

β -Lapachone ameliorates L-DOPA-induced dyskinesia in a 6-OHDA-induced mouse model of Parkinson's disease

YOUNG-KYOUNG RYU^{1,2}, HYE-YEON PARK^{1,3}, JUN GO¹, IN-BOK LEE¹,
YOUNG-KEUN CHOI¹, CHUL-HO LEE^{1,4} and KYOUNG-SHIM KIM¹

¹Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141;

²Department of Biological Sciences, College of Biosciences and Biotechnology, Chung-Nam National University, Daejeon 34134; ³Department of Brain and Cognitive Sciences, Ewha Womans University, Seoul 03760;

⁴Department of Functional Genomics, University of Science and Technology, Daejeon 34113, Republic of Korea

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Abstract. The dopamine precursor 3,4-dihydroxyphenyl-L-alanine (L-DOPA) is the most widely used symptomatic treatment for Parkinson's disease (PD); however, its prolonged use is associated with L-DOPA-induced dyskinesia in more than half of patients after 10 years of treatment. The present study investigated whether co-treatment with β -Lapachone, a natural compound, and L-DOPA has protective effects in a 6-hydroxydopamine (6-OHDA)-induced mouse model of PD. Unilateral 6-OHDA-lesioned mice were treated with vehicle or β -Lapachone (10 mg/kg/day) and L-DOPA for 11 days. Abnormal involuntary movements (AIMs) were scored on days 5 and 10. β -Lapachone (10 mg/kg) co-treatment with L-DOPA decreased the AIMs score on both days 5 and 10. β -Lapachone was demonstrated to have a beneficial effect on the axial and limb AIMs scores on day 10. There was no significant suppression in dopamine D1 receptor-related and ERK1/2 signaling in the DA-denervated striatum by β -Lapachone-cotreatment with L-DOPA. Notably, β -Lapachone-cotreatment with L-DOPA increased phosphorylation at the Ser9 site of glycogen synthase kinase 3 β (GSK-3 β), indicating suppression of GSK-3 β activity in both the unlesioned and 6-OHDA-lesioned striata. In addition, astrocyte activation was markedly suppressed by β -Lapachone-cotreatment with L-DOPA in the striatum and substantia nigra of the unilateral 6-OHDA model. These findings suggest that β -Lapachone cotreatment with L-DOPA

therapy may have therapeutic potential for the suppression or management of the development of L-DOPA-induced dyskinesia in patients with PD.

Introduction

Parkinson's disease is characterized by motor symptoms such as tremor, postural instability, and bradykinesias caused by the progressive loss of dopaminergic neurons in the substantia nigra (1,2). Although the motor symptoms of PD can be managed with dopamine replacement therapy using L-DOPA (3), long-term levodopa treatment leads to motor complications involving dyskinesia and motor fluctuations tend to occur within a few years of L-DOPA treatment initiation (4). L-DOPA-induced dyskinesia affects quality of life and requires intervention.

The neurological mechanisms of L-DOPA-induced dyskinesia (LID) remain largely unclear. Among the several factors that play a role in the onset and severity of LID, abnormalities in connectivity between the striatum and the motor cortex induced by the loss of dopaminergic neurons are considered to be critical elements (5). Numerous studies have reported alterations in the basal ganglia circuitry involving excessive release of dopamine (DA) and hyper-activation of striatal DA receptors and related signaling pathways (5-7). In addition to neuronal targets for normalizing DA D1 receptor signaling, such as extracellular signaling-regulated kinase 1/2 (ERK1/2), mTOR, Δ FosB, and the M4 muscarinic receptor (7-9), non-neuronal mechanisms for regulating glial cells have also been suggested to contribute to the development of LID (10). Reactive microglia and astrocytes have been observed in postmortem brain samples of PD patients (11,12), and an upregulation of astrocytosis in the striatum of PD animal models displaying LID has been reported (13). Higher DA induced by prolonged L-DOPA treatment results in the production of toxic products in astrocytes (10). With these premises, modulating glial-mediated neuroinflammation may be a new promising target for treating LID in PD.

β -Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) is a quinone-containing compound that was originally isolated from a lapacho tree in South America (14).

Correspondence to: Dr Chul-Ho Lee or Dr Kyoung-Shim Kim, Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology, Gwahak-ro 125, Yuseong-gu, Daejeon 34141, Republic of Korea
E-mail: chullee@kribb.re.kr
E-mail: kskim@kribb.re.kr

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The therapeutic effects of β -Lapachone on rheumatoid arthritis and metabolic syndrome have been reported (15,16). Recently, in neurological disorders such as cerebral ischemia, multiple sclerosis, and Huntington's disease, the neuroprotective effects of β -Lapachone have also been reported (17-20). β -Lapachone is known as an anticancer drug candidate that can facilitate quinone oxidoreductase-1 (NQO1)-dependent oxidation of NADP(H) (21). NQO1, a phase II antioxidant enzyme, is involved in cytoprotective and detoxification processes (22). In addition, NQO1 has also been reported to play a regulatory role in the dopaminergic system of rodents (22,23). β -Lapachone has strong anti-inflammatory and anti-oxidative effects *in vitro* and *in vivo* (24,25). The neuroprotective effect of β -Lapachone, which works by upregulating the pAMPK/NRF/HO-1 signaling pathway in astrocytes, has been shown in an MPTP-induced PD mouse model (20). However, the effect of β -Lapachone cotreatment on L-DOPA therapy in PD has not been elucidated. We hypothesized that β -Lapachone co-treatment with L-DOPA alleviates the dyskinesia induced by chronic L-DOPA treatment. In the present study, we examined the behavioral AIMs associated with LID in an animal model of PD and the effects of β -Lapachone on the DIR signaling pathway, astrocyte activation, and GSK-3 β phosphorylation in the 6-OHDA mouse model of PD.

