Mitochondrial dysfunction and increased oxidative stress have been shown to be implicated in dopaminergic cell degeneration in Parkinson’s disease (Jenner, 2003). In this disease, the major mitochondrial defect seems to be associated with complex I at the electron transport chain. Impairment of complex I activity leads to excess ROS formation, which causes mitochondrial dysfunction and cell death (Fl-eury et al., 2002; Jenner, 2003). Implication of oxidative stress in the pathogenesis and progression of Parkinson’s disease is supported by the decrease in GSH content, increase in levels of lipid peroxidation products, increased production of ROS, and increase in iron content in substantia nigra (Olanow and Tatton, 1999).

MPTP produces an irreversible and severe parkinsonian-like syndrome in human and nonhuman primates (Przedborski and Jackson-Lewis, 1998; Przedborski et al., 2000). The inhibition of complex I in the mitochondrial electron transport chain induced by MPP⁺, the active metabolite of MPTP, results in impaired ATP production, loss of mitochondrial membrane potential, and formation of ROS (Cassarino et al., 1997; Schulz et al., 1997). The membrane permeability transition of mitochondria is known as a central event in the course of toxic and oxidative forms of cell injury, as well as apoptosis (Mignotte and Vaysseïre, 1998). Along with respiratory chain inhibition, MPP⁺-induced neuronal cell death is suggested to be mediated by formation of the mitochondrial permeability transition, which leads to the release of cytochrome c and activation of caspases, and by disturbance of intracellular Ca²⁺ homeostasis (Cassarino et al., 1999; Lee et al., 2002, 2005).

Licorice root is a traditional herbal remedy that has been used for the treatment of various pathologic conditions, in-
including chronic hepatitis and gastric ulcer (Shibata, 2000). Increasing evidence indicates that glycyrrhizin, a triterpenoid saponin found in *Glycyrrhiza glabra*, and its hydrolyzed metabolite, 18β-glycyrrhetinic acid (GA), reveal anti-inflammatory, antiancancer, and antipathotoxic effects (Jeong et al., 2002; Matsui et al., 2004; Agarwal et al., 2005). GL and GA have antioxidant ability and reduce oxidative damage due to carbon tetrachloride, tert-butyl hydroperoxide, or ischemia-reperfusion injury (Nagai et al., 1991; Jeong et al., 2002; Kinjo et al., 2003). GA attenuates tumor necrosis factor-α, lipopolysaccharide-induced cell death in human hepatoblastoma cell line, and cultured liver cells (Yoshikawa et al., 1999; Zheng and Lou, 2003).

Mitochondrial dysfunction, increased oxidative stress, and inflammation are involved in neurodegenerative process in Parkinson’s disease (Olanow and Tatton, 1999; Jenner, 2003). Therefore, the compounds that have properties, such as mitochondrial function enhancement, anti-inflammatory activity, and antioxidant ability may provide the neuroprotective effect on the degeneration of striatal dopaminergic neurons (Bonuccelli and Del Dotto, 2006). GA and GL are known to exhibit an antioxidant and anti-inflammatory effect. However, the effect of licorice compounds against neuronal cell injury due to exposure of parkinsonian toxin MPP⁺ remains uncertain. Therefore, the aim of the present study was to assess the preventive effect of GL and GA against the neurotoxicity of MPP⁺ in relation to the mitochondria-mediated cell death process and role of oxidative stress.

**Materials and Methods**

Materials. TiterTACS colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD), Quantitine M rat/mouse cytochrome c assay kit were from R&D systems (Minneapolis, MN), anti-cytochrome c (A-8) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), horseradish peroxidase-conjugated anti-mouse IgG was from EMD-Calbiochem (La Jolla, CA), SuperSignal West Pico chemiluminescence substrate for cytochrome *c* from each sample. PC12 cells (5 x 10⁶ cells/ml) were treated with MPP⁺ for 24 h at 37°C, and the pellets were suspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (BD Biosciences, San Jose, CA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

**Measurement of Apoptosis in Cells.** Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. PC12 cells (1 x 10⁶) were treated with MPP⁺ for 24 h at 37°C, washed with phosphate-buffered saline (PBS), and fixed with formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3’ ends of DNA fragments using terminal deoxynucleotidyl transferase, and the nucleotide was detected using a streptavidin-horseradish peroxidase and TACS-Sapphire according to the TiterTACS protocol. Data were expressed as absorbance at 450 nm.

**Flow-Cytometric Measurement of Mitochondrial Transmembrane Potential.** Changes in the mitochondrial transmembrane potential during the MPP⁺-induced apoptosis in PC12 cells were quantified by flow cytometry with the cationic lipophilic dye DiOC₆(3) (Berthier et al., 2004). Cells (1 x 10⁶/ml) were treated with neurexin toxin for 24 h at 37°C, DiOC₆(3) (40 nM) was added to the medium, and cells were incubated for 15 min at 37°C. After centrifugation at 412g for 10 min, the supernatants were removed, and the pellets were suspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (BD Biosciences, San Jose, CA) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

**Measurement of Cytochrome *c* Release.** The release of cytochrome *c* from mitochondria into the cytosol was assessed by performing Western blot analysis and a solid phase, enzyme-linked immunosorbent assay. PC12 cells (5 x 10⁶ cells/ml for Western blotting and 5 x 10⁶ cells/ml for ELISA) were harvested by centrifugation at 412g for 10 min, washed twice with PBS, suspended in buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.5 mM diithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES-KOH at pH 7.5) and homogenized further by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at 100,000g for 30 min, and the supernatant was used for analysis of cytochrome *c*. Protein concentration was determined by the method of Bradford according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA).

For Western blotting, supernatants were mixed with SDS-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min. Samples (30 µg protein/well) were loaded onto each lane of 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (GE Healthcare Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% nonfat dried milk. The membranes were labeled with anti-cytochrome *c* (diluted 1:1000 in TBS containing 0.1% Tween 20) overnight at 4°C with gentle agitation. After four washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) for 2 h at room temperature. Protein bands were treated with SuperSignal West Pico chemiluminescence substrate and identified by detecting the enhanced chemiluminescence in Luminescent image analyzer (Lite for Las-1000 plus version 1.1; Fuji Photo Film Co., Tokyo, Japan).

For ELISA for cytochrome *c*, the supernatants and cytochrome *c* conjugate were added into the 96-well microplates coated with monoclonal antibody specific for rat/mouse cytochrome *c*. The procedure formazan. Absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340; Molecular Devices Co., Sunnyvale, CA). Cell viability was expressed as a percentage of the value in control cultures.

**Morphological Observation of Nuclear Change.** PC12 cells (1 x 10⁶ cells/ml) were treated with MPP⁺ for 24 h at 37°C, and the nuclear morphological change was assessed using the Hoechst dye 33258 (Oberhammer et al., 1992). Cells were incubated with 1 µg/ml Hoechst 33258 for 3 min at room temperature, and nuclei were observed using an Olympus Microscope with a WU excitation filter (Olympus, Tokyo, Japan).
was performed according to the manufacturer's instructions. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding diluted solutions of cytochrome c standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as nanograms/milliliter by reference to the standard curve.

Measurement of Caspase-3 Activity. The activation of caspase-3 that occurred during the apoptotic process in cells was assessed (Migmotte and Vayssiere, 1998). PC12 cells (2 × 10⁶ cells/ml) were pretreated with rotenone for 24 h at 37°C, and caspase-3 activity was determined according to the user's manual for the ApoAlert CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) and incubated for 1 h at 37°C. Absorbance of the chromophore p-nitroanilide produced was measured at 405 nm. The standard curves were obtained from the absorbance of p-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of p-nitroanilide.

Measurement of Intracellular ROS Formation. The dye DCFH₂-DACH, which is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu et al., 1998). PC12 cells (4 × 10⁶) were treated with MPP⁺ for 24 h at 37°C, washed, suspended in PBS-free RPMI 1640 medium, incubated with 50 μM dye for 30 min, and washed with PBS. The cell suspensions were centrifuged at 412 g for 10 min, the medium were removed, and cells were dissolved with 1% Triton X-100. Fluorescence changes were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (Spectrafluor; TECAN, Salzburg, Austria).

Measurement of Total Glutathione. The total glutathione (reduced form GSH + oxidized form glutathione disulfide) was determined using glutathione reductase (van Klaveren et al., 1997). PC12 cells (4 × 10⁶) were treated with MPP⁺ for 24 h at 37°C and centrifuged at 412 g for 10 min in a microplate centrifuge, the media were removed, and cells were washed twice with PBS. Cells were dissolved with 2% 5-sulfosalicylic acid (100 μl) and incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM 5,5'-dithio-bis-(2-nitrobenzoic acid), and 105 mM NaH₂PO₄, pH 7.5, at 37°C. Glutathione reductase (20 μl of 100 U/ml) was added, and the mixture was incubated for an additional 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples.

