Acacetin Protects Dopaminergic Cells against 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Neuroinflammation in vitro and in Vivo

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Acacetin (5,7-dihydroxy-4′-methoxy flavone), a constituent of flavone naturally present in plants, has anti-cancer and anti-inflammatory activities. Neuroinflammation is thought to be one of the major pathological mechanisms responsible for Parkinson’s disease (PD), and has been a primary target in the development of treatment for PD. In the present study, we evaluated the neuroprotective effect of acacetin in PD induced by 1-methyl-4-phenylpyridine (MPP+) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and examined the related pathways in vitro and in vivo. In primary mesencephalic culture, acacetin protected dopaminergic (DA) cells and inhibited production of inflammatory factors such as nitric oxide, prostaglandin E2, and tumor necrosis factor-α against MPP+-induced toxicity in a dose-dependent manner. Then, we confirmed the effect of acacetin (10 mg/kg/d for 3 d, per os (p.o.)) in a mouse model of PD induced by MPTP (30 mg/kg/d for 5 d, intraperitoneally (i.p.)). In the behavioral test (pole test), the acacetin-treated mice showed decreased time of turning and locomotor activity, which were longer in MPTP-only treated mice. In addition, the acacetin-treated group inhibited degeneration of DA neurons and depletion of dopamine level induced by MPTP toxicity in the substantia nigra and striatum of the brain. Moreover, the acacetin-treated group inhibited microglia activation, accompanied by production of inducible nitric oxide synthases and cyclooxygenase-2. These results suggest that acacetin can protect DA neurons against the neurotoxicity involved in PD via its anti-inflammatory action.

Key words
acacetin; Parkinson’s disease; neuroprotection; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; anti-inflammatory

Parkinson’s disease (PD), a progressive neurodegenerative disease (ND), is accompanied by loss of dopaminergic (DA) neurons in the substantia nigra (SN) to the striatum (ST); thus, it involves symptoms such as resting tremor, rigidity, bradykinesia, and postural instability.2 PD affects ca. 2% of the population over the age of 60; the proportion increases steadily with age.3 However, the exact mechanism of pathogenesis of PD and other NDs remains incompletely understood. It is believed that inflammation, oxidative stress, mitochondrial dysfunction, apoptosis, or proteasomal impairment directly or indirectly induces neuronal death.3

One of the key players in the pathogenesis of ND is neuroinflammation. It results mostly from the abnormal microglial response in the brain, and activated microglia play a major role in neuroinflammatory mechanisms. They also exert a deleterious influence on neuronal cells by secreting cytotoxic pro-inflammatory factors such as nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β.4,5 The neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its active metabolite 1-methyl-4-phenylpyridine (MPPP), are commonly used in in vitro and in vivo models of PD that exhibit dopaminergic neuronal degeneration and Parkinsonian symptoms mediated by inflammatory processes; both activate microglia in the SN.6 This is associated and co-localized with an increase in inducible nitric oxide synthases (iNOS) expression and cyclooxygenase-2 (COX-2) induction, leading to DA neurodegeneration.6–8 In addition, there exists some proof of activated microglia in ND models and inhibition of inflammatory mediators has been suggested as a potential therapeutic application of ND.9 Thus, it is possible that regulating the inflammatory response could be an important part of treatment of NDs such as PD.10

Acacetin (5,7-dihydroxy-4′-methoxy flavone) is an O-methylated flavone and is naturally present in plants such as chrysanthemum,10 safflower,10 and Calamintha11 and Linaria species.12 It has long been considered to have anti-cancer activity. For example, acacetin inhibits cell growth and cell cycle progression13 and induces apoptosis in gastric carcinoma cells,14 breast cancer MCF-7 cells,15 prostate cancer DU145 cells,16 and lung cancer AS49 cells via activating the caspase cascade and regulating other molecular factors related to apoptosis.17 Other studies have revealed its anti-inflammatory effects; acacetin inhibits the transcriptional activation of iNOS and COX-2 in Raw264.7 cells activated by lipopolysaccharide via interference with Akt and inhibitor of kappa B (IκB) phosphorylation.18 Although various activities of acacetin has been demonstrated, the effect of it on PD has not been examined. Therefore, we evaluated the protective effect of acacetin against MPP+- and MPTP-induced toxicity, and explored its possible mechanisms by performing anti-inflammatory assays in primary mesencephalic cells and in a mouse model of PD.

The authors declare no conflict of interest.
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MATERIALS AND METHODS

Materials  Acacetin was kindly provided by Dr. Sun Yeou Kim from Graduate School of East-West Medical Science, Kyung Hee University. Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone laboratories Inc. (Logan, UT, U.S.A.). Minimum essential medium (MEM) was purchased from Gibco Industries Inc. (Auckland, NZ). MPP\(^+\), MPTP, poly-L-lysine (PLL), glucose, glutamine, sodium nitrite (NaNO\(_2\)), phosphoric acid, naphthylethylene diamide, sulfanilamide, paraformaldehyde (PFA), 3,3-diaminobenzidine (DAB), phosphate buffer (PB), phosphate buffered saline (PBS), glycercin, ethylene glycol, triton X-100, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Rabbit anti-tyrosine hydroxylase (TH) affinity-purified polyclonal antibody and rat anti-CD11b monoclonal antibody were obtained from Chemicon International Inc. (Temecula, CA, U.S.A.). Biotinylated anti-rabbit and rat antibodies, normal goat serum, and avidin–biotin peroxide complex (ABC) standard kit were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Rat TNF-\(\alpha\) assay kit, Trizol reagent, and moloney murine leukemia virus (MMLV) reverse transcriptase were purchased from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.) and QuantiTect SYBR Green polymerase chain reaction (PCR) kit was purchased from Qiagen (Valencia, CA, U.S.A.). Prostaglandin E\(_2\) (PGE\(_2\)) ELISA kit and dopamine research enzyme-linked immunosorbent assay (ELISA) kit were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, U.S.A.) and Labor Diagnostika Nord GmbH and Co., KG (Am Eichenhain 1, Nordhorn, Germany), respectively. iNOS, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in oligonucleotide primers were purchased from Bioneer (Daejeon, Korea).

Culture of Primary Mesencephalic Cells and Sample Treatment  The cell cultures were prepared from the ventral mesencephalon of 14-d embryos of timed pregnant Sprague–Dawley rats (Orient Bio., Osan, Korea). Briefly, after the mesencephalons were dissected, collected, and dissociated, the cells were seeded onto PLL-coated cover slips in 24-well plates at a density of 1.5×10\(^5\) cells/well in MEM supplemented with 6.0g/L glucose, 2mM glutamine and 10% FBS. Cultures were maintained in a water-saturated atmosphere of 5% CO\(_2\) at 37°C. Seven-day-old cultures were used for treatment. The composition of the cells at the time of treatment was 45% astrocytes, 5% microglia and 50% neurons with 6% of the neurons being DA neurons. The cells were treated with acacetin (50, 100, 200 nm) for 1 h and then with 10\(\mu\)M MPP\(^+\) for an additional 23 h. After culture supernatants were collected separately, the treated cells were fixed with 4% PFA or 30 min at room temperature for immunohistochemistry (IHC).

Measurement of Extracellular NO, PGE\(_2\), and TNF-\(\alpha\)  The accumulated level of NO in culture supernatants was measured using the colorimetric reaction with the Griess reagent for 10 min at room temperature in a dark. Absorbance was measured at a wavelength of 570nm using a spectrophotometer (Versamark microplate reader; Molecular Devices, Sunnyvale, CA, U.S.A.) and determined concentrations of NO by NaNO\(_2\) standard curve. PGE\(_2\) and TNF-\(\alpha\) assays were performed according to the each manufacturer’s protocol using culture supernatants. Briefly, levels of PGE\(_2\) and TNF-\(\alpha\) were measured using spectrophotometer at 405 nm and 450 nm, and then data were expressed as standard curves of PGE\(_2\) (pg/mL) and TNF-\(\alpha\) (pg/mL), respectively.

Animals and Sample Treatment  Animal maintenance and treatment were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Seoul, Korea. Male C57BL/6 mice (7 weeks) were purchased from Samtako Inc. (Osan, Korea). Animals were housed at an ambient temperature of 23±1°C and relative humidity 60±10% under a 12 h light/dark cycle, allowed free access to water and food. Animals were assigned into three groups; (1) control group (n=10), (2) MPTP group (n=10), and (3) MPTP+acacetin group (n=10). MPTP (30mg/kg, intraperitoneally (i.p.)) dissolved in normal saline were treated for 5d and acacetin (10mg/kg/d, per os (p.o.)) dissolved in 10% DMSO was administered for the last 3d of MPTP treatment. MPTP was injected two hours after acacetin treatment and equal volumes of vehicles were given to the control and MPTP groups.

Behavioral Test  The pole test is a useful method in mice PD model to measure bradykinesia. We performed the pole test on the seventh day after the last MPTP injection. The mice were held on the top of the pole (diameter 8mm, height 55cm, with rough surface). The time that mice needed to turn down completely and climbed down and four feet reached the floor was recorded. Each trial had the cut-off limit of 30 s.

Tissue Preparation  For performing IHC, the mice were anesthetized with Rumpun\textsuperscript{®} and Zoletil\textsuperscript{®} solution (3:1 ratio, 1mL/kg) intramuscularly on the seventh day after the last MPTP injection and then perfused transcardially with PBS, followed by ice cold 4% PFA in 0.1 M PB. Then brains were rapidly taken out, post fixed overnight in 4% PFA solution, and were processed through a cryoprotection in 30% sucrose at 4°C. Frozen brains were cut into 30\(\mu\)m coronal section using a microtome cryostat (CM3000, Leica, Wetzlar, Germany) and sections were stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M phosphate buffer) at 4°C until use. For reverse transcription (RT)-PCR and DA level assay, mice were decapitated and their brains were rapidly excised on the first day and seventh day after the last MPTP injection, respectively. Tissues including striatum (ST) and SN were stored at −80°C until use.

Immunohistochemistry and Quantification  Fixed mesencephalic cells on cover slips and free-floating brain sections were rinsed in PBS before immunostaining and they were pre-treated with 1% hydrogen peroxide for 15 min to remove endogenous peroxidase activity. Then they were incubated overnight with anti-TH antibody (1:2000 dilution) and anti-CD11b antibody (1:1000 dilution) for detection of DA cells and microglia, respectively, at 4°C. They were then incubated for 90min with biotinylated secondary antibody (1:200 dilution), followed by incubation in ABC solution for 1h at room temperature. The color was developed with DAB. After every incubation step, they were washed three times with PBS. The mesencephalic cells on cover slips were mounted on gelatin-coated slides and air dried. The free-floating brain tissue sections were mounted on gelatin-coated slides, dehydrated with an ascending alcohol, cleared with xylene, and cover slipped using Histomount\textsuperscript{TM} (National Diagnostics, Atlanta, GA, U.S.A.). Quantifications of the effects in primary mesencephalic DA cells were performed by counting.
the TH-immunoreactive (TH-IR) cells in entire areas of five wells of each group at ×100 magnification and by measuring TH-IR neurite length in ten representative areas per well in five wells of each group at ×200 magnification. Quantification of effect in brain tissue sections were performed by counting the numbers of the TH-IR cells in the SN and measuring the optical density of TH-IR fibers in ST at ×100 and ×40 magnifications, respectively, using Stereoinvestigator software (MBF Bioscience Inc., Williston, VT, U.S.A.) and then presented as a percent of the control group values. The images were photographed with a research microscope (BX51T-32F01; Olympus Corporation, Tokyo, Japan).

**Measurement of Dopamine Level** The level of dopamine in ST was measured according to the manufacturer’s instructions. After every reaction was finished with stop solution, result was detected by spectrophotometer at a wavelength of 450 nm and calculated concentration of dopamine using a standard curve (ng/mL).

**RT Real-Time PCR** Total RNA was isolated using Trizol reagent according to the manufacturer’s protocol. RNA (1 µg) was reverse-transcribed in a 20 µL reaction mixture using MMLV reverse transcriptase. For real-time PCR, cDNAs (0.2 µL) were amplified using a QuantiTect SYBR Green PCR kit in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The cycle profile used was an initial denaturation at 95°C for 10 min followed by 40 cycles of at 95°C for 15 s, 60°C for 10 s, and 72°C for 20 s. As a control for normalizing sample loading, PCR amplification of the housekeeping gene GAPDH was included for each sample in each run. Nucleotide sequences of the primers were based on published cDNA sequences of mouse iNOS (iNOS forward, 5′-ggacacctaggtgcaccattactg-3′; iNOS reverse, 5′-ggacacctagtcacctggatgct-3′), COX-2 (COX-2 forward, 5′-cctgttggccatgag-3′; COX-2 reverse, 5′-gattgacacccacacat-3′), and GAPDH (GAPDH forward, 5′-gaagaagactctactcactc-3′; GAPDH reverse, 5′-gaagaagactctactcactc-3′). Fluorescence measurements were obtained online and were analyzed with Rotor-Gene 3000 software (ver. 6.0; Corbett Research). Gene expression was quantitated using the comparative Ct (threshold cycle) method as described.23)

**Statistical Analyses** All quantitative data derived from this study were analyzed statistically. The results are expressed as the mean ± standard error of mean (S.E.M.). The mean and S.E.M. for each group was compared by one-way analysis of variance followed by the least significant difference test using SPSS 12.0 K for windows (SPSS Inc., Chicago, IL, U.S.A.). The p-values less than 0.05 were deemed to be statistically significant.

**RESULTS**

**Effect of Acacetin on MPP⁺-Induced DA Cell Damage in Primary Mesencephalic Culture** To assess the protective effects of acacetin on DA neurons, we counted TH-immunoreactive (IR) neurons. Treatment with MPP⁺ reduced the number of TH-IR neurons and the length of them by 51.29% and 42.85% compared to the control group. However, treatment of acacetin at 50–200 nM prevented DA cell loss induced by MPP⁺ toxicity, showing 66.41% ± 2.13% of the control group dose-dependently (Fig. 1). Also, acacetin preserved morphological changes of DA cells including the shortening of dendrites from MPP⁺ toxicity by showing an elongation of 75.37% ± 4.75% to 94.51% ± 5.35% of the control group dose-dependently (Fig. 1).

**Effect of Acacetin on MPP⁺-Induced Production of Inflammatory Factors in Primary Mesencephalic Culture** To investigate the effect of acacetin on inflammatory response induced by MPP⁺ toxicity, we examined levels of NO, PGE2, and TNF-α using cell supernatants. These factors have important roles in the cellular system; NO, is an important cellular messenger molecule involved in many physiological and pathological processes,24) and PGE₂ is a primary mediator of febrile response, is excessively released by molecular signaling in inflammatory condition and upregulates COX-2.
expressions. TNF-α, a cytokine involved in systemic inflammation, are excessively produced on abnormal conditions by toxins such as MPP⁺. In this study, MPP⁺-treated group showed significant increase of NO, PGE₂, and TNF-α levels by 169.78±10.32, 375.62±84.69 and 284.89±27.26% compared to the control group, respectively. In contrast, treatment with acacetin at 50–200 nM dose-dependently suppressed the increased levels, to 127.82±7.24–115.83±5.01, 225.55±104.85–96.65±16.43, and 202.89±18.14–137.07±4.89% compared to the control group, respectively (Fig. 2). From these data, it is suggested that protective effect of acacetin in DA cells is due to regulating inflammatory response, by reducing NO, PGE₂, and TNF-α levels.

Effects of Acacetin on MPTP-Induced Movement Impairment and DA Neuronal Degeneration in Mice To confirm the effect of acacetin on DA neurons in a mouse model of PD, we performed a behavioral test and stereological analysis after acacetin and/or MPTP treatment. In the pole test, the MPTP group showed bradykinesia, showing 14.91±1.85 s throughout turning on the top and climbing down the pole. However, the acacetin-treated group showed recovery effect on MPTP-induced movement impairment, showing 8.71±2.28 s (Fig. 3). Also, this group inhibited dopamine depletion by MPTP treatment, resulting 38.04±2.96 and 46.56±1.12% of the control groups in the SN and the ST, respectively, whereas acacetin treatment recovered the depletion by 63.88±2.71 and 70.15±7.33% of the control in the SN and the ST, respectively.

Then, we carried out TH-IHC to make sure the effect of acacetin on DA neurons in this model. As shown in Fig. 4, the MPTP induced a significant DA neurodegeneration, showing 51.26±1.25% decrease in the number of TH-IR neurons compared to the control group in the SN. Acacetin administration at a dose of 10 mg/kg/d for 3 d significantly inhibited the MPTP-induced loss of DA neurons by 68.59±1.16% of the control group. Furthermore, the optical density of TH immunoreactivity in the ST of mouse brains showed that acacetin inhibited the DA cell fibril loss against MPTP stress. These results suggest that acacetin recovered DA neuronal damage from MPTP induced neurotoxicity.

Effects of Acacetin on MPTP-Induced Microglial Activation and iNOS and COX-2 Expression in Mice To examine whether acacetin affects microglial system or not, we performed IHC using CD11b antibody. In this study, MPTP-treated group showed increase of activated microglia in the SN by 252.00±38.85 of CD11b-IR cell numbers and 112.51±1.97% of CD11b-IR brightness compared to the control group.
However, acacetin inhibited the MPTP-induced microglial activation in the SN, showing 52.38 ± 7.04 and 89.18 ± 1.61% of CD11b-IR cell numbers and brightness, respectively, compared to the MPTP-treated group (Fig. 5). In addition, we measured iNOS and COX-2 expression levels which are released by inflammatory signal using real time RT-PCR. MPTP-treated group significantly increased level of iNOS by 29.36 ± 1.77 and 36.20 ± 2.03 folds of the control in the SN and the ST, respectively. In contrast, treatment with acacetin suppressed it to 15.30 ± 1.03 and 14.20 ± 1.23 folds of the control, respectively. Similar to the result of iNOS, COX2 is excessively expressed by MPTP toxicity, by 34.90 ± 1.83 and 40.70 ± 1.97 folds of the control in the SN and the ST whereas it was suppressed by treatment of acacetin, showing 21.50 ± 1.93 and 24.70 ± 1.93 fold of the control, respectively (Fig. 6). Taken together, acacetin could regulate the inflammatory response through inhibiting microglial activation and levels of iNOS and COX-2 expression.