Materials and methods

Animals. C57BL/6J mice (8-9 weeks-old male, 22-27 g) used in the experiment were purchased from Laboratory Animal Resource Center of KRIBB (Korea). The living environment of mice is controlled with day and night cycles (light on at 7:00 A.M. and light off at 7:00 P.M) and the temperature (21-23°C) and humidity (50-60%) are kept constant. Gamma-irradiated laboratory chow (Envigo Teklad) and autoclaved water were provided. The animal room was maintained under specific pathogen-free conditions. The mice were habituated for 7 days before surgery. 6-hydroxydopamine (6-OHDA) was injected into the substantia nigra pars compacta (SNc) of mice. To investigate the effect of β -Lapachone on L-DOPA-induced dyskinesia in PD, we generated a PD mouse model by inducing unilateral 6-OHDA lesions (n=25 animals). Two weeks after inducing the 6-OHDA lesion, ipsilateral turning behavior, induced by d-amphetamine, was observed in all unilateral 6-OHDA-lesioned mice. Based on the number of d-amphetamine-induced rotations, mice were randomly assigned to two groups: Vehicle (0.9% NaCl)-treated group (n=12 animals), and β -Lapachone (10 mg/kg/day)-treated group (n=13 animals). The threshold number of ipsilateral rotations induced by d-amphetamine was 300. β -Lapachone was orally administered 30 min before L-DOPA injection for 11 days (Fig. 1A). Finally, only mice with at least an 80% reduction in tyrosine hydroxylase (TH)-positive cells in the 6-OHDA-lesioned SNc and TH-immunoreactive fibers in the 6-OHDA-lesioned striata relative to the striata without lesions were included in the analyses. All animal experiments were approved by the Institutional Animal Care and Use Committee of the KRIBB.

Drugs. 6-Hydroxydopamine (6-OHDA), desipramine hydrochloride, 3,4-Dihydroxy-L-phenylalanine (L-DOPA), and

benserazide hydrochloride (peripheral dopa decarboxylase inhibitor) were purchased from Sigma-Aldrich Co. LLC; Merck KGaA. 6-OHDA was dissolved in 0.2% ascorbic acid in 0.9% NaCl, stored at -20°C and diluted to 5 μ g/ μ l with 0.2% ascorbic acid before use. Desipramine, L-DOPA, and benserazide hydrochloride were dissolved in 0.9% NaCl. β -Lapachone was purchased from Tocris Bioscience and dissolved in 0.9% NaCl. D-amphetamine (D-AMPH) was purchased from United States Pharmacopeia and then diluted in 0.9% NaCl and stored at -20°C.

Intra-nigral injection of 6-OHDA. The injection of 6-OHDA was performed as described previously (26). 25 min after the intraperitoneal administration of desipramine (25 mg/kg), a mixed anesthetic of ketamine hydrochloride (71.34 mg/kg) and xylazine hydrochloride (6.14 mg/kg) was administered intraperitoneally (26,27). After anesthesia, mice were placed in a stereotactic frame (Stoeling Europe) with a mouse warming pad, as previously described (26). Mice were injected with 3 μ l of 6-OHDA (5 μ g/ μ l, at the injection speed of 1 μ l/min) into the left SNc at the following coordinates: Anteroposterior, -3.0 mm; median lateral, -1.3 mm; and dorsoventral, -4.7 mm. Mice were left on the warmer (37°C) until they woke up from anesthesia. To avoid dehydration, the mice were subcutaneously administered sterile glucose-saline solution (50 mg/ml, 0.1 ml/10 g body weight) immediately after surgery and once a day for 3 days. Food pellets were mixed with 15% sugar/water solution and placed in a shallow vessel on the floor of the cage for 7 days.

Cylinder test. After 3 weeks of 6-OHDA injection and the first injection of L-DOPA, a cylinder test was performed to determine the sensorimotor abnormalities manifested by unilateral 6-OHDA injection and the protective effects of L-DOPA on sensorimotor function. On the first day of L-DOPA, the cylinder test was performed after 1 h of vehicle or β -Lapachone treatment and 30 min after injection of L-DOPA. Mice were placed in a transparent acrylic cylinder (diameter, 15 cm; height, 27 cm). The number of contacts with both forelimbs touching the wall was counted for 5 min. The use of the impaired (right) forelimb was expressed as a percentage of the total number of supporting wall contacts.

D-amphetamine-induced rotation test. A d-amphetamine [5 mg/kg, intraperitoneally (i.p.)]-induced rotation test was used to measure the unilateral 6-OHDA-lesion-induced asymmetry of mice. The unilateral lesion of the nigro-striatal dopamine system induced a profound asymmetry in motor performance. The amphetamine-induced rotation reflects dopaminergic cell loss in the 6-OHDA-lesioned side of the brain (26,27). After 2 weeks of 6-OHDA injections, d-amphetamine-induced rotations of mice were recorded in a cylinder (diameter, 20 cm; height, 13 cm) for 60 min. The number of ipsilateral rotations was analyzed using the SMART video tracking program (Panlab).

Abnormal involuntary movement test. To determine the effects of β -Lapachone cotreatment with L-DOPA in dyskinesia, both the vehicle and 10 mg/kg β -Lapachone groups were cotreated with L-DOPA in 6-OHDA-lesioned mice 4 weeks

after the 6-OHDA lesion was induced. 6-OHDA injected mice were treated with β -Lapachone and L-DOPA (20 mg/kg, i.p.) and benserazide (12 mg/kg, i.p., a selective inhibitor of the peripheral dopa decarboxylase) for 11 days after 4 weeks of 6-OHDA injection. Mice were individually placed in a separate glass cylinder, and dyskinetic behaviors were scored for 1 min (monitoring period) every 20 mins block for a period of 120 min on days 5 and after L-DOPA injection. The AIM score corresponds to the sum of the individual scores for each AIM subtype. A composite score was obtained by the adding the scores for axial, limb, and orofacial (ALO) AIMS in consideration of the report that composite AIM scores more closely reflect human dyskinetic behavior compared with the locomotive (LOC) AIM score. The score was measured from 1 to 4 points for each subtype. 0 point indicates no abnormal behavior, 1 point means that abnormal behavior appears once or twice, 2 point means that abnormal behavior appears repeatedly more than 2 times, 3 point means that abnormal behavior is repeated for more than 30 sec in 1 min, and 4 point means that abnormal behavior is repeated for more than 30 sec in 1 min and it does not stop even if a stimulation, such as sound, is given (8).

