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Short Communication

Protective Effect of Sauchinone on Methamphetamine-Induced Neurotoxicity in Mice

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Abstract. Sauchinone is a lignan isolated from *Saururus chinensis* known to suppress nitric oxide (NO) activity. Previous studies demonstrate that NO plays a key role in methamphetamine-induced neurotoxicity. Thus, we hypothesized that sauchinone could have a suppressive effect on the neurotoxicity induced by methamphetamine. Repeated injections of methamphetamine cause degeneration of dopaminergic nerve terminals, whereas sauchinone treatment significantly prevented this degeneration. Sauchinone treatment also inhibited the methamphetamine-induced activation of glia cells and the production of NO via a blockade of inducible NO synthase protein expression. Our results suggest that sauchinone can prevent methamphetamine-induced neurotoxicity through the suppression of NO production.

Keywords: methamphetamine, sauchinone, nitric oxide

Saururus chinensis has been traditionally used in oriental medicine for the treatment of hepatitis, edema, jaundice, and gonorrhea (1). *S. chinensis* has a variety of active compounds, including diastereomeric lignans such as sauchinone, sauchinone A, and 1′-epi-sauchinone. Among these active compounds, sauchinone has been reported to have a variety of biological effects such as hepatoprotective, anti-inflammatory, and antioxidant activity in several cell types (2, 3). Sauchinone has been shown to inhibit the production of lipopolysaccharide (LPS)-induced inflammatory factors such as nitric oxide (NO) in Raw 264.7 cells (3). Based on the protective and anti-inflammatory effects of sauchinone, the current study was designed to examine the effects of sauchinone on methamphetamine-induced neurotoxicity in mice.

Methamphetamine (MAP) is a powerful psychostimulant and a highly abused drug worldwide. In addition to its psychotic effects, MAP may cause long-lasting neural damage and degeneration of dopaminergic (DA) and serotonergic nerve terminals in several brain areas. This degeneration leads to a reduction in DA functions, including long-term decreases in tyrosine hydroxylase (TH) and dopamine transporter (DAT) activity. MAP also activates glial cells in the striatum, which may increase the level of glial cell marker proteins such as glial fibrillary acidic protein (GFAP) and CD11b antigens (4). The mechanisms by which MAP could induce damage to the DA nerve endings have been extensively studied, yet much remains unknown. Accumulating data suggests that there are three mechanisms involved in MAP-induced neurotoxicity: oxidative mechanisms, temperature homeostasis, and ion dysregulation. Oxidative mechanisms have historically been the prevalent hypothesis for MAP neurotoxicity (5). Oxidative mechanisms have two pathways: oxygen-based pathways and NO-based pathways (6). In NO-based pathways, MAP triggers the production of NO, which is transformed to peroxynitrite, a major neurotoxin. NO has been shown to inactivate TH (7) and DAT (8) proteins by peroxynitrite-induced nitration, to bind to DA, and to induce the oxidation of DA (9). NO synthase (NOS) inhibitors have a protective effect on MAP toxicity, although they do not affect MAP-induced hyperthermia (10). Thus, NO is thought to be involved in the increased vulnerability of DA neurons in MAP neurotoxicity. In the current study, we hypothesized that sauchinone could prevent MAP-induced neurotoxicity through a blockade of NO production. We examined the

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effects of sauchinone on DA degeneration and microglial activation induced by repeated injections of MAP in mice. In addition, we examined NO production and inducible NOS (iNOS) and neuronal NOS (nNOS) protein expression in the striatum of mice.

Male C57/BL6 mice (Hyuchang, Pusan, South Korea) weighing 20 – 25 g were used in the present study. All mice were kept on ad libitum food and water and maintained on a 12-h light/dark cycle throughout the course of the study. All animals used procedures that were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the provisions of the NIH Guideline for the Care and Used of Laboratory Animals. MAP hydrochloride, obtained from the Korean Food & Drug Administration (Chungcheongbuk-do, South Korea), was dissolved in saline. Sauchinone was provided by Dr. S.C. Kim (Daegu Haany University, South Korea) and dissolved in a vehicle (40% polyethylene glycol in saline). It was isolated from the *n*-hexane fraction of *S. chinensis* by successive silica gel chromatography and reverse-phase high-pressure liquid chromatography as previously described (2).

Mice were injected repeatedly with MAP (5 mg/kg × 4, i.p. with 2-h intervals) or saline. Sauchinone (5 or 10 mg/kg in vehicle) or vehicle (40% PEG in saline) was orally administered 1 h prior to the 1st and 3rd MAP/saline injections. The animals were returned to the same home cage after the final measurement of rectal temperature. Mice were sacrificed 72 h after the last injection of MAP or saline for immunohistochemistry, western blotting, and NO measurement.

Mice were transcardially perfused with saline followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under pentobarbital sodium anesthesia (50 mg/kg, i.p.). After the perfusion, the brains were rapidly removed from the skull, post-fixed for 24 h in a fixative containing 4% paraformaldehyde in 0.1 M PB (pH 7.4), and then cryoprotected in 20% sucrose in PB for 48 h. Brains frozen with OCT compound were cut coronally into 30-μm-thick sections on a cryostat at a level containing the mid-striatum (+0.6 to +1.0 mm from bregma). The sections were soaked in 0.5% H_2O_2 in 10 mM PBS containing 0.2% Triton X-100 (PBST) for 30 min at room temperature. After washing with PBST (5 min × 5), the sections were incubated in 1% normal goat serum for 30 min. The sections were exposed to rat anti-DAT monoclonal antibody (1:1000 in PBST; Chemicon, Billerica, MA, USA) or rat anti-CD11b monoclonal antibody (1:200 in PBST, Chemicon) for 18 h at 4°C. After incubation with the primary antibody, sections were washed for 5 min × 5 in PBST before incubation with a biotinylated secondary antibody (diluted 1:250; Santa Cruz, Santa Cruz, CA, USA) for 2 h at room temperature. Following washes in PBST (10 min × 3), the sections were incubated with an avidin–biotin peroxidase complex (diluted 1:2000; Vector Lab, Burlingame, CA, USA) for 1 h at room temperature. Immunopositive signals were visualized by 3,3′-diaminobenzidine, nickel, and H_2O_2. Brain tissues were homogenized in a lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail. Homogenates were kept on ice for 20 min and then centrifuged for 15 min at 12,000 rpm at 4°C. Total proteins were fractionated by 10% – 12% gel electrophoresis and electrophoretically transferred to a PVDF membrane. Membranes were incubated with the following primary antibodies overnight at 4°C: mouse anti-GFAP antibody (1:1000, Chemicon), rat anti-DAT antibody (1:1000, Chemicon), rabbit anti-TH antibody (1:5000, Santa Cruz), anti-iNOS antibody (1:500, Santa Cruz), or anti-nNOS antibody (1:500, Santa Cruz). Membranes were incubated with an appropriate secondary antibody (1:2000, Santa Cruz) and developed using enhanced chemiluminescent detection methods (ECL kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). The measurement of brain nitrate was previously described (11). In brief, brain nitrate was measured after its reduction to nitrite, followed by the Griess reaction. The striatum was homogenized in ice-cold lysis buffer (50 mM potassium phosphate, 0.25 M sucrose, 0.1 mM EDTA). The homogenate was centrifuged at 8000 × *g* for 30 min. Supernatants were incubated in the dark for 30 min with 4 mM NAPDH and 0.04 U nitrate reductase in potassium phosphate buffer. Thereafter, the supernatant was mixed with a Griess reagent. Absorbance was measured at 540 nM after incubation for 20 min.

Repeated injections of MAP produced significant hyperthermia in mice. MAP causes the degeneration of striatal DA nerve terminals, and the loss of DA nerve terminals can be indicated by a decrease in striatal DAT protein. DAT protein expression in the striatum is shown in Fig. 1A. A reduction in striatal DAT expression was observed 3 days after the final MAP injection. This reduction in striatal DAT signal was significantly attenuated by pre-treatment with sauchinone at a dose of 10 mg/kg (Fig. 1A). There were no significant changes in striatal DAT signals in the group treated with sauchinone alone. We confirmed and quantified the expression of DAT and TH by western blotting. As shown in Fig. 1B, repeated MAP injections decreased striatal DAT and TH protein expression by approximately 80% and 60%, respectively. Sauchinone treatment prior to the MAP injections attenuated the reduction of DAT and TH protein expression in dose-dependent manner. In contrast, no significant change was detected in the group treated with

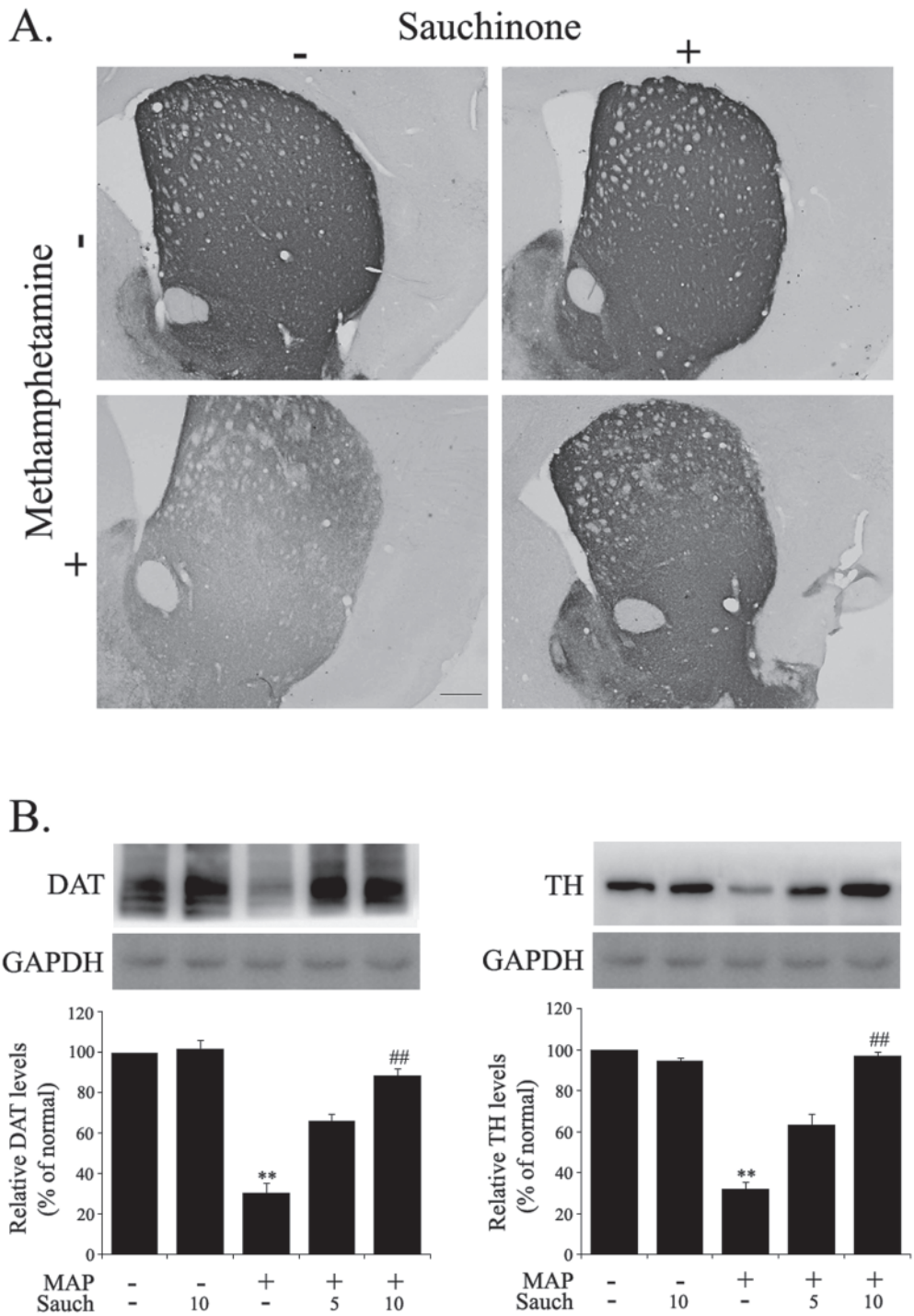

Fig. 1. Effect of sauchinone on dopaminergic neuron terminal damage induced by MAP. A) Representative photomicrographs of DAT-immunoreactive signals in the mouse striatum (40 ×). Scale bar = 200 μm. B) Quantification of DAT and TH expression by western blotting. The intensity of the positive signal was normalized with that of GAPDH. The relative intensity is expressed as a percentage of the normal group [i.e., no MAP or sauchinone (Sauch) treatment]. The values given are the mean ± S.E.M. (n = 5 – 7). Data were analyzed using Tukey's test. $^{**}P < 0.001$, normal vs. MAP; $^{\#\#}P < 0.001$, MAP vs. MAP + sauchinone (10 mg/kg).

sauchinone alone.

Because repeated MAP administration is known to induce activation of glial cells in the striatum (4), we examined the effects of sauchinone on the MAP-induced activation of microglial cells (Fig. 2). Repeated MAP injections significantly increased the number of CD11b-positive activated microglial cells in the striatum 3 days after the final injection (Fig. 2A). However, pre-treatment with a 10 mg/kg dose of sauchinone inhibited the MAP-induced CD11b expression of activated microglial cells in the striatum (Fig. 2A). There were no changes in the number of CD11b-positive microglial cells in the striatum after sauchinone treatment alone. The western blot data were consistent with those of the immunohistochemistry. Repeated MAP injections induced about 500% increase in CD11b and GFAP protein expression, whereas