**DISCUSSION**

In this study, we evaluated the neuroprotective effect of acacetin in primary mesencephalic cells and in mice stressed by MPP⁺ and MPTP, respectively. The mechanism of the effect was investigated in terms of regulation of microglial activation and pro-inflammatory factor production.

First, we examined the effect of acacetin on neuronal damage induced by MPP⁺ in cultured primary DA cells, which are found primarily in the midbrain. Loss of these cells is observed in PD.28,29) Thus, assessing the effect on DA neurons in the midbrain is required to discover any positive effect in a PD experimental system. In cultured mesencephalic cells, MPP⁺ significantly reduced the number of TH-IR cells, causing shrinkage of cell bodies and withering of neurite. However, acacetin treatment significantly protected DA cells from this toxicity, resulting in an increased number of TH-IR cells and preservation of the neuronal morphology of DA cells (Fig. 1). Then, to confirm the protective effect of acacetin in an MPTP-induced mouse PD model, we performed a behavioral test, determined dopamine levels, and a stereological analysis. Reduction of DA cells in the SN causes severe dopamine depletion in the ST, followed by motor dysfunction.30) MPTP-induced behavioral impairment was recovered by acacetin treatment, which also showed significantly increased dopamine levels in the ST and the SN. In addition, acacetin treatment reduced MPTP-induced loss of DA cell bodies in the SN.
and its fibers in the ST (Figs. 3, 4). Taken together, these in vitro and in vivo data provide strong evidence that acacetin exerts neuroprotective effects in PD models.

Next, we investigated the mechanism underlying acacetin-induced neuroprotection by assessing its anti-inflammatory effect in the same models. Previous studies have determined that treatment with MPP+/MPTP causes neuronal death through release of pro-inflammatory factors, such as cytokines and NO. Therefore, we measured NO, PGE2, and TNF-α levels using supernatants from primary mesencephalic cells consisted of DA neurons and neighboring rich glial cells. The acacetin-treated group significantly inhibited the overproduction of NO, PGE2, and TNF-α induced by MPP+ (Fig. 2). We next examined CD11b-immunoreactivity to detect microglia activation and iNOS and COX-2 expressions in mouse brain tissue. Similar to previous reports that activated microglia not only damage neurons but also overproduce proinflammatory factors, the MPTP-treated group showed significant increases in the number of CD11b-IR cells and iNOS and COX-2 mRNA expression. However, acacetin inhibited microglial activation and suppressed iNOS and COX-2 expression (Fig. 6). Similarly, previous study reported that acacetin significantly inhibited inflammation in over-activated microglial cells in a lipopolysaccharide-induced mouse model of neuroinflammation. Therefore, the protective effect of acacetin on DA neurons resulted from control of microglial activation, resulting in regulation of inflammatory factors.

Flavones, a subclass of flavonoids, suppress neuroinflammation, resulting in a neuroprotective effect within the brain. For instance, famous flavones such as baicalein, wogonin, and scutellarin from Scutellaria baicalensis, which is commonly used in Oriental medicine, have various pharmacological activities, including neuroprotection via attenuation of microgliosis, astrolosis, NO production, iNOS and COX-2 expression, and cytokine release. Also, other flavones such as apigenin and luteolin protect neuronal cells via inhibition of inflammation pathways; apigenin suppressed p38 mitogen-activated protein kinase and c-Jun N-terminal kinase (JNK) phosphorylation without affecting the activity of extra-cellular signal-regulated kinase ERK. Moreover, luteolin suppressed iNOS expression and interleukin-6 (IL-6) production through the JNK pathway. Because inflammation in the brain is one of the key players in the pathogenesis for PD, a drug that controls the inflammatory response or, more specifically, regulates glial activation and secretion of inflammatory factors, is needed to treat PD and other NDs. Therefore, development of a novel effective agent remains; acacetin is a potential candidate for preventing or treating PD.

In summary, this study is the first to report a neuroprotective effect of acacetin in a PD model. Acacetin protected DA cells against MPP+ and MPTP-induced neurotoxicity by inhibiting microglial activation and pro-inflammatory factor production in vitro and in vivo models of PD. These results suggest that acacetin may be a useful neuroprotective candidate agent for treatment of PD.

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REFERENCES