Immunohistochemistry. Immunohistochemistry was conducted as previously described (26). Briefly, 1 h after vehicle or β -Lapachone administration and 30 min after chronic L-DOPA injections, mice were euthanized by quick cervical dislocation and their brains are removed. The mouse brains were fixed with 4% paraformaldehyde in PBS for more than one day and then carefully cut into 40 μ m coronal sections on a vibratome (Vibratome VT1000A). Free-floating sections were blocked with 5% horse serum for 1 h at room temperature. Samples were incubated in primary antibodies overnight at 4°C. The primary antibodies used were rabbit polyclonal antibodies for tyrosine hydroxylase (TH; Pel-Freez), ionized calcium-binding adapter molecule 1 (Iba-1), and astrocytes (GFAP, Dako). The secondary antibody, a biotinylated secondary anti-rabbit IgG (1:200, Vector Laboratories), was administered for 1 h at room temperature, and then samples were rinsed 3 times in 1X TBST. Immunohistochemistry was subsequently performed using avidin-biotinylated peroxidase complex (ABC kit, Vector Laboratories) for 1 h at room temperature and then rinsed 3 times in 1X TBST, followed by incubation in 3,3'-diaminobenzidine (Sigma-Aldrich Co.; Merck KGaA) for 10 min at room temperature; samples were then attached to the slide. Since the levels of TH depletion in the striatum and SNc could affect the behavioral analysis, only mice that showed TH depletion levels of >80% were included in the final analysis of this study. In our previous study, TH depletions >80% in the SNc and striatum showed the absence of a correlation between TH depletion and AIM scores in 6-OHDA-lesioned mice (8). When 6-OHDA is directly injected into the SNc, a significant loss of dopaminergic neurons occurs 2-3 days later (28,29). In this study, dopaminergic cell death was assessed at the end of all experiments. TH-stained neurons in the left and right SNc (-3.6 to -3.0 mm from the bregma) were counted for two sections per animal. GFAP-stained astrocytes and Iba-1-stained microglia in the left and right SNc (-3.6 to -3.0 mm from the bregma)

were counted for one section per animal. To avoid double counting of neurons with unusual shapes, TH-stained cells were counted only when their nuclei were visualized in a focal plane using the MetaMorph image analyzer (Molecular Devices Inc.). Qualitative evaluations of immunoreactive cells were performed in a blinded manner.

Western blot analysis. Western blot analysis was performed as described previously (8). 30 min after completion of the L-DOPA with β -Lapachone treatment schedule, brain tissue was quickly removed and homogenized in homogenization buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.1% sodium deoxycholate) containing a cocktail of protease inhibitors (Roche Diagnostics GmbH). Equal protein samples were resolved by SDS-PAGE and then transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc.) using a semi-dry transfer system (Trans-Blot SD, Bio-Rad Laboratories, Inc.). The blots were incubated overnight at 4°C with the following primary antibodies (unless otherwise stated, all antibodies were used at 1:1,000 dilution); rabbit polyclonal antibodies for TH (1:2,000, Pel-Freez), extracellular signal-regulated kinases 1/2 (ERK1/2, 1:2,000, Cell Signaling Technology, Inc.), pERK1/2 (Thr202/Tyr204; Cell Signaling Technology, Inc.), GSK3 β (Cell Signaling Technology, Inc.), pGSK3 β (Ser9, Cell Signaling Technology, Inc.), AMPA receptor subunit GluR1 (Abcam), pGluR1 (Ser845, Millipore), FosB (Cell Signaling Technology, Inc.), c-Fos (Santa-Cruz Biotechnology, Inc.), GFAP (Dako; Agilent Technologies, Inc.), and actin (1:10,000, Millipore), respectively. After incubation with horseradish peroxidase-conjugated secondary antibodies (Jax ImmunoResearch), the blots were developed using an enhanced chemiluminescence kit (ATTO Corporation) and quantified using Quantity One 1-D analysis software, version 4.6.1 (Bio-Rad Laboratories, Inc.).

Statistical analysis. GraphPad PRISM (GraphPad Software, Inc.) software was used to perform the statistical analyses. Two-sample comparisons were carried out using Student's t-test (unpaired t-test), while multiple comparisons were made using one-way ANOVA followed by Tukey-Kramer's post hoc test and two-way ANOVA followed by bonferroni's post hoc test. All data were presented as the mean \pm standard error of the mean (SEM) and statistical differences were accepted at the 5% level unless otherwise indicated.

Results

Generation of 6-OHDA-induced mouse model of PD. Based on the ipsilateral rotations and forelimb use, the mice were separated into the vehicle/L-DOPA group, and 10 mg/kg β -Lapachone/L-DOPA group. The ipsilateral rotation and forelimb use did not differ between the two groups (Fig. 1F; 609.73 \pm 45.00% in vehicle/L-DOPA and 589.70 \pm 73.96% in 10 mg/kg β -Lapachone/L-DOPA, and G; 38.48 \pm 0.52% in vehicle/L-DOPA, and 38.41 \pm 0.66% in 10 mg/kg β -Lapachone/L-DOPA). The nigral dopaminergic cell bodies were depleted by 92.08 \pm 1.44% in vehicle/L-DOPA group and 95.60 \pm 0.33% in 10 mg/kg β -Lapachone/L-DOPA group (Fig. 1B and C). The striatal dopaminergic dendritic fibers were depleted by 93.20 \pm 1.02% in vehicle/L-DOPA

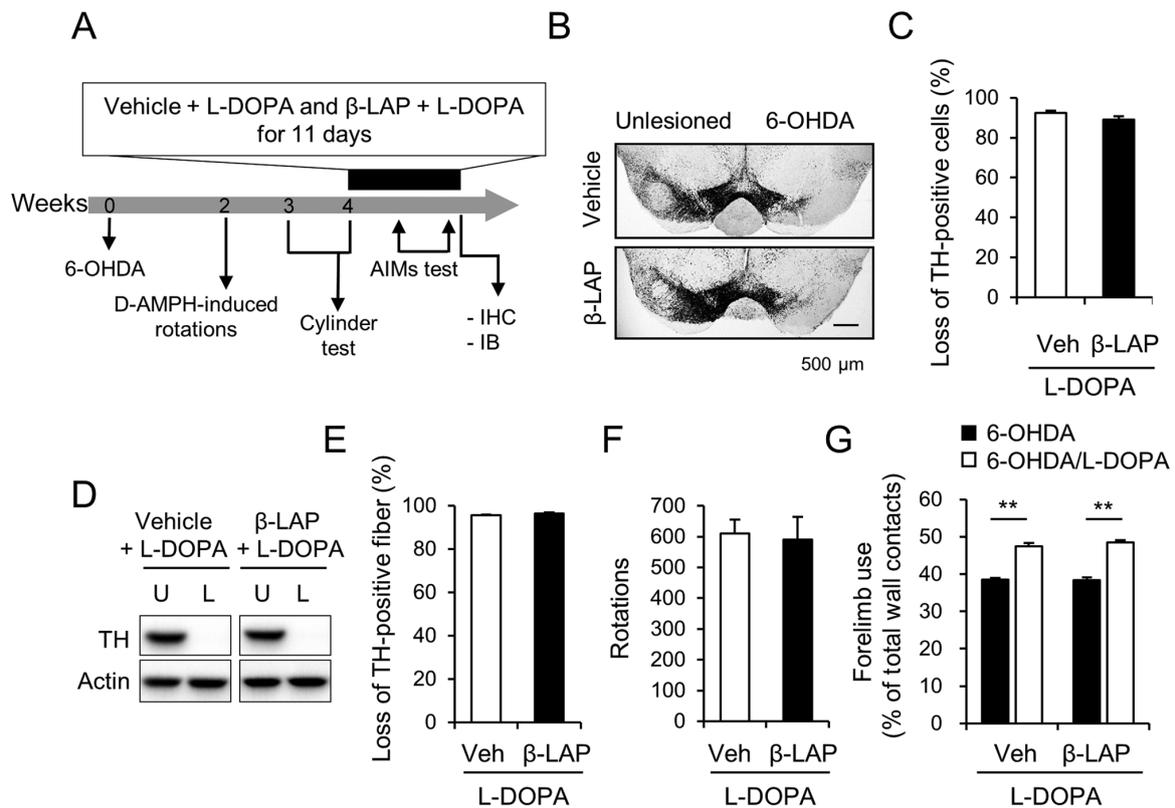


Figure 1. Unilateral 6-OHDA-lesioned model of Parkinson's disease was established in C57BL/6J mice. (A) A schematic of the experimental procedure. (B) Photomicrograph showing TH-immunoreactive cells in the SNc and (C) % loss of TH-positive cells in the lesioned side compared with those in the intact side of the SNc (n=11 animals/group). Scale bar, 500 μ m. The protein extract from the striatum of vehicle + L-DOPA group and 10 mg/kg β -Lapachone + L-DOPA group were subjected to western blot analyses using TH antibody (n=11 animals/group) and (D) representative blots are provided. (E) Semi-quantification of the western blotting data. (F) D-amphetamine-induced rotations for 60 min in the vehicle + L-DOPA (n=11 animals) and 10 mg/kg β -Lapachone + L-DOPA (n=11 animals) groups. (G) Right forelimb use in the cylinder test after 6-OHDA lesioning (6-OHDA) and 30 min after the first treatment with L-DOPA (6-OHDA/L-DOPA) and vehicle or 10 mg/kg β -Lapachone. ** P <0.01 (Student's t -test). Data are presented as the mean \pm SEM. 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase; SNc, substantia nigra pars compacta; Veh, vehicle + L-DOPA; β -LAP, β -Lapachone + L-DOPA; AIMs, abnormal involuntary movements; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; U, unlesioned; L, 6-OHDA-lesioned; IHC, immunohistochemistry; IB, immunoblot.

group and $96.24 \pm 0.62\%$ in 10 mg/kg β -Lapachone/L-DOPA group (Fig. 1D and E).

β -Lapachone mitigates the development of LID in the 6-OHDA mouse model. On the first day of β -Lapachone and L-DOPA treatment, the therapeutic effect of L-DOA on motor deficits was not affected by β -Lapachone treatment (Fig. 1G; $47.45 \pm 0.86\%$ in vehicle/L-DOPA group and $48.57 \pm 0.58\%$ in the 10 mg/kg β -Lapachone/L-DOPA group). The forelimb use was significantly improved by L-DOPA treatment in all groups (P <0.01).

To compare dyskinesia in vehicle-treated and β -Lapachone-treated mice, the AIMs test was performed on days 5 and 10. On day 5, the total AIM scores were $20.82 \pm 0.58\%$ in the vehicle/L-DOPA group, and $15.09 \pm 0.98\%$ in the 10 mg/kg β -Lapachone/L-DOPA group (Fig. 2A). β -Lapachone treatment significantly decreased total AIM scores ($t_{(21)}=5.339$, P <0.01). In addition, both LOC and ALO scores were also decreased by β -Lapachone treatment (Fig. 2B; $t_{(21)}=5.367$, P <0.01, and Fig. 2C; $t_{(21)}=2.934$, P <0.01). In the ALO subtypes, axial AIMs were significantly decreased by β -Lapachone treatment (Fig. 2D; $t_{(21)}=2.329$, P <0.05), but not limb ($t_{(21)}=1.793$, P >0.05) or orolingual ($t_{(21)}=0.6778$, P >0.05) scores (Fig. 2E and F).

On day 10, the total AIM scores were $32.36 \pm 1.26\%$ in the vehicle group, and $22.09 \pm 2.21\%$ in the 10 mg/kg β -Lapachone/L-DOPA group (Fig. 3A). β -Lapachone treatment significantly decreased total AIM scores (Fig. 3B; $t_{(21)}=4.589$, P <0.01). In addition, both LOC and ALO scores were also decreased by β -Lapachone treatment (Fig. 3A; $t_{(21)}=3.942$, P <0.01, and Fig. 3C; $t_{(21)}=3.819$, P <0.01). In the ALO subtypes, axial and limb AIMs were significantly decreased by β -Lapachone treatment (Fig. 3D; axial, $t_{(21)}=2.516$, P <0.05, and Fig. 3E; limb, $t_{(21)}=3.499$, P <0.01), but not orolingual ($t_{(21)}=1.590$, P >0.05) scores (Fig. 3F).

β -Lapachone does not alter hyperactivation of the DIR signaling pathway in LID. To examine the possible involvement of the DA D1 receptor and ERK1/2 signaling in the beneficial role of β -Lapachone treatment in LID, we performed western blotting using the unlesioned and 6-OHDA lesioned striata of vehicle/L-DOPA-treated and 10 mg/kg β -Lapachone/L-DOPA-treated group. The phosphorylation of GluR1 at Ser845 and ERK1/2 at Thr202/Tyr204 and the expression of Δ FosB and c-Fos were increased by chronic treatment with L-DOPA in the 6-OHDA-lesioned side of the striatum compared to the unlesioned side of striatum (Fig. 4A and B). However, the enhanced level of phosphorylation and expression was not

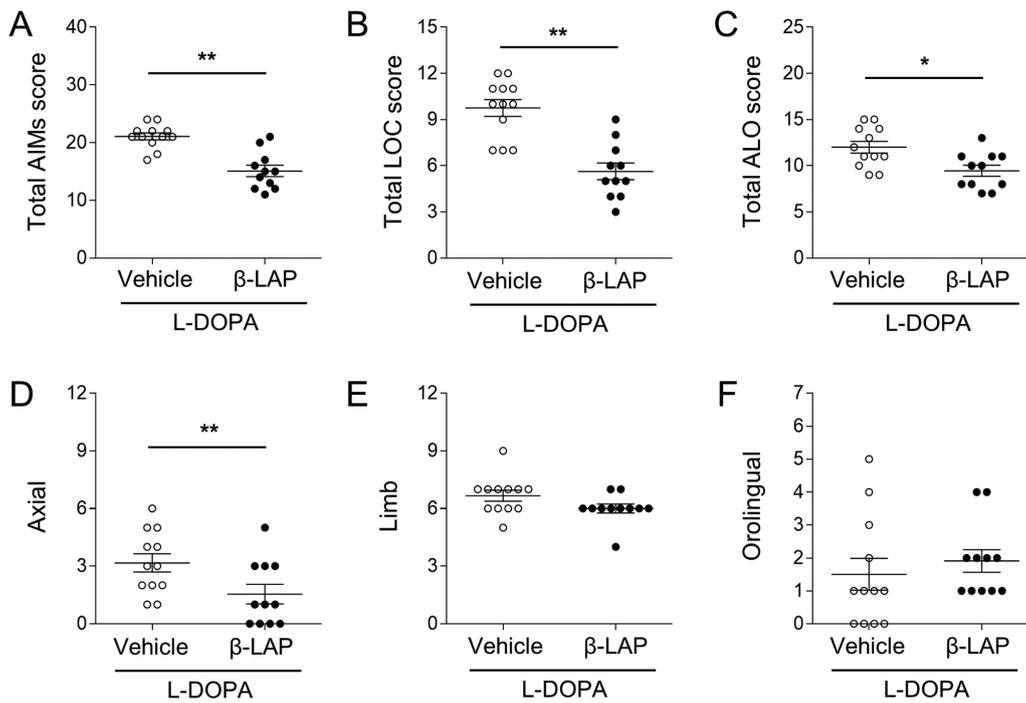


Figure 2. Anti-dyskinetic effects of β -Lapachone in L-DOPA-induced dyskinesia on day 5. (A) Total AIMs, (B) LOC and (C) ALO scores for 120 min after L-DOPA injection on day 5 in the vehicle + L-DOPA (n=11 animals) and 10 mg/kg β -Lapachone + L-DOPA (n=11 animals) groups. Cumulative ALO AIM scores for 120 min after L-DOPA treatment were subdivided into (D) axial, (E) limb and (F) orolingual subscores on day 5. *P<0.05, **P<0.01 (Student's t-test). Data are presented as the mean \pm SEM. AIMs, abnormal involuntary movements; LOC, locomotive; ALO, axial-limb-orofacial; β -LAP, β -Lapachone; L-DOPA, 3,4-dihydroxyphenyl-L-alanine.

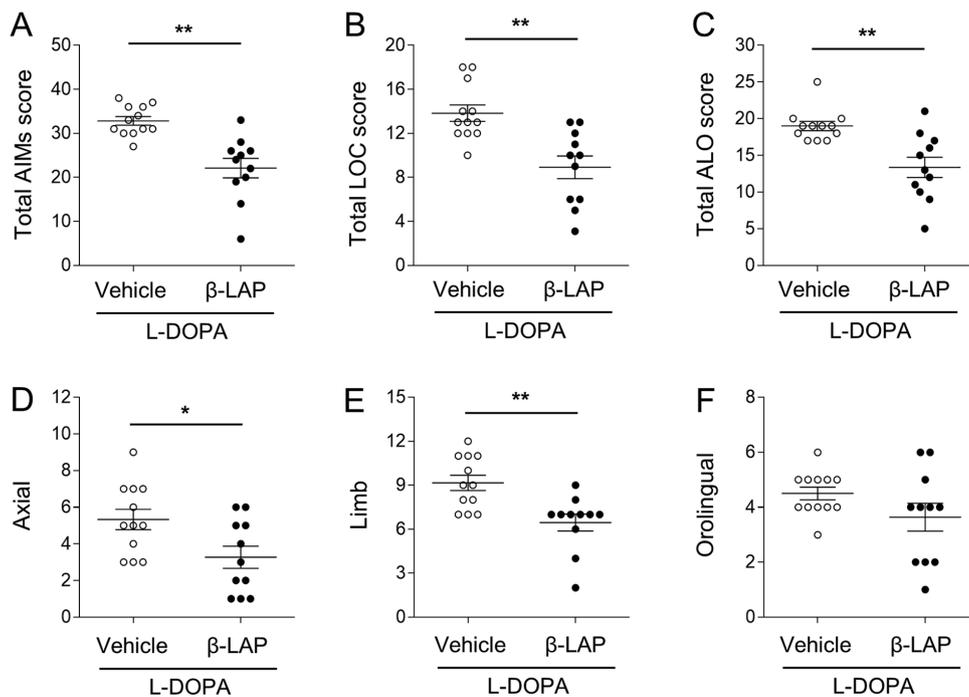


Figure 3. Anti-dyskinetic effects of β -Lapachone in L-DOPA-induced dyskinesia on day 10. (A) Total AIMs, (B) LOC and (C) ALO scores for 120 min after L-DOPA injection on day 10 in the vehicle + L-DOPA (n=11 animals) and 10 mg/kg β -Lapachone + L-DOPA (n=11 animals) groups. Cumulative ALO AIM scores for 120 min after L-DOPA treatment were subdivided into (D) axial, (E) limb and (F) orolingual subscores on day 10. *P<0.05, **P<0.01 (Student's t-test). Data are presented as the mean \pm SEM. AIMs, abnormal involuntary movements; LOC, locomotive; ALO, axial-limb-orofacial; β -LAP, β -Lapachone; L-DOPA, 3,4-dihydroxyphenyl-L-alanine.

decreased by co-administration of β -Lapachone and L-DOPA. The pERK1/2 level was increased rather than reduced on

both sides of the striatum in the β -Lapachone-treated group compared to vehicle-treated group (Fig. 4A and B, P<0.01).

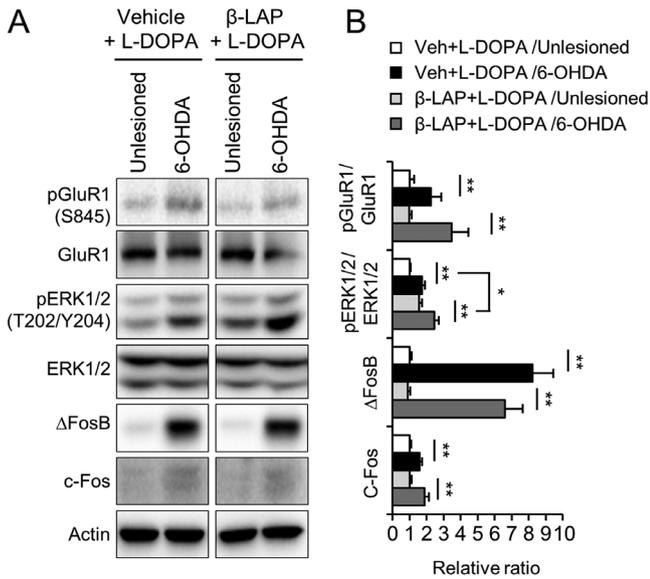


Figure 4. Effects of β -Lapachone on the D1 receptor and ERK1/2 signaling in the L-DOPA-induced dyskinesia. Protein extracts from the striatum of the vehicle + L-DOPA and 10 mg/kg β -Lapachone + L-DOPA groups were subjected to western blot analysis using pGluR1, GluR1, pERK1/2, ERK1/2, Δ FosB and c-Fos antibodies (n=11 animals) and (A) representative blots are shown. (B) Semi-quantification of western blotting data. Values were normalized to actin values, and are presented relative to vehicle + L-DOPA in the unlesioned striatum. * P <0.05, ** P <0.01 (n=11 animals; Student's t-test and one-way ANOVA). Data are presented as the mean \pm SEM. 6-OHDA, 6-hydroxydopamine; β -LAP, β -Lapachone; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; GluR1, AMPAR subunit glutamate receptor 1; pGluR1, phospho-GluR1 at Ser845; ERK1/2, extracellular signal regulated kinase 1/2; pERK1/2, phospho-ERK1/2 at Thr202/Tyr204; Δ FosB, deltaFosB; c-Fos, proto-oncogene c-fos.

β -Lapachone regulated phosphorylation of GSK3 β in both the intact and 6-OHDA-lesioned striatum. Accumulating evidence suggests that glycogen synthase kinase-3 β (GSK-3 β) is involved in the development of LID (30,31). GSK-3 β is inactivated by phosphorylation at the N-terminal Ser9 (32). However, the regulatory effect of β -Lapachone on GSK-3 β in the brain has not previously been investigated. We examined whether co-treatment with β -Lapachone and L-DOPA affected GSK-3 β phosphorylation in the LID of the 6-OHDA mouse model. The phosphorylation of GSK-3 β was significantly increased in the 6-OHDA lesioned side of the striatum compared to the unlesioned side of the striatum in the vehicle-treated group (Fig. 5A and B, $t_{(10)}=4.046$, P <0.01, paired t-test). Two-way ANOVA showed significant main effects on the 6-OHDA lesion ($F_{(1,20)}=6.35$, P <0.05) and β -Lapachone ($F_{(1,20)}=20.06$, P <0.01), but not on the 6-OHDA lesion or β -Lapachone interaction ($F_{(1,20)}=0.03$, P >0.05).

β -lapachone relieves astrocyte activation in dopamine depleted regions. To determine the effect of β -Lapachone on astrocyte and microglia activation, we performed immunohistochemistry and immunoblotting using GFAP and Iba-1 antibodies, markers of astrocyte and microglia activation respectively. Immunohistochemistry revealed that, the percentage of the areas occupied by GFAP immunoreactive astrocytes was significantly increased by 6-OHDA lesions in both groups in the SNc (Fig. 6A and B). Of note, the percentage of area occupied by GFAP immunoreactive

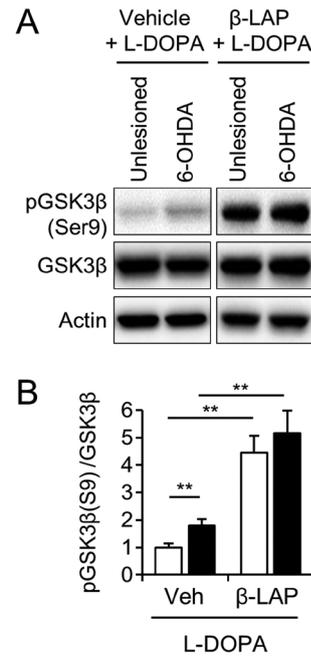


Figure 5. Effects of β -Lapachone on phosphorylation of GSK3- β in L-DOPA-induced dyskinesia. The protein extracts from the striatum of the vehicle + L-DOPA and 10 mg/kg β -Lapachone + L-DOPA groups were subjected to western blot analyses using p-Ser9-GSK3 β and GSK3 β antibodies (n=11 animals) and (A) representative blots are shown. (B) Semi-quantification of western blotting data. Values were normalized to actin, and are presented relative to vehicle + L-DOPA in the unlesioned striatum. ** P <0.01 (Student's t-test and two-way ANOVA followed by Bonferroni post hoc test). Data are presented as the mean \pm SEM. 6-OHDA, 6-hydroxydopamine; β -LAP, β -Lapachone; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; p, phosphorylated.

astrocytes was significantly decreased by β -Lapachone co-treatment with L-DOPA compared to vehicle treatment with L-DOPA in the 6-OHDA-lesioned SNc (Fig. 6A and B). In addition, western blot results showed a significant decrease in the protein expression of GFAP in the β -Lapachone + L-DOPA group compared to the vehicle + L-DOPA group in the 6-OHDA-lesioned striatum (Fig. 6C and D, two-way ANOVA showed the main effect of lesion: $F_{(1,20)}=3.71$, P >0.05; drug effect: $F_{(1,20)}=12.63$, P <0.01, interaction, $F_{(1,20)}=12.63$, P <0.01). However, the activation of microglia was not decreased by β -Lapachone treatment in either the intact or lesioned side of SNc (Fig. 6E and F, two-way ANOVA showed a main effect of lesion: $F_{(1,20)}=118.78$, P <0.01; drug effect: $F_{(1,20)}=0.20$, P >0.05, interaction, $F_{(1,20)}=1.14$, P <0.05).

Discussion

In the present study, we found that β -Lapachone treatment with L-DOPA can suppress the development of dyskinesia associated with long-term treatment of L-DOPA in a mouse model of PD. The hyperactivation of dopamine D1 receptor signaling pathway caused by repeated administration of L-DOPA in a dopamine depletion situation was not regulated by β -Lapachone co-treatment. Of note, the inhibitory effect of β -Lapachone on GSK3 activation was revealed in LID. Moreover, we demonstrated that β -Lapachone co-treatment with L-DOPA could suppress astrocyte activation in the

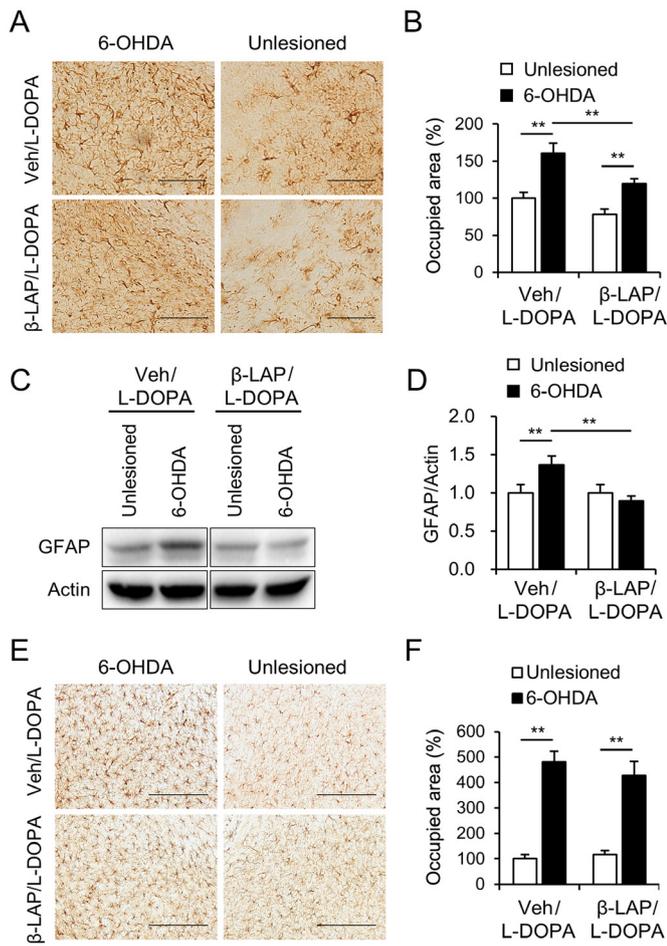


Figure 6. Effects of β -Lapachone on the activation of the astrocyte and microglia. (A) Photomicrograph showing GFAP-immunoreactive cells in the SNc and (B) the percentage of the areas occupied by GFAP-immunoreactive astrocytes in intact and 6-OHDA lesioned SNc of the Veh + L-DOPA and β -Lapachone + L-DOPA groups (n=11 animals). Quantification of GFAP-positive astrocytes reactivity was measured, and was presented relative to veh + L-DOPA in unlesioned SNc. Scale bar, 100 μ m. The protein extracts from the striatum of the vehicle + L-DOPA and 10 mg/kg β -LAP + L-DOPA groups were subjected to western blot analysis using the GFAP antibody (n=11 animals) and (C) representative blots are provided. (D) Expression levels of GFAP in dorsal striatum were analyzed using western blotting. Values were normalized using actin, and are presented relative to vehicle + L-DOPA in unlesioned striatum. (E) Photomicrograph showing Iba-1 immunoreactive cells in the SNc and the (F) percentage of the areas occupied by Iba-1 immunoreactive astrocytes in intact and 6-OHDA lesioned SNc of the vehicle + L-DOPA group and β -Lapachone + L-DOPA group (n=11 animals). Quantification of Iba-1-positive microglia reactivity was measured, and was presented relative to veh + L-DOPA in unlesioned SNc. Scale bar, 200 μ m. **P<0.01 (two-way ANOVA followed by a post hoc test). Data are presented as the mean \pm SEM. 6-OHDA, 6-hydroxydopamine; β -LAP, β -Lapachone; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; GFAP, glial fibrillary acidic protein; SNc, substantia nigra pars compacta; Veh, vehicle; Iba-1, ionized calcium-binding adapter molecule 1.

chronic treatment of L-DOPA in the 6-OHDA lesioned striatum and SNc.

Dyskinesia is a serious motor complication that occurs when L-DOPA is administered to patients with PD over a long period (5). The striatum is thought to be important for dyskinesia development. A direct output pathway expressing the D1 dopamine receptor in the striatum is overstimulated by L-DOPA treatment, and the modulation of these signaling pathways can effectively suppress LID in animal models (7,33,34).

The phosphorylation of GluR1 and ERK1/2 and the expression of Δ FosB and c-Fos mediating the D1R signaling pathway was not suppressed by β -Lapachone. Rather, ERK1/2 activation was enhanced by β -Lapachone cotreatment in both the intact and 6-OHDA-lesioned striata. These results indicate that the effects of β -Lapachone on LID were not due to down-regulation of the D1 receptor signaling pathway.

It was reported that the inhibition of GSK-3 β promotes the induction of long-term potentiation in neurons (35). Moreover, the role of GSK-3 β in the phosphorylation of several substrates involved in synaptogenesis and neurite stabilization has been suggested (36,37). In PD, an abnormal increase in GSK-3 β activity can affect dendrite degeneration (38). The protective effects in GSK-3 β inhibition on LID have been reported (30). Ser9-phosphorylation of GSK-3 β enhanced by a single treatment with L-DOPA but decreased in response to repeated administration of L-DOPA indicating enhancement of GSK-3 β activity in prolonged L-DOPA treatment (26). In our study, β -Lapachone co-treatment with L-DOPA markedly increased phosphorylation of GSK-3 β at Ser9 in the striatum. These data suggest that prolonged treatment with β -Lapachone and L-DOPA can inactivate GSK-3 β in the striatum of LID in PD.

Anti-inflammatory effects of β -Lapachone on lipopolysaccharide-activated *in vivo* and *in vitro* models have been reported (1,24). β -Lapachone showed anti-inflammatory properties by inhibiting NF- κ B activation, blocking IkappaB α degradation in microglia (1). β -Lapachone co-treatment with L-DOPA did not alter microglial activation in the 6-OHDA-lesioned SNc, indicating that microglia have no effect on LID. Accumulating evidence suggests that both PD-related inflammation and LID-related inflammation states could lead to astrogliosis (10). Astrogliosis, which is caused by increasing neuroinflammation in the brain, can be modulated by LID (39,40). The activation of astrocytes was markedly inhibited by β -Lapachone co-treatment with L-DOPA in the 6-OHDA-lesioned striatum and SNc in LID. Increases in astrocyte activation and number were restricted to the hemisphere ipsilateral to the 6-OHDA-lesioned side in mice receiving L-DOPA. These results indicate that astrocyte activity can be regulated by β -Lapachone co-treatment with L-DOPA in the striatum and SNc in LID of PD.

This work provides the first evidence for the therapeutic utility of β -Lapachone in the LID of PD. Altogether, our results indicate that dyskinesia caused by prolonged L-DOPA treatment in 6-OHDA-lesioned mice is attenuated by β -Lapachone with L-DOPA. Therefore, the results collectively suggest that β -Lapachone may be a candidate for treating dyskinesia in PD. These behavioral and neurobiological studies in a 6-OHDA-lesioned mouse model of PD should be further investigated using other *in vivo* and *in vitro* systems and patients with PD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YKR, CHL and KSK designed the research. YKR, HYP, JG, IBL and YKC performed the experiments. YKR, HYP, JG and KSK analyzed data. YKR, CHL and KSK interpreted data and wrote the paper. YKR, CHL, and KSK critically revised the manuscript. YKR and KSK assessed and confirmed the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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