

Protective Effects of the Antiparkinsonian Drugs Talipexole and Pramipexole against 1-Methyl-4-phenylpyridinium-Induced Apoptotic Death in Human Neuroblastoma SH-SY5Y Cells

YOSHIHISA KITAMURA, TADASHI KOSAKA, JUN-ICHI KAKIMURA, YASUJI MATSUOKA, YASUKO KOHNO, YASUYUKI NOMURA, and TAKASHI TANIGUCHI

Department of Neurobiology (Y.Ki., T.K., J.K., Y.M., T.T.), Kyoto Pharmaceutical University, Kyoto 607-8412, Japan, Product Management Department (Y.Ko.), Marketing Division, Nippon Boehringer Ingelheim, Hyogo 666-0193, Japan, and Department of Pharmacology (Y.N.), Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

Received April 28, 1998; Accepted August 28, 1998

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Treatment of human neuroblastoma SH-SY5Y cells with 1 mM 1-methyl-4-phenylpyridinium (MPP⁺) for 3 days induced production of reactive oxygen species (ROS), followed by caspase-3 activation, cleavage of poly(ADP-ribose) polymerase (PARP), and apoptotic cell death with DNA fragmentation and characteristic morphological changes (condensed chromatin and fragmented nuclei). Simultaneous treatment with 1 mM talipexole slightly inhibited the MPP⁺-induced ROS production and apoptotic cell death. In contrast, pretreatment with 1 mM talipexole for 4 days markedly protected the cells against MPP⁺-induced apoptosis. However, this protective effect might not be mediated by dopamine receptors. The talipexole pretreatment induced an increase in antiapoptotic Bcl-2 protein level but had no effect on levels of proapoptotic Bax, Bak, and Bad. It also inhibited MPP⁺-induced ROS production, p53 ex-

pression, and cleavages of caspase-3 and PARP. Similarly, pramipexole pretreatment increased Bcl-2 and inhibited MPP⁺-induced apoptosis. Although pretreatment with bromocriptine also had a protective effect against MPP⁺-induced apoptosis, it had no effect on the protein levels of Bcl-2 family members. On the other hand, N⁶,2'-O-dibutyl cAMP or calphostin C induced a decreased Bcl-2 level and enhanced MPP⁺-induced cell death. These results suggest that talipexole has dual actions: (1) it directly scavenges ROS, affording slight protection against MPP⁺-induced apoptosis, and (2) it induces Bcl-2 expression, thereby affording more potent protection, if it is administered before MPP⁺. Pramipexole has similar effects, whereas bromocriptine seems to exhibit the former but not the latter effect.

MPTP produces an irreversible and severe parkinsonian-like syndrome that causes selective degeneration of the nigrostriatal dopaminergic neurons in humans (Davis *et al.*, 1979; Langston *et al.*, 1983). This neurotoxin has been used to create animal models of Parkinson's disease (Langston and Irwin, 1986). MPTP is converted by monoamine oxidase B in glial cells such as astrocytes to MPP⁺, which is accumulated intracellularly in neurons via a dopaminergic transporter (Snyder and D'Amato, 1986). Thus, MPP⁺ is an active metabolite of MPTP and a neurotoxin for dopaminergic neurons. The MPP⁺-induced neuronal death is caused by apoptosis in rat mesencephalic and striatal neurons (Mochizuki *et al.*, 1994) and human neuroblastoma SH-SY5Y cells (Itano and Nomura, 1995). It also has been shown that apoptotic death

of neurons occurs in the brain of patients with Parkinson's disease (Mochizuki *et al.*, 1996). The characteristic morphological and biological features of apoptosis are cellular shrinkage, membrane blebbing, chromatin condensation, and fragmentation of chromatin DNA into nucleosomal fragments of ~180 bp.

In recent animal studies, dopamine receptor agonists such as bromocriptine and pergolide, which are used for symptomatic therapy of Parkinson's disease, showed possible neuroprotective effects under a variety of neurodegenerative conditions (Lange *et al.*, 1994; Liu *et al.*, 1995). Great interest, therefore, is focused on the neuroprotective effects of dopamine receptor agonists as candidates for the current and future treatment of Parkinson's disease. The human neuroblastoma cell line SH-SY5Y, which was subcloned from the SK-N-SH cell line, often is used as a model of human dopaminergic neurons (Itano and Nomura, 1995; Ross and Biedler, 1985). The azepine

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture in Japan (Y. Ki, Y.M., Y.N., T.T.).

ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; diBu-cAMP, N⁶,2'-O-dibutyl cAMP; C-DCDHF-DA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester; MPP⁺, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; ANOVA, analysis of variance.

derivatives talipexole (Arnt *et al.*, 1986) and pramipexole (Mierau *et al.*, 1995) are agonists for D_2/α_2 receptors and D_2/D_3 receptors, respectively, both lacking the ability to stimulate the D_1 receptor. In the current study, we examined the protective effects of talipexole and pramipexole, which are novel drugs for the treatment of Parkinson's disease, along with bromocriptine, which is the most widely used ergot-derivative dopamine receptor agonist, against MPP⁺-induced apoptotic death in human neuroblastoma SH-SY5Y cells.

Experimental Procedures

Materials. Talipexole (B-HT 920CL₂; 6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]azepine dihydrochloride) and pramipexole [SND 919CL₂Y; (-)-2-amino-4,5,6,7-tetrahydro-6-(propylamino)benzothiazole dihydrochloride] were obtained from Boehringer-Ingelheim (Ingelheim, Germany). Bromocriptine mesilate was kindly donated by

Sandoz Pharma A.G. (Basel, Switzerland). MPP⁺ and dopamine receptor antagonists were from Research Biochemicals International (Natick, MA). diBu-cAMP was from Wako (Osaka, Japan). Calphostin C was from Kyowa Hakko Kogyo (Tokyo, Japan). PMA was from Sigma Chemical (St. Louis, MO). MTT was from Dojindo Laboratories (Kumamoto, Japan). Hoechst 33258 bis-benzimide (H-1398) and C-DC-DHF-DA (C-2938) were from Molecular Probes (Eugene, OR).

Primary antibodies included mouse monoclonal antibodies to human Bcl-2 (clone 124, M887) from DAKO (Copenhagen, Denmark), human Bak (clone TC100, AM03) from Oncogene Research Products (Cambridge, MA), mouse Bad (clone 48, B36420) and human caspase-3 (clone 19, C31720) from Transduction Laboratories (Lexington, KY), human p53 (DO-1, sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit polyclonal antibodies to human Bcl-x (B22630) from Transduction Laboratories, human Bax (N-20, sc-493) from Santa Cruz Biotechnology, proenzyme/active fragments of human caspase-3 (65906E) from PharMingen (San Diego, CA), and the caspase-3 cleavage site of human PARP (06-557) from Upstate Biotechnology (Lake Placid, NY). An enhanced chemiluminescent detection system (ECL kit) from Amersham (Buckinghamshire, England) was used for immunodetection.

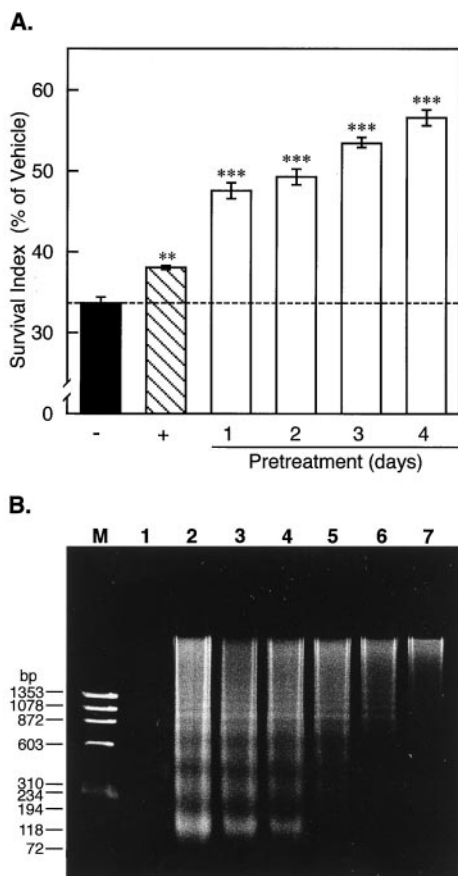


Fig. 1. Effects of simultaneous treatment and pretreatment with talipexole on MPP⁺-induced cell death (A) and DNA fragmentation (B). SH-SY5Y cells were treated without (100% activity, lane 1) or with 1 mM MPP⁺ for 3 days in the absence (black column, lane 2) or presence (hatched column, lane 3) of 1 mM talipexole. Alternatively, cells were pretreated with 1 mM talipexole for 1–4 days before treatment for 3 days with 1 mM MPP⁺ in the absence of talipexole (B, lanes 4–7, respectively). A, Cell survival was measured by MTT assay. Each value is the mean \pm standard error (%) of five determinations, based on the vehicle control as 100%. The F value in ANOVA was $F(5,24) = 129.589$ ($p < 0.0001$). In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. **, $p < 0.01$; ***, $p < 0.001$ versus no pretreatment with talipexole (-) (Bonferroni/Dunn test). B, DNA fragmentation in $\sim 2 \times 10^6$ cells was assessed. M, DNA size markers. Simultaneous treatment with talipexole afforded slight but significant protection against cell death (A, hatched column) and inhibition of DNA fragmentation (B, lane 3). Pretreatment with talipexole markedly inhibited MPP⁺-induced cell death and DNA fragmentation.

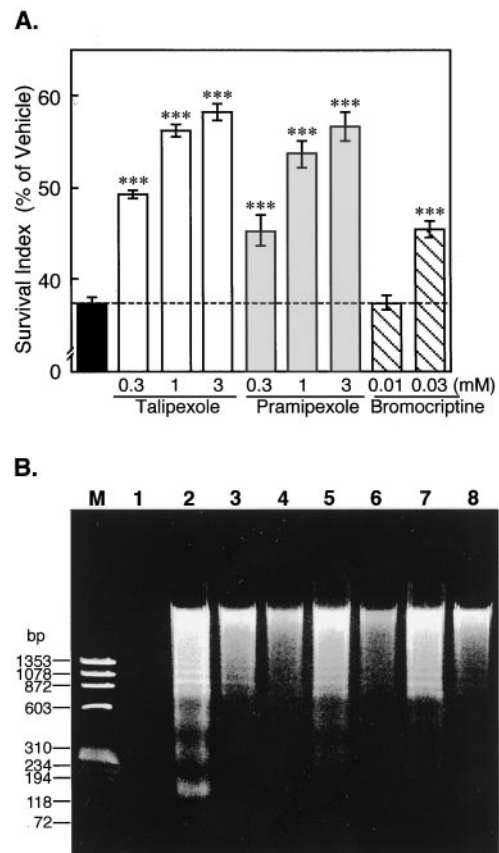


Fig. 2. Protective effects of pretreatment with talipexole, pramipexole, and bromocriptine on MPP⁺-induced cell death and DNA fragmentation. SH-SY5Y cells were pretreated for 4 days with vehicle (black column), talipexole (at 0.3, 1, or 3 mM; open column), pramipexole (at 0.3, 1, or 3 mM; gray column), or bromocriptine (at 0.01 or 0.03 mM; hatched column) and then further treated with 1 mM MPP⁺ for 3 days. A, Cell survival was measured by MTT assay. Each value is the mean \pm standard error (%) of six determinations, based on the vehicle control as 100%. The F value in ANOVA was $F(8,45) = 86.520$ ($p < 0.0001$). In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. ***, $p < 0.001$ versus pretreatment with vehicle (black column; Bonferroni/Dunn test). B, DNA fragmentation in $\sim 2 \times 10^6$ cells was assessed: no treatment (lane 1); MPP⁺ treatment after pretreatment with vehicle (lane 2), talipexole at 1 and 3 mM (lanes 3 and 4), pramipexole at 1 and 3 mM (lanes 5 and 6), or bromocriptine at 0.01 and 0.03 mM (lanes 7 and 8), respectively. M, DNA size markers.

Cell culture and treatment with drugs. The human neuroblastoma cell line SH-SY5Y (Ross and Biedler, 1985) was used in the current experiments. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 50 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin and were kept at 37° in humidified 5% $\text{CO}_2/95\%$ air. SH-SY5Y cells were treated with drugs as follows. (1) For simultaneous treatment, SH-SY5Y cells were simultaneously treated for 24 hr or 3 days with 1 mM MPP^+ and 1 mM talipexole. (2) For pretreatment, the cells were pretreated for 1–4 days with vehicle, talipexole, pramipexole, bromocriptine, diBucAMP, calphostin C, or PMA in the absence or presence of a dopaminergic antagonist. The cells then were washed with fetal calf serum-free Dulbecco's modified Eagle's medium and further treated with 1 mM MPP^+ for 3 days in the absence of drugs. Treated cells were subjected to cell survival assay, DNA fragmentation analysis, immunoblotting, and fluorescence measurement.

MTT assay. MTT is converted in living cells to formazan, which has a specific absorption maximum. After pre- and post-treatments of SH-SY5Y cells as described, the culture medium was changed to the medium containing 5 mg/ml MTT, and the cells were incubated further for 4 hr. Then, they were mixed thoroughly with an equal volume of isopropanol/0.04 M HCl. After centrifugation at $10,000 \times g$ for 5 min, the absorbance of the supernatant was measured at 570 nm.

Assay of DNA fragmentation. After treatment of $\sim 2 \times 10^6$ cells with vehicle or MPP^+ in 60-mm-diameter dishes, the cells were

scraped from the dishes using a rubber policeman, centrifuged at $800 \times g$ for 10 min, and resuspended in 100 μl of lysis buffer of 10 mM Tris-HCl, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100. After incubation for 10 min at 4°, lysates were centrifuged at $15,000 \times g$ for 30 min. The supernatants thus obtained were supplemented with 2 μl of RNase A (20 mg/ml) and then incubated at 37° for 1 hr. Next, 2 μl of proteinase K (20 mg/ml) was added, and incubation was continued at 37° for 1 hr. After these incubations, 20 μl of 5 M NaCl and 120 μl of isopropanol were added, and the mixture was held overnight at -20° . After centrifugation at $15,000 \times g$ for 20 min, DNA pellets were resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. DNA fragments thus obtained were electrophoretically separated on a 2% agarose gel for 90 min at 50 V. The gel was stained with ethidium bromide and photographed under UV transillumination. DNA size markers (pX174 RF DNA/*Hae*III fragments) are 1353, 1078, 872, 603, 310, 271/281, 234, 194, 118, and 72 bp.

Hoechst 33258 staining. Chromosomal condensation and DNA fragmentation were determined using the chromatin dye Hoechst 33258. After pre- and post-treatments of SH-SY5Y cells, the cells were harvested and fixed with 4% paraformaldehyde in PBS for 30 min at 4°. After three rinses with the same buffer, cells were stained with 1 μM Hoechst 33258 for 5 min and analyzed under a fluorescence microscope (Axioplan; Carl Zeiss, Jena, Germany) with excitation at 352 nm. Fluorescence micrographs were scanned with a high resolution camera (ProgRes 3008, Carl Zeiss).

C-DCDHF-DA staining. To detect ROS production, we used the redox-sensitive dye C-DCDHF-DA, which is readily taken up by cells. After simultaneous treatment or pretreatment of SH-SY5Y cells in uncoated glass-bottomed microwells (inner diameter, 18 mm; MatTek, Ashland, MA), C-DCDHF-DA was added to the cell culture to a final concentration at 2 μM for 10 min at 37°. After two rinses with serum-free medium, samples were scanned under a confocal microscope (LSM410, Carl Zeiss). The optimal vertical position at the middle of the cells was set, and then the field was rapidly scanned. Because illumination at the excitation wavelength of 488 nm caused increased fluorescence because of oxidation of this dye (Greenlund *et al.*, 1995), each field was exposed to light for exactly the same time, and the laser intensity was set at only 3% of maximum. After scanning (excitation, 488 nm; emission, over 515 nm), the average relative fluorescence intensity for every cell in each field was evaluated. Fluorescence micrographs were printed with a full-color digital photo printer (Pictography 3000; Fuji Film, Tokyo, Japan).

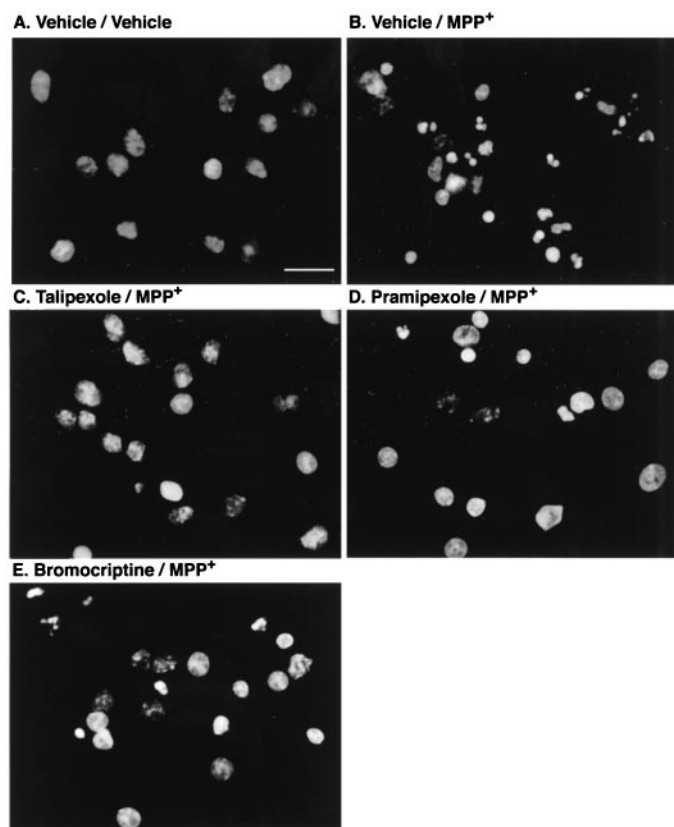


Fig. 3. Protective effects of pretreatment with talipexole, pramipexole, and bromocriptine against MPP^+ -induced chromatin destruction. SH-SY5Y cells were pretreated for 4 days with vehicle (A and B), 1 mM talipexole (C), 1 mM pramipexole (D), or 0.03 mM bromocriptine (E) and then further treated with vehicle (A) or 1 mM MPP^+ (B–E) for 3 days. Nuclei were stained with Hoechst 33258. Numerous cells treated with MPP^+ after vehicle pretreatment showed apoptotic features (chromatin condensation and DNA fragmentation) (B). Many nuclei appeared normal in cells pretreated with talipexole (C), pramipexole (D), and bromocriptine (E). Bar (in A) = 20 μm .

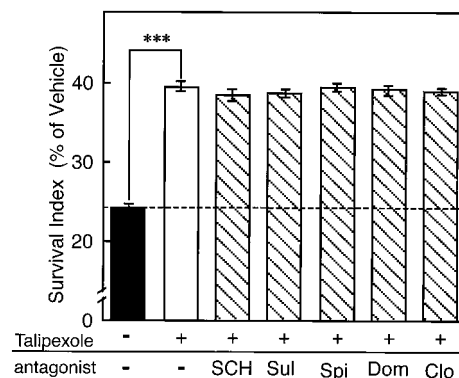


Fig. 4. Lack of effect of dopaminergic antagonists on talipexole pretreatment-induced protection. SH-SY5Y cells were pretreated for 4 days with 0.5 mM talipexole in the presence of 0.1 mM SCH23390 (SCH), 0.3 mM sulpiride (Sul), 0.01 mM spiperone (Spi), 0.01 mM domperidone (Dom), or 0.01 mM clozapine (Clo) and then further treated with 1 mM MPP^+ for 3 days. Subsequently, cell survival was measured by MTT assay. Each value is the mean \pm standard error (%) of four determinations, based on the vehicle control as 100%. The F value in ANOVA was $F(6,21) = 137.141$ ($p < 0.0001$). When the Bonferroni/Dunn test was performed for *post hoc* comparisons, dopaminergic antagonists had no significant effect.

Immunoblotting assay for Bcl-2 family members, p53, caspase-3, and PARP. Cell lysates were dissolved in Laemmli's sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%, 10%, or 8% polyacrylamide gels). Immunoblotting was performed by transferring proteins from a slab gel to a sheet of polyvinylidene difluoride membrane (BioRad Laboratories, Richmond, CA) by electroelution at a constant voltage of 50 V for 2 hr at 4°. The PVDF membrane was incubated with Tris-buffered saline, pH 8.0, containing 0.3% Triton X-100 (TBS-T) and 5% dehydrated skim milk (Difco Laboratories, Detroit, MI) to block nonspecific protein binding. The membrane then was incubated with primary antibodies, including mouse monoclonal antibodies to Bcl-2 (diluted 1:300), Bak (1:400), Bad (1:300), p53 (1:2000), and procaspase-3 (1:1000) or rabbit polyclonal antibodies to Bcl-x (1:1000), Bax (1:300), active caspase-3 (1:1000), and PARP (1:600), followed by horseradish peroxidase-linked antibodies against either rabbit or mouse immunoglobulins (each diluted 1:1000) as secondary antibodies. Bound HRP-labeled antibodies were detected by chemiluminescence assay (ECL kit, Amersham). The protein bands that reacted with the antibodies were detected on radiographic film (X-Omat JB-1; Kodak, Rochester, NY) after exposure for 5–60 sec. The bands of Bcl-2, Bcl-x, p53, and caspase-3 on radiographic films were scanned and densitometrically analyzed by a dual-wavelength flying-spot scanner (CS-9000; Shimadzu, Kyoto, Japan). Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards (BioRad) were used as molecular mass markers. Apparent molecular masses of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were 112, 84, 53.2, 34.9, 28.7, and 20.5 kDa, respectively, according to the manufacturer's labeling.

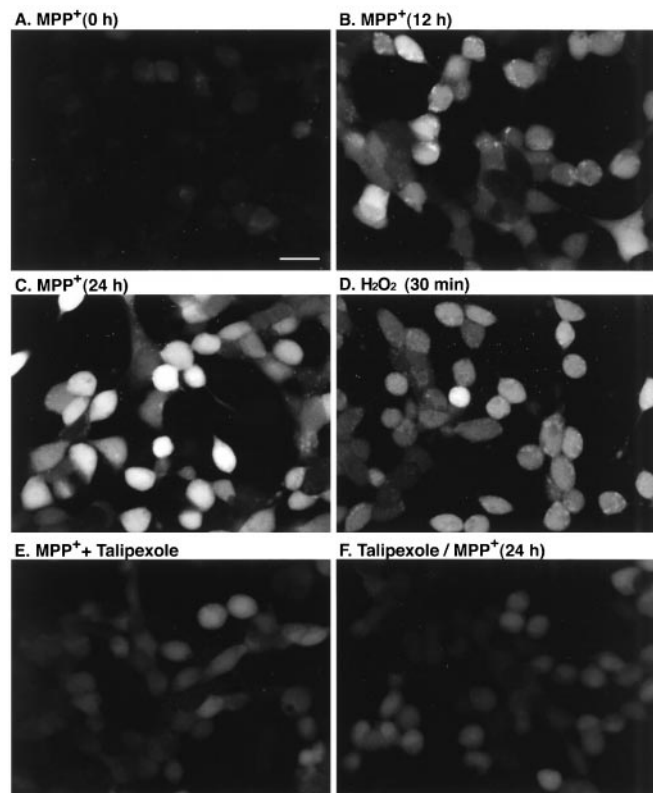


Fig. 5. Confocal analysis of MPP⁺-induced ROS production. SH-SY5Y cells were treated with 1 mM MPP⁺ for 0 hr (A), 12 hr (B), or 24 hr (C). As a positive control, cells were treated with 5 mM H₂O₂ for 0.5 hr (D). Other cells were simultaneously treated for 24 hr with 1 mM talipexole and 1 mM MPP⁺ (E) or for 24 hr with 1 mM MPP⁺ after pretreatment for 4 days with 1 mM talipexole (F). Treated cells were loaded with C-DCDHF-DA (2 μM, for 10 min) and analyzed for ROS production by using a laser scanning confocal microscope. Bar (in A) = 20 μm.

Statistical evaluation. Results in MTT assay and densitometric analysis of the immunoblots are given as mean ± standard error values. Statistical significance of differences was determined by mean values of the ANOVA. Further statistical analysis for *post hoc* comparisons was done by using the Bonferroni/Dunn test.

Results

MPP⁺-induced apoptotic death in SH-SY5Y cells. Treatment with MPP⁺ caused a time- and concentration-dependent increase in cell death, and delayed DNA fragmentation was observed (data not shown). In particular, SH-SY5Y cells treated with 1 mM MPP⁺ for >3 days exhibited marked cell death and DNA fragmentation (Fig. 1). To confirm the morphological change, we further examined cells under a fluorescence microscope using a chromatin dye, Hoechst 33258. At 3 days after MPP⁺ treatment, the cell body and nucleus were shrunken and compacted, with condensed chromatin and fragmented nuclei (Fig. 3B). In addition, MPP⁺ induced cleavages of 32-kDa caspase-3 and 116-kDa PARP to 20/17-kDa active fragments and an 85-kDa fragment, respectively (Fig. 10). These morphological and biochemical changes are characteristic of apoptosis.

Protective effects of talipexole and pramipexole against MPP⁺-induced apoptotic death of SH-SY5Y cells. Preliminary treatment of SH-SY5Y cells for 4 days with talipexole or pramipexole at 0.01–1 mM did not markedly alter cell growth, but bromocriptine treatment at >0.1 mM decreased the cell viability (data not shown). Bromocriptine has ~10-fold higher affinity for the D2-receptor than

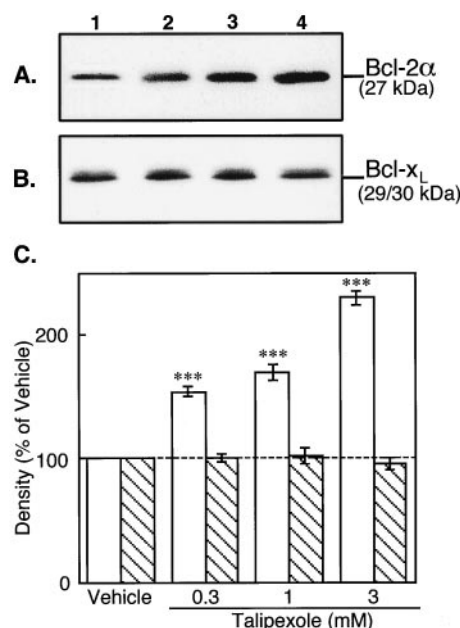


Fig. 6. Changes of protein levels of antiapoptotic Bcl-2 and Bcl-x induced by treatment with talipexole. SH-SY5Y cells were treated for 4 days with vehicle (lane 1) or talipexole at 0.3 (lane 2), 1 (lane 3) and 3 (lane 4) mM and then lysed. Samples (10 μg protein/lane) were subjected to immunoblotting with antibodies against Bcl-2 (A) and Bcl-x (B). C, Protein levels of Bcl-2α (open column) and Bcl-x_L (hatched column) were measured by densitometric analysis of the immunoblots. The density of each protein band is given as the mean ± standard error (%) of three determinations, based on the vehicle control as 100%. The F values for protein levels of Bcl-2α and Bcl-x_L in ANOVA were F(3,8) = 150.797 (*p* < 0.0001) and 0.475 (*p* = 0.7084), respectively. In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. ***, *p* < 0.001 versus the level in the corresponding vehicle control (Bonferroni/Dunn test).

talipexole and pramipexole, but the agonistic potency of bromocriptine is lower than that of talipexole. Therefore, we used talipexole or pramipexole at 0.01–3 mM and bromocriptine at 0.01–0.03 mM.

Simultaneous treatment with 1 mM MPP⁺ and 1 mM talipexole for 3 days (without talipexole pretreatment) resulted in slight but significant protection against cell death and DNA fragmentation compared with 1 mM MPP⁺ alone (Fig. 1). In addition, talipexole pretreatment at the same concentration inhibited MPP⁺-induced cell death and DNA fragmentation in a pretreatment time- and concentration-dependent manner (Figs. 1 and 2). Pretreatment with other D₂ receptor agonists, pramipexole and bromocriptine, also inhibited MPP⁺-induced cell death and DNA fragmentation (Fig. 2). Although MPP⁺ treatment alone induced many apoptotic features such as condensed chromatin and fragmented nuclei (Fig. 3B), pretreatment with talipexole, pramipexole, and bromocriptine markedly decreased these features (Fig. 3, C–E, respectively).

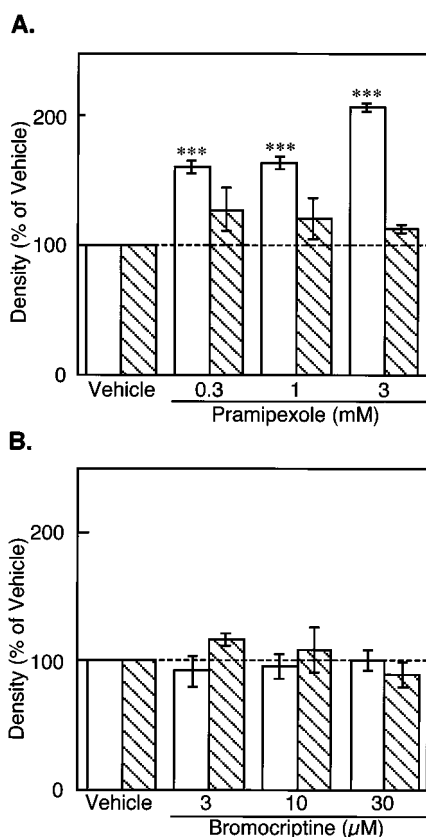


Fig. 7. Changes in protein levels of antiapoptotic Bcl-2 and Bcl-x induced by pretreatment with pramipexole and bromocriptine in SH-SY5Y cells. SH-SY5Y cells were treated for 4 days with vehicle, pramipexole (A), or bromocriptine (B). Samples (10 μg protein/lane) were subjected to immunoblotting with antibodies against Bcl-2 and Bcl-x. Protein levels of Bcl-2α (open column) and Bcl-x_L (hatched column) were measured by densitometric analysis of the immunoblots. Each value is the mean ± standard error (%) of three determinations, based on the density of the band in the vehicle control as 100%. In pramipexole treatment, F values for protein levels of Bcl-2α and Bcl-x_L in ANOVA were F(3,8) = 135.326 ($p < 0.0001$) and 1.035 ($p = 0.4275$), respectively. In bromocriptine treatment, F values for protein levels of Bcl-2α and Bcl-x_L in ANOVA were F(3,8) = 1.763 ($p = 0.2318$) and 1.450 ($p = 0.2992$), respectively. In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. ***, $p < 0.001$ versus the level in the corresponding vehicle control (Bonferroni/Dunn test).

Because the D₂ receptor is expressed in SH-SY5Y cells (Farooqui, 1994), we examined whether the protective effect of talipexole is mediated by dopaminergic receptors. However, various selective or nonselective antagonists for dopaminergic receptor subtypes, SCH23390 (D₁/D₅), sulpiride (D₂/D₃), spiperone (D₂/D₄), domperidone (D₂/D₃/D₄), and clozapine (D₄), did not inhibit the talipexole-induced protection (Fig. 4).

Effects of talipexole and pramipexole on MPP⁺-induced ROS production in SH-SY5Y cells. We further performed confocal analysis with a ROS-specific fluorogen, C-DCDHF-DA, to examine whether MPP⁺ induces ROS production and, if so, what is the effect of talipexole. The fluorescence intensity of SH-SY5Y cells showed an early increase when the cells were treated for 30 min with H₂O₂ as a positive control (Fig. 5D). In contrast, a delayed increase of the intensity was observed after 12 and 24 hr in the cells treated with 1 mM MPP⁺ (Fig. 5, B and C, respectively).

Compared with the fluorescence intensity 24 hr after treatment with 1 mM MPP⁺ alone, that with simultaneous treatment with 1 mM talipexole (Fig. 5E) was much lower, being close to the basal level (Fig. 5A). Similarly, simultaneous treatment with pramipexole or bromocriptine inhibited MPP⁺-induced fluorescence (ROS production; data not shown). Pretreatment with 1 mM talipexole for 4 days also markedly inhibited MPP⁺-induced ROS production (Fig. 5F).

Effects of talipexole and pramipexole on protein levels of Bcl-2 family members in SH-SY5Y cells. Bcl-2 family members regulate apoptotic cell death, and SH-SY5Y cells natively express antiapoptotic members such as Bcl-2 and Bcl-x (Dole *et al.*, 1995; Reed *et al.*, 1991) and proapoptotic members such as Bax, Bak, and Bad (Kitamura *et al.*, 1998b). Therefore, we examined whether the protein levels of Bcl-2 family members are influenced by treatment with talipexole, pramipexole, and bromocriptine. For this purpose, we used each specific antibody for immunoblotting of 27-kDa Bcl-2α, 29/30-kDa Bcl-x_L, 21-kDa Bax, 30-kDa Bak, or 23-kDa Bad (Figs. 6 and 8). Treatment with talipexole or pramipexole for 4 days induced significant enhancement of the antiapoptotic Bcl-2α protein level but did not change the Bcl-x_L level (Figs. 6C and 7A, respectively), whereas treatment with bromocriptine for 4 days did not markedly change the levels of either antiapoptotic protein (Fig. 7B). On the other hand, protein levels of proapoptotic members such as

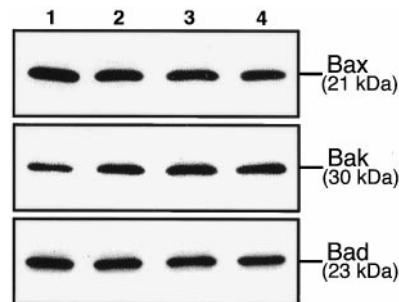


Fig. 8. No change in protein levels of proapoptotic Bax, Bak, and Bad in SH-SY5Y cells treated with talipexole, pramipexole or bromocriptine. SH-SY5Y cells were treated for 4 days with vehicle (lane 1), 1 mM talipexole (lane 2), 1 mM pramipexole (lane 3), and 0.03 mM bromocriptine (lane 4). Samples (10 μg protein/lane) were subjected to immunoblotting with antibodies against Bax, Bak, and Bad. These proapoptotic protein levels were not markedly changed in cells treated with talipexole, pramipexole, or bromocriptine.

Bax, Bak, and Bad were not markedly changed by treatment for 4 days with talipexole, pramipexole, or bromocriptine (Fig. 8). Although treatment for 3 days with MPP⁺ alone did not significantly change the Bcl-2 α level, the protein level was significantly increased even 3 days after MPP⁺ treatment by pretreatment with talipexole for 4 days (Fig. 9).

Inhibitory effects of talipexole against MPP⁺-induced p53 expression and cleavages of caspase-3 and PARP in SH-SY5Y cells. p53 protein and caspase-3 (CPP32) act as a transcription factor and a key enzyme for apoptosis, respectively (Ko and Prives, 1996; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). In the case of caspase-3, the 32-kDa proenzyme is cleaved by other proteases to generate the active form, which is composed of 12-kDa and active 20/17-kDa fragments. Subsequently, 116-kDa PARP is cleaved by active caspase-3 in cells undergoing apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Therefore, we further examined p53 expression and cleavages of caspase-3 and PARP.

Although treatment with MPP⁺ for 3 days markedly in-

duced expression of p53 protein, pretreatment with talipexole for 4 days inhibited the MPP⁺-induced p53 expression (Fig. 9). Using mouse monoclonal anti-caspase-3 antibody (C31720; Transduction Laboratories), the 32-kDa proenzyme of caspase-3 was decreased by MPP⁺ treatment, but 20/17-kDa active fragments were undetectable. Talipexole pretreatment inhibited the MPP⁺-induced degradation of pro-caspase-3, and the level of the protein was similar to that in the control (Fig. 9). Using rabbit polyclonal anti-caspase-3 antibody (65906E; PharMingen), 20/17-kDa active fragments cleaved from 32-kDa proenzyme were detected. MPP⁺-induced cleavage of caspase-3 was inhibited by pretreatment with talipexole or pramipexole (Fig. 10A). In addition, MPP⁺-induced PARP cleavage to an 85-kDa fragment also was detected using polyclonal antibody against the caspase-3 cleavage site of human PARP (06-557; Upstate Biotechnology). This PARP cleavage also was inhibited by pretreatment with talipexole or pramipexole (Fig. 10B).

Decrease of Bcl-2 level and enhancement of MPP⁺-induced apoptosis by diBu-cAMP or calphostin C. We previously found that activation of cAMP-dependent PKA or inhibition of PKC induced a decrease in Bcl-2 level and then enhanced NO-induced apoptosis (Kitamura *et al.*, 1998a). In the current study, treatment for 4 days with 1 mM diBu-cAMP or 30 nM calphostin C induced a decrease in antiapoptotic Bcl-2 and Bcl-x but did not markedly change the levels of proapoptotic Bax, Bak, and Bad (Fig. 11A). These pretreatments enhanced MPP⁺-induced cell death (Fig. 11B) and cleavages of caspase-3 and PARP (data not shown). On the other hand, treatment with 100 nM PMA induced an increase in Bcl-2 and Bcl-x but did not change Bax, Bak, and Bad (Fig. 11A). In addition, MPP⁺-induced cell death (Fig. 11B) and cleavages of caspase-3 and PARP were inhibited by PMA pretreatment (data not shown).

Discussion

The brains of patients with Parkinson's disease show evidence of inhibition of mitochondrial complex I (Schapira *et al.*, 1989), generation of oxidative stress (Castellani *et al.*, 1996), and induction of apoptosis (Mochizuki *et al.*, 1996). Similar phenomena are observed in MPTP-treated animal

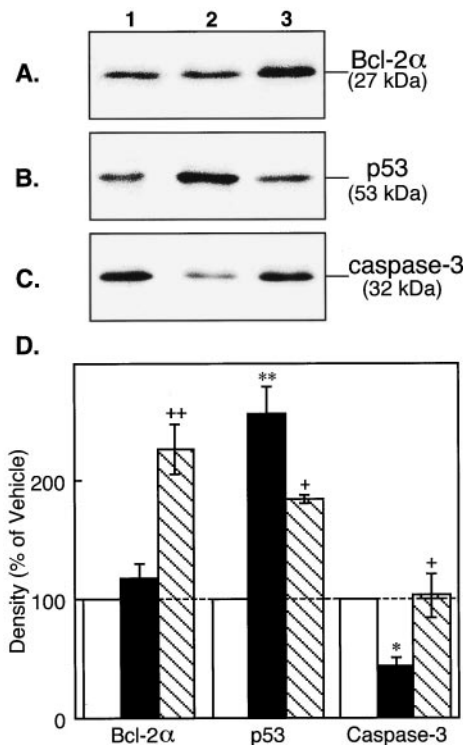


Fig. 9. Changes of protein levels of Bcl-2, p53, and caspase-3 induced by MPP⁺ treatment after talipexole pretreatment. SH-SY5Y cells were pretreated for 4 days with vehicle (lanes 1 and 2) or 3 mM talipexole (lane 3) and then further treated for 3 days with 1 mM MPP⁺ in the absence of talipexole (lanes 2 and 3) and lysed. Samples (10 μ g protein/lane) were subjected to immunoblotting with antibodies against Bcl-2 (A), p53 (B), and caspase-3 (C31720, Transduction Laboratories; C). Protein levels of Bcl-2 α , p53 and caspase-3 were measured by densitometric analysis of the immunoblots. D, Open column, control (vehicle treatment after vehicle pretreatment); filled column, MPP⁺ treatment after vehicle pretreatment; hatched column, MPP⁺ treatment after talipexole pretreatment. Each value is the mean \pm standard error (%) of three determinations based on the density of the band in the vehicle control as 100%. The F values for protein levels of Bcl-2 α , p53, and caspase-3 in ANOVA were F(2,6) = 23.972 (p = 0.0014), 18.707 (p = 0.0026), and 8.091 (p = 0.0198), respectively. In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. *, p < 0.05; **, p < 0.01 versus Control (open column); +, p < 0.05, ++, p < 0.01 versus Vehicle + MPP⁺ (filled column) (Bonferroni/Dunn test).

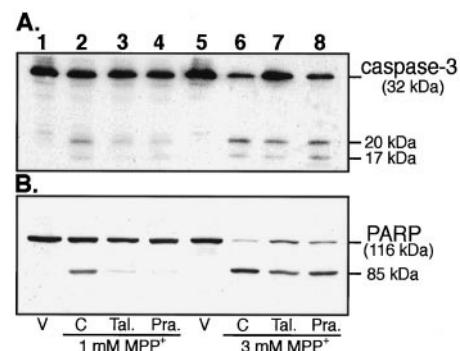


Fig. 10. MPP⁺-induced cleavages of caspase-3 and PARP and inhibition by talipexole or pramipexole. SH-SY5Y cells were pretreated for 4 days with vehicle (lanes 1, 2, 5, and 6; V and C), 1 mM talipexole (lanes 3 and 7; Tal.), or 1 mM pramipexole (lanes 4 and 8; Pra.) and then further treated with 1 mM MPP⁺ for 3 days (lanes 2-4) or 3 mM MPP⁺ for 2 days (lanes 6-8). Samples (10 μ g protein/lane) were subjected to immunoblotting with antibodies against proenzyme/active fragments of caspase-3 (65906E; PharMingen; A) and the caspase-3 cleavage site of human PARP (06-557; Upstate Biotechnology; B).

models (Tatton and Kish, 1997; Sriram *et al.*, 1997). It is known that levodopa and dopamine exhibit neurotoxicity (Ogawa, 1994), whereas D₂ receptor agonists such as bromocriptine have neuroprotective effects against MPTP-induced and ischemic insults (Lange *et al.*, 1994; Liu *et al.*, 1995). We recently found that the administration of talipexole or pramipexole inhibited MPTP-induced degeneration of dopaminergic neurons in C57BL/6 mouse, but talipexole did not inhibit the uptake of MPP⁺ into striatal synaptosomes (Kitamura *et al.*, 1997). Thus, the mechanisms of these neuroprotective effects remain unclear. In the current study, we therefore focused on intracellular events related to MPP⁺ cytotoxicity, and we examined the protective effects of talipexole and pramipexole on the MPP⁺-induced changes.

In human neuroblastoma SH-SY5Y cells, MPP⁺ treatment induced delayed ROS production in comparison with the immediate production induced by H₂O₂ (Fig. 5). This was followed by apoptotic cell death with the characteristic DNA fragmentation and morphological changes (condensed chromatin and fragmented nuclei). It is known that MPP⁺ is

concentrated in mitochondria (Ramsay and Singer, 1986; Mizuno *et al.*, 1995), where it inhibits mitochondrial complex I (Mizuno *et al.*, 1995; Nicklas *et al.*, 1987) and that mitochondrial dysfunction causes apoptosis (Kroemer *et al.*, 1997). Therefore, delayed ROS production by MPP⁺ in SH-SY5Y cells may be mediated through inhibition of mitochondrial complex I, leading to apoptotic death.

Because simultaneous treatment with talipexole inhibited MPP⁺-induced ROS production (Fig. 5E) and slightly inhibited MPP⁺-induced apoptosis (Fig. 1), talipexole may have direct scavenging effects against ROS. On the other hand, pretreatment with talipexole markedly protected the cells against MPP⁺-induced apoptosis in a treatment time- and concentration-dependent manner (Figs. 1 and 2), and this protective effect might not be mediated by dopamine receptors (Fig. 4). This pretreatment also inhibited H₂O₂-induced DNA fragmentation (data not shown). Furthermore, talipexole treatment induced an increase in antiapoptotic Bcl-2 protein level but did not change the Bcl-x level (Fig. 6), whereas protein levels of proapoptotic Bax, Bak, and Bad were not markedly changed by talipexole treatment (Fig. 8). This pretreatment also inhibited MPP⁺-induced ROS production (Fig. 5F). In this case, MPP⁺ treatment was conducted after talipexole had been washed out, so the protection was not mediated by direct ROS scavenging but probably via another pathway, such as induction of Bcl-2 expression. Pramipexole pretreatment also enhanced the Bcl-2 level (Fig. 7A) and inhibited MPP⁺-induced apoptosis (Fig. 2). Furthermore, talipexole pretreatment inhibited the MPP⁺-induced p53 expression and decrease in a 32-kDa proenzyme of caspase-3 (Fig. 9). Induction of p53 protein is usually considered one of the primary events in the apoptotic cascade after DNA damage (Ko and Prives, 1996). It is also known that 32-kDa caspase-3 is activated through cleavage to 12-kDa and active 20/17-kDa fragments by apoptotