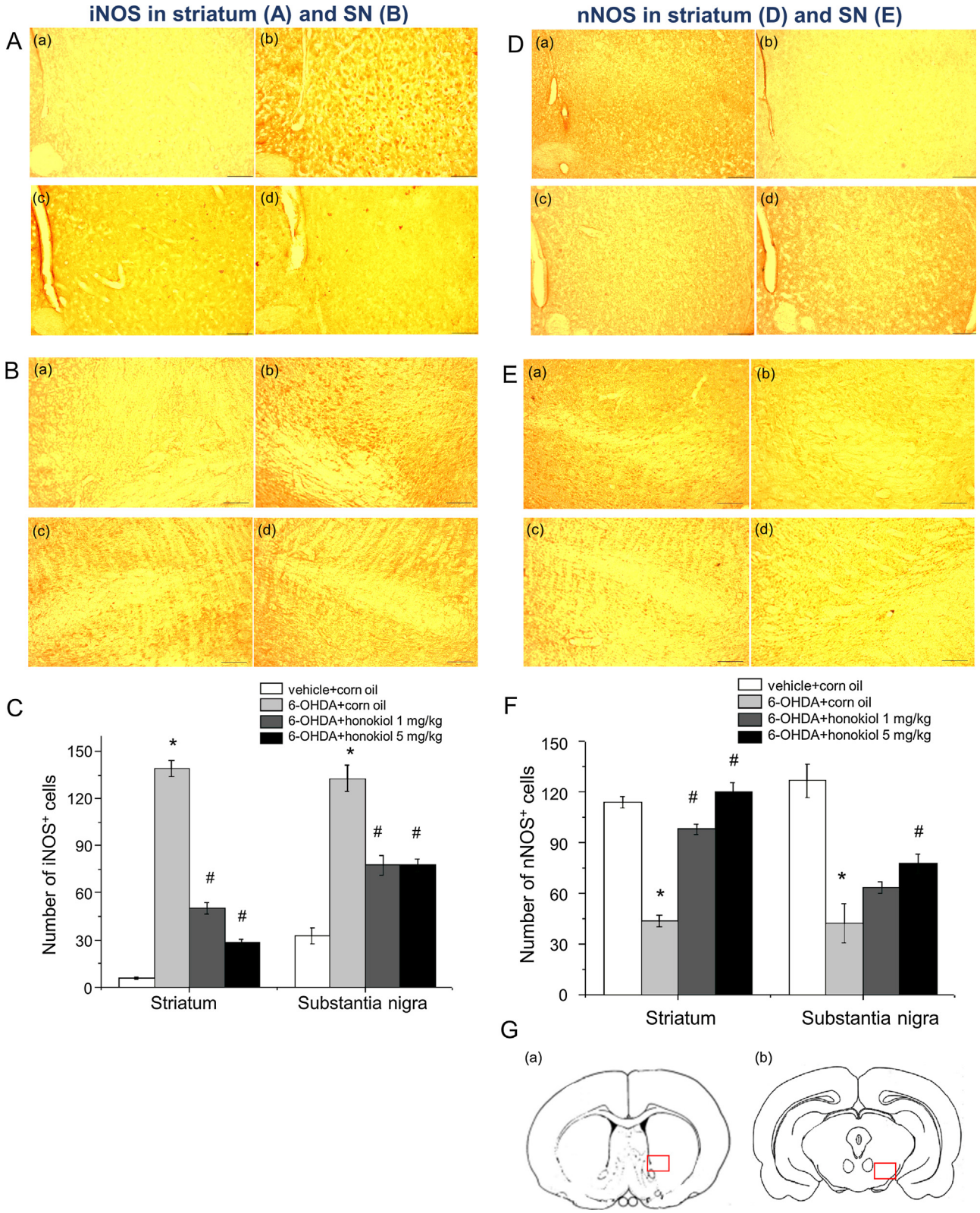

Fig. 5. Effects of subchronic post-treatment with honokiol on the nigrostriatal iNOS and nNOS expression in 6-OHDA lesioned mice. Honokiol was given on day 8 after 6-OHDA-induced unilateral lesion and continually applied daily for 14 days. Photomicrographs illustrate the experimental groups: vehicle + corn oil (a), 6-OHDA + corn oil (b), 6-OHDA + honokiol (1 mg/kg, c), and 6-OHDA + honokiol (5 mg/kg, d). The (A and B) iNOS and (D and E) nNOS positive cells were present in 6-OHDA-lesioned (A and D) striatum and ipsilateral (B and E) SN. Scale bar 20 μm. The bar graph illustrates the immunohistochemical staining (C) iNOS and (F) nNOS expression from the striatum and SN in 6-OHDA-lesioned mice. Values are mean ± SEM from 4 to 5 animals (n = 4-5). $^{*}p < 0.05$ compared with vehicle + corn oil groups; $\#p < 0.05$ compared with 6-OHDA + corn oil groups (two-way ANOVA followed by Bonferroni *post-hoc* analysis). (G) Scheme of coronal sections of the striatum (a) and SN (b). The squares represent the sampled fields for stereological measurements of the iNOS/nNOS immunoreactive profiles in the striatum and SN.

24 h after the last honokiol administration (21 days after 6-OHDA injection). Honokiol (1 and 5 mg/kg) posttreatment improved the contralateral rotation behaviors induced by apomorphine in hemi-parkinsonian mice (Fig. 4A).

Neuropathological examination revealed that TH-ir fibers tremendously decreased in the lesioned striatum and ipsilateral SN, compared with intact site and sham (vehicle + corn oil) group. However, posttreatment with honokiol for 14 days significantly recovered the 6-OHDA-induced neuronal loss in unilateral striatal (Fig. 4B) and SN (Fig. 4C) neurons. There was no significant difference in TH-ir in the contralateral striatum and SN among all the tested groups.

Distinct effects of honokiol on iNOS and nNOS expression in 6-OHDA lesioned mice

Immunohistochemistry analysis showed an increased number of iNOS but reduced nNOS positive cells in nigrostriatum lesioned by 6-OHDA, compared to those in vehicle + corn oil treated mice (Fig. 5). Honokiol (1 and 5 mg/kg) post-treatment for 14 days significantly reversed the tremendous increases in iNOS (Fig. 5C) and decreases in nNOS (Fig. 5F) protein expression induced by 6-OHDA. These results demonstrated that posttreatment with honokiol could ameliorate 6-OHDA-elicited neurotoxicity, at least in part, through the NOS signaling.

Discussion

The current pharmacological treatment against PD remains limited to symptomatic relieving and neuroprotective effects. It appears that these available agents cannot stop the progression of PD or facilitate the recovery of dopamine neurons. The present study investigated the protective and therapeutic effects of honokiol on unilateral striatal 6-OHDA-lesioned mouse PD model by means of motor functions and histopathological staining. Our results demonstrate that honokiol treatment, starting 30 min prior to 6-OHDA injection, for 14 days significantly prevented motor deficits and reduced progressive loss of nigrostriatal DA neurons. Moreover, honokiol posttreatment, beginning on day 8 after 6-OHDA lesion, for 14 days rescued motor deficits and recovered the TH-ir neuronal loss in lesioned striatum and ipsilateral SN. These results support that honokiol may possess both preventive and therapeutic potential for treatment of motor dysfunctions and neurodegeneration in PD.

Most frequently, 6-OHDA represents one of the most common neurotoxins utilized to degenerate the DA neurons, particularly the nigrostriatal system, in the rodent models. It results in behavioral and pathological signs in animals similar to those of observed in PD patients [17]. The mouse PD model has been crucially applied in developing therapeutic strategies to relieve the motor symptoms of PD, since 6-OHDA causes nigrostriatal DA neurodegeneration as it is directly administrated into striatum or SN [18,19]. Consistent with previous reports [19,20], the mice injected with 6-OHDA into unilateral striatum exhibited motor abnormalities. Following the delivery of 6-OHDA into striatum, the spontaneous ipsilateral turning and motor imbalance and incoordination were observed on the first day, which could result from the acute toxicity of 6-OHDA leading to impairment of striatal DA neurons. It also indicated that the striatal DA neuronal damage occurred immediately in this 6-OHDA-lesioned mouse [15]. The reduction in motor activities of 6-OHDA-lesioned mice could be prevented by honokiol pretreatment, suggesting that honokiol might exert the neuroprotective action in the impaired DA system. On days 7 and 21, hemi-parkinsonian mice represent a tremendous increase in apomorphine-induced rotation. Indeed, this rotational behavior is an outcome of nigrostriatal DA neuronal loss and maximal DA depletion. Moreover, honokiol posttreatment for 2 weeks significantly attenuated apomorphine-induced rotation. Accordingly, it suggests that honokiol is capable of protecting and reversing the motor dysfunction in nigrostriatum lesioned by 6-OHDA. Thus, the far-reaching protective and therapeutic effects of honokiol may be potential useful in treatment of PD and other neurodegenerative disorders.