Statistical Analysis. Data are expressed as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

Results

Prevention of MPP⁺-Induced Cell Death and Nuclear Damage by Licorice Compounds. The preventive effect of licorice compounds GL and GA on the cytotoxicity of MPP⁺ was assessed in PC12 cells that are differentiated by nerve growth factor. The incidence of cell death after exposure to 500 μM MPP⁺ for 24 h was approximately 46%. GL (1–100 μM) significantly reduced the 500 μM MPP⁺-induced cell death with a maximal inhibitory at 50 μM; beyond this concentration, the inhibitory effect declined (Fig. 1). We also examined the effect of GA, a metabolite of GL, against the MPP⁺-induced cell viability loss in PC12 cells. The current data revealed that GA showed a maximal inhibitory effect at 10 μM; beyond this concentration, the inhibitory effect declined (Fig. 1). Although GA at 100 μM and GA at 25 μM caused approximately 3 and 11% cell death, respectively, they prevented the cell death due to MPP⁺. The preventive effect of licorice compounds against cell death was also observed in PC12 cells treated with rotenone, a mitochondrial complex I inhibitor. Treatment with 2.5 μM rotenone for 24 h caused approximately 44% cell death. As expected, GL and GA significantly reduced the rotenone-induced cell death and exhibited a maximal inhibitory effect at 10 and 5 μM, respectively (Fig. 2).

To examine whether the toxic effect of MPP⁺ against PC12 cells is mediated by oxidative stress, we assessed the effect of various scavengers. Treatment with 1 mM thiol compound N-acetylcysteine, 10 μg/ml catalase (a scavenger of hydrogen peroxide), 20 μM Trolox (a scavenger of hydroxyl radicals and peroxyxinitrite), 25 μM carboxy-PTIO (a scavenger of nitric oxide), and 30 μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics superoxide dismutase) prevented cell death due to 500 μM MPP⁺ exposure (Fig. 3).

To assess apoptotic cell death due to MPP⁺ and clarify the preventive effect of licorice compounds against the cytotoxicity of MPP⁺, we investigated the effect of GL and GA on the nuclear morphological changes observed in the MPP⁺-treated cells. Nuclear staining with Hoechst 33258 demonstrated that control PC12 cells had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were demonstrated in

Fig. 1. Prevention of MPP⁺-induced cell death by licorice compounds. PC12 cells were pretreated with licorice compounds (1–100 μM GL in A or 1–25 μM GA in B) for 15 min and exposed to 500 μM MPP⁺ for 24 h, and cell viability was determined. Data represent mean ± S.E.M. (n = 6). *, P < 0.05 compared to control (percentage of control); **, P < 0.05 compared to MPP⁺ alone.
cells treated with 500 μM MPP⁺. As seen in the current data, the MPP⁺-induced nuclear damage was attenuated by the addition of 50 μM GL and 10 μM GA (Fig. 4A).

During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. To clarify the preventive effect of licorice compounds on the MPP⁺-induced nuclear damage, we further assessed the protective effect by performing the quantitative analysis for DNA fragmentation. The amount of fragmented DNA was measured by monitoring the binding of dNTP to the 3’ ends of DNA fragments and detected by a quantitative colorimetric assay. PC12 cells were treated with 500 μM MPP⁺ in the presence or absence of licorice compounds. Control cells showed absorbance of 0.219 ± 0.006 (mean ± S.E.M. of six experiments), whereas exposure to 500 μM MPP⁺ for 24 h increased the absorbance approximately 2.5-fold (Fig. 4B). The addition of GL and GA (50 and 10 μM each) significantly prevented the fragmentation of DNA due to MPP⁺ exposure.

Preventive Effect of Licorice Compounds on MPP⁺-Induced Changes in Mitochondrial Membrane Permeability. We assessed the preventive effect of licorice compounds against the MPP⁺-induced cell viability loss by investigating the effect on changes in the mitochondrial membrane permeability. Changes in the mitochondrial transmembrane potential in PC12 cells exposed to MPP⁺ were quantified by flow cytometry with the dye DiOC₆(3). When PC12 cells were treated with 500 μM MPP⁺ for 24 h, the percentage of cells with depolarized mitochondria (characterized by low values of the transmembrane potential) increased. As seen in the present data, GL and GA (50 and 10 μM each) significantly attenuated the MPP⁺-induced increase in cells with depolarized mitochondria (Fig. 5A).

Opening of the mitochondrial permeability transition pore causes the release of cytochrome c from mitochondria into the cytosol and subsequent activation of caspases as one of the mitochondria-mediated cell death signaling events (Kim et al., 2006). The MPP⁺-induced change in the mitochondrial membrane permeability was assessed by measuring the cytochrome c release and caspase-3 activation. In Western blot analysis, PC12 cells treated with MPP⁺ for 24 h showed an increase in the cytosolic cytochrome c levels. Addition of 50 μM GL and 10 μM GA prevented the MPP⁺-induced increase in cytochrome c levels (Fig. 5B). We confirmed the preventive effect of licorice compounds on the MPP⁺-induced cytochrome c release by performing the ELISA-based quantitative analysis. The present data revealed that GL (10 and 50 μM) and GA (1 and 10 μM) significantly attenuated the release of cytochrome c (Fig. 5B). We examined the activation of caspase-3 in PC12 cells exposed to MPP⁺. Cells treated with MPP⁺ exhibited an increase in caspase-3 activity, whose response was significantly depressed by the addition of 10 to 50 μM GL and 1 to 10 μM GA (Fig. 5C). Treatment
with 50 μM GL or 10 μM GA alone did not cause significant changes in cytochrome c release and caspase-3 activity.

**Prevention of MPP⁺-Induced Formation of ROS and Depletion of GSH by Licorice Compounds.** To determine whether the preventive effect of licorice compounds against the cytotoxicity of MPP⁺ was ascribed to the depressant effect on oxidative stress, we investigated the effect on the formation of ROS and the depletion of GSH in PC12 cells. The formation of ROS within cells was determined by monitoring a conversion of DCFH₂-DA to DCF. PC12 cells treated with 500 μM MPP⁺ showed a significant increase in DCF fluorescence. As seen in cell death findings, GL and GA (1–100 μM) prevented the MPP⁺-induced increase in DCF fluorescence and showed a maximal inhibitory effect at 50 and 10 μM, respectively; beyond these concentrations, the inhibitory effect declined (Fig. 6).

Reduction of cellular GSH levels increases the sensitivity of neurons to toxic insults and induces changes in mitochondrial function (Hall, 1999). The work determined whether the preventive effect of licorice compounds on the cytotoxicity due to MPP⁺ exposure was ascribed to the inhibitory effect on the depletion of GSH. The thiol content in the control PC12 cells was 5.33 ± 0.12 nmol/mg protein. Treatment with 500 μM MPP⁺ for 24 h depleted GSH contents by 47%. The present data showed that GL and GA (1–100 μM) significantly prevented the MPP⁺-induced depletion of GSH, and GL had a maximal inhibitory at 50 μM and GA at 10 μM, respectively (Fig. 7). Similar to their effects on cell death and...
formation of ROS, beyond those concentrations, the inhibitory effect declined. Although GA at 100 μM and GA at 25 μM caused decrease in GSH contents by approximately 3 and 14%, respectively, they prevented the MPP⁺-induced depletion of GSH.

**Discussion**

The toxic effect of MPP⁺ was assessed in differentiated PC12 cells by measuring the nuclear damage, mitochondrial dysfunction, and cell death. PC12 cells upon the nerve growth factor stimulation not only display abundant neuritic growth but also adopt a neurochemical dopaminergic phenotype (Kadota et al., 1996). Formation of the mitochondrial permeability transition causes a release of cytochrome c from mitochondria and subsequent activation of caspase-3 that is involved in apoptotic cell death (Mignotte and Vayssieres, 1998). In PC12 cells treated with MPP⁺, the condensation and fragmentation of nuclei and significant increase in caspase-3 activity indicate apoptotic cell death. MPP⁺ induces apoptosis in neuronal cells by causing loss of the mitochondrial transmembrane potential, leading to the release of mitochondrial cytochrome c and subsequent activation of caspase-3 (Cassarino et al., 1999; Lee et al., 2005). Consistent with this finding, in this study, the MPP⁺-induced apoptotic cell death seemed to be mediated by the loss of the mitochondrial membrane potential and cytochrome c release that resulted in activation of caspase-3.