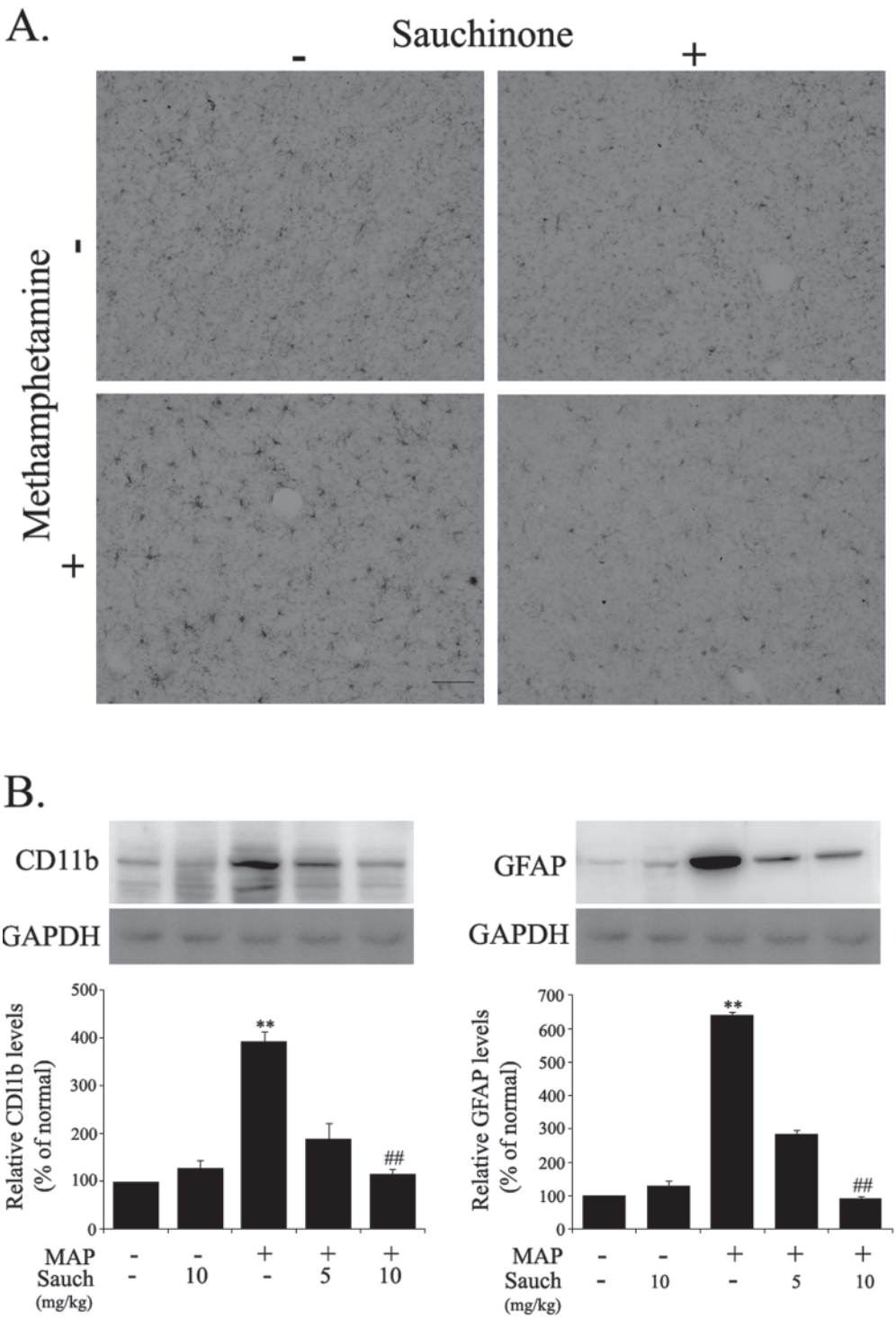

Fig. 2. Effect of sauchinone on MAP-induced glial cell activation. A) Representative photomicrographs of CD11b-immunoreactive signals in the mouse striatum (40 ×). Scale bar = 200 μm. B) Quantification of GFAP and CD11b expression by western blotting. The intensity of the positive signal was normalized with that of GAPDH. The relative intensity is expressed as a percentage of the normal group [no MAP or sauchinone (Sauch) treatment]. The values given are the mean ± S.E.M. (n = 5 – 7). Data were analyzed using Tukey's test. $^{**}P < 0.001$, normal vs. MAP; $^{\#\#}P < 0.001$, MAP vs. MAP + sauchinone (10 mg/kg).

sauchinone treatment blocked this increase in CD11b and GFAP protein expression induced by MAP (Fig. 2B). There were no significant changes in CD11b or GFAP expression after sauchinone treatment alone.

As shown in Fig. 3A, repeated MAP injections stimulated the expression of iNOS and nNOS proteins. Pretreatment with sauchinone inhibited the increase in MAP-induced iNOS protein expression. However, sauchinone treatment had little effect on MAP-induced nNOS protein expression. In addition, repeated MAP injections increased NO production in the striatum of mice, and sauchinone blocked this increase of NO production in the MAP-treated striatum (Fig. 3B).

A major finding of the current study is that sauchinone

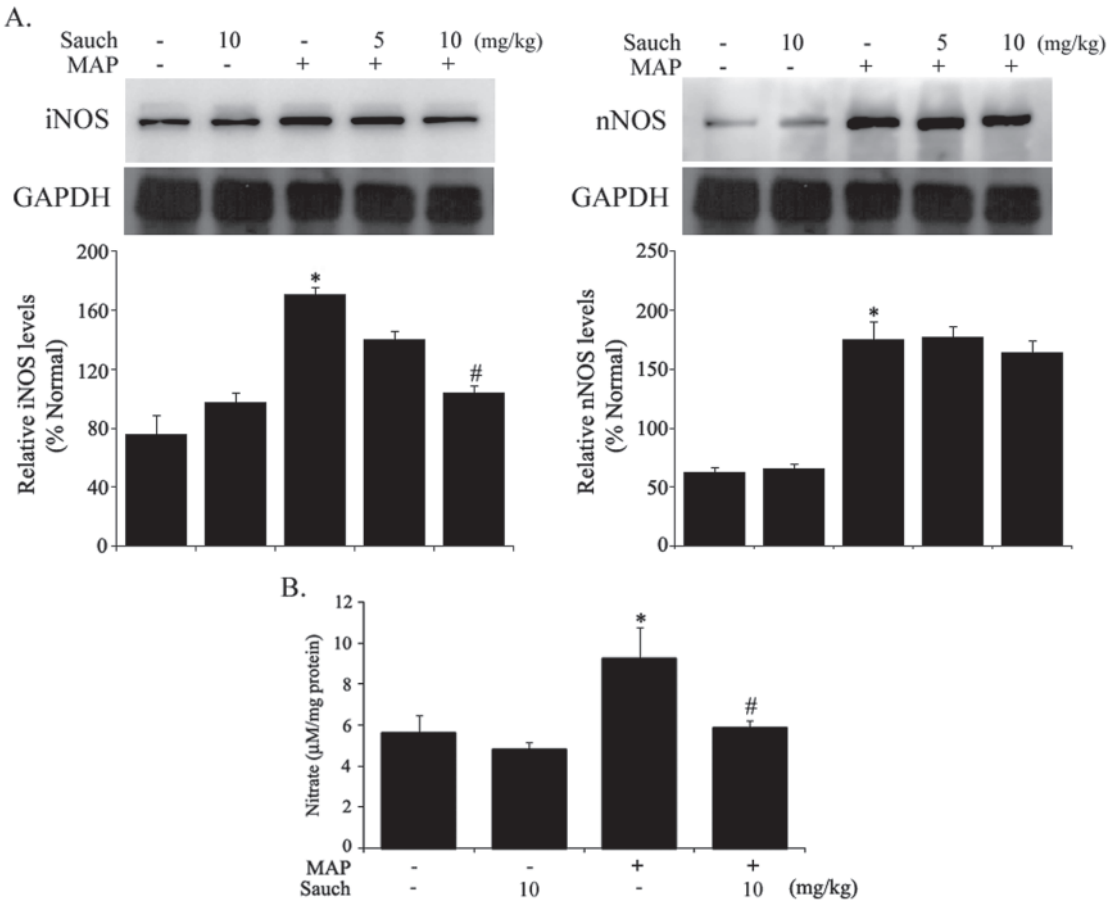

Fig. 3. Effect of sauchinone on the expression of NOS and the production of NO in the mouse. A) Quantification of iNOS and nNOS expression by western blotting. The intensity of the positive signal was normalized with that of GAPDH. The relative intensity is expressed as a percentage of the intensity in the normal group [no MAP or sauchinone (Sauch) treatment]. The values given are the mean ± S.E.M. (n = 5 – 7). B) Quantification of NO production data. The values given are the mean ± S.E.M. (n = 5 – 7). Data were analyzed using a one-way ANOVA followed by Tukey's test. $^{*}P < 0.005$, normal vs. MAP; $^{\#}P < 0.005$, MAP vs. MAP + sauchinone (10 mg/kg).

treatment prior to multiple injections of MAP prevented MAP-induced striatal degeneration of DA neurons. In animal models, MAP intoxication causes morphological and structural brain abnormalities and long-lasting depletions of DAergic nerve endings, resulting in a reduction in DAT and TH immunoreactivity compared to controls (4). In addition, repeated MAP injections induce the activation of glial cells, indicated by the up-regulation of GFAP and CD11b expression. Consistent with previous reports, our results showed that repeated MAP administration increased GFAP and CD11b levels and reduced striatal DAT and TH levels. However, sauchinone treatment significantly prevented these depletions of DAergic nerve endings and activations of glial cells in the mouse striatum. In particular, sauchinone decreased the accumulation of microglia cells in the MAP-treated striatum.

The mechanisms of MAP-induced neurotoxicity are not well understood. However, accumulating evidence suggests that the production of NO plays a critical role in MAP-induced neurotoxicity (12). The NOS inhibitor 7-nitroindazole (13) and several flavonoids, which can attenuate iNOS expression (14), can block MAP-induced neurotoxicity. Sauchinone has been known to suppress LPS-induced NO production through a blockade of iNOS expression (3). In the current study, repeated MAP injections triggered an increase in striatal NO production, and sauchinone suppressed this increase in NO production. Inhibition of NO production by sauchinone was accompanied by a decrease of iNOS protein expression. Thus, these results suggest that the protective effects of ISL against MAP-induced neurotoxicity may be due to its suppressive effects on oxidative stress, which were mediated by the repression of NO production through a blockade of iNOS expression.

In conclusion, sauchinone protected DA end terminals

from MAP-induced neurotoxicity via suppression of NO production in the striatum. Therefore, we suggest the potency of sauchinone as a novel therapeutic agent to prevent MAP-induced neurotoxicity.

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