TH is a rate-limiting enzyme for the biosynthesis of dopamine and usually applied as the biomarker for detecting the integrity of DA neurons in PD [21]. Numerous findings have demonstrated that the decreased TH in midbrain usually accompanies the occurrence of motor complications and neurodegeneration in PD. Accumulating evidence further reveals that the intrastriatal 6-OHDA administration could provoke the reduction of TH-ir cells, resulting in nigrostriatal DA neuronal death and dysfunction [18,22]. The eventual loss of nigrostriatal DA neurons became significant one week after intrastriatal 6-OHDA lesion in mice [15]. Furthermore, striatal injection of 6-OHDA induced a progressive and sustained retrograde degeneration of DA neurons in the ipsilateral SN that should require 1–3 weeks to accomplish [23,24]. It has been revealed that one week after the unilateral striatal 6-OHDA lesion, apomorphine-induced contralateral rotation behavior increased linearly with greater loss of TH-ir expression in striatum. At the third week post-lesioning by 6-OHDA, there was more serious loss of TH-ir fiber, reflecting the long-term damage of DA neurons [25]. In agreement with these previous reports, our results also showed that the nigrostriatal TH-ir were enormously reduced in the ipsilateral side lesioned by 6-OHDA. Moreover, the reduction in TH expression was restored by both honokiol treatment paradigms. Honokiol could recover the expression of PD biomarker TH-ir in nigrostriatal DA neurons, suggesting that this compound is potentially useful for counteracting neuronal progressive damage to achieve the functional behavioral outcomes.

NO, derived from iNOS and/or nNOS, has been recognized as the key pathological factor in the inflammatory processes and has been linked to neuronal death in PD patients and parkinsonism animal models [13,26]. The NO-NOS signaling pathway in nigrostriatum is considered as a new target for PD treatment. However, it is still debating the beneficial or detrimental effects and the relative roles of iNOS, highly expressed in activated glial cells, and constitutive nNOS, present in intrinsic nigrostriatal neurons, under pathological conditions in CNS [27]. Several *in vivo* and *in vitro* studies have demonstrated that the neuronal toxic effects of 6-OHDA result from NO overproduction *via* up-regulation of iNOS and nNOS expression [28,29]. The increases in iNOS and nNOS activity inversely correlated with DA neuronal loss in the 6-OHDA-induced PD models [30]. However, our results showed that 6-OHDA exposure reduced the nNOS expression in lesioned striatum and the ipsilateral SN. In line with our findings, it has been reported that the degeneration of nigrostriatal DA neurons induced by unilateral 6-OHDA injection into MFB leads to the decreased expression of nNOS in the ipsilateral SN [31]. Thus, nNOS is suggested to play a dual role in DA neuronal survival. As nNOS is produced endogenously, it exhibits protective activity against neurotoxicity. Inversely, nNOS may turn into neurotoxic to cells when it is overexpressed.

One of main findings in this study is that honokiol posttreatment reversed the enhanced iNOS and decreased nNOS expression induced by 6-OHDA in nigrostriatum. It appears that distinct expressions of iNOS and nNOS in PD mice and in response to honokiol treatment correlated inversely with the severity of neurotoxicity induced by 6-OHDA, suggesting that iNOS and nNOS under NO signaling might act oppositely. Thus, selective enhancement of nNOS expression may positively contribute to the restorative action of honokiol on neuronal survival and functional recovery. Moreover, inhibition of the increased iNOS expression by honokiol has the beneficial effects on the inflammatory

neurotoxicity and motor dysfunction in 6-OHDA-lesioned PD mice. Actually, we have observed that honokiol had a potent anti-inflammatory activity by inhibiting NO production and iNOS upregulation induced by cytokine in BV2 microglial cells [10]. Thus, targeting the ongoing neuroinflammation may be one of the pharmacological actions of honokiol to delay or retard the progressive neurodegeneration. It is further considered that the nitrative and inflammatory products released from the DA neurons lesioned by 6-OHDA may activate glial cells. Therefore, attenuation of glial activation may be an alternative mechanism by which honokiol exhibits its therapeutic action in PD. Although honokiol is proposed to act at multiple targets, the precise mechanisms underlying its neuroprotective effects and improvement of the progressive neurodegeneration remain unclear. Further investigation is required to reveal its pharmacological mechanisms and efficacy for the treatment of motor symptoms and neurodegeneration in PD.

Conclusions

The present study demonstrated that honokiol treatment exhibited preventive and therapeutic actions in restoration of DA degeneration as well as motor dysfunctions, at least in part through the NOS signaling pathway, in a PD mouse model. Accordingly, honokiol may be a potentially valuable neuroprotective and therapeutic agent to improve the pathological conditions and behavioral deficits for PD treatment.

Author contributions

HH Chen and MH Chan designed research; PC Chang performed research; C Chen contributed the synthetic honokiol; HH Chen and MH Chan analyzed data and wrote the paper. The authors declare that there is no conflict of interests regarding the publication of this paper.

Conflict of interest statement

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