Licorice compound GA attenuates the tumor necrosis factor-induced apoptotic cell death without cell surface Fas activation (Yoshikawa et al., 1999). However, it has been shown that GA induces cell death in various cancer cell lines (Hi-basami et al., 2006). Furthermore, GL enhances Fas-mediated apoptosis without alteration of caspase-3-like activity (Ishiwata et al., 1999). In addition, it is uncertain whether the preventive effect of licorice compounds comes from their inhibitory action on formation of the mitochondrial permeability transition. Therefore, the present study was conducted to assess the effect of GL and GA against the MPP⁺-induced cell damage in relation to the mitochondrial-mediated cell death process. As seen in the present data, GL up to 100 μM and its metabolite GA up to 25 μM significantly prevented the MPP⁺-induced cell viability loss in PC12 cells. Although there is a difference of inhibitory potency on the basis of concentration, a metabolite GA as well as GL exhibits a cytoprotective effect. The protective effect of GL and GA against cell damage was also detected in the rotenone-treated PC12 cells. Intravenous administration of GL achieved maximal plasma concentration of 36 μM in healthy men and 120 μM in hepatitis patients (Yamamura et al., 1992; van Rossum et al., 1999). In patients with chronic hepatitis, the administration with GL suppository (a mixture of 300 mg glycyrrhizinic ammonium salt and 60 μg of sodium capric acid) yielded approximately 6 μM serum GL levels (Fujikura et al., 2003). Oral administration of licorice achieved 0.4 to 10 μM plasma GA levels in humans (de Groot et al., 1988; Yamamura et al., 1992). The current work was performed under acute exposure to high concentration of MPP⁺. Therefore, at the concentrations achievable in humans, both compounds seemed to exert an effective depressant effect against the neurotoxicity of parkinsonian toxins, such as MPP⁺ and rotenone. The present data suggest that GL less than 100 μM and GA less than 25 μM may prevent the toxicity of MPP⁺ against PC12 cells by suppressing loss of the mitochondrial transmembrane potential, release of mitochondrial cytochrome c, and activation of caspase-3. Meanwhile, the toxicity at the high concentrations seems to nullify the protective effect of licorice compounds.

The MPP⁺ treatment causes the respiratory chain inhibition, leading to the formation of ROS and nitrogen species (Jenner, 2003). ROS act upon mitochondria, causing a disruption of mitochondrial membrane potential and the release of cytochrome c (Fleury et al., 2002). The formation of ROS in PC12 cells exposed to MPP⁺ and the inhibitory effect of antioxidants, including Trolox and carboxy-PTIO, suggest that MPP⁺ induces the formation of ROS and nitrogen species that is involved in mitochondrial dysfunction and cell death. Meanwhile, the inhibitory effect of catalase indicates that the cytotoxicity of MPP⁺ seems to be mediated by extracellularly released hydrogen peroxide, a cell-permeable oxidant. GL and GA attenuate either renal injury due to ischemia-reperfusion or FeCl₂ plus ascorbate-induced lipid peroxidation in liver homogenates through scavenging action on free radicals (Yokozawa et al., 2000; Jeong et al., 2002). However, GA induces apoptosis of HL60 cells by increasing production of ROS (Makino et al., 2006). These reports represent uncertainty whether licorice compounds have antioxidant ability and reveal a cytoprotective effect. We examined whether the preventive effect of licorice compounds against the mitochondrial damage was ascribed to their inhibitory action on oxidative stress. An increased ROS production causes changes in the levels of intracellular antioxidants,
such as GSH, NADH, or NADPH, that result in impairment of mitochondrial function (Fleury et al., 2002). The oxidation at two separate sites.

The inhibitory effect of licorice compounds on the MPP+-induced viability loss in PC12 cells by suppressing the mitochondrial permeability transition pore, leading to the activation of caspase-3. The preventive effect may be accomplished by the inhibitory action on the formation of ROS and depletion of GSH. The findings suggest that licorice compounds seem to exhibit a protective effect against neuronal cell injury, which is mediated by oxidative mitochondrial damage. GL and GA may provide a beneficial protective effect against the neurotoxicity due to exposure of parkinsonian toxins.

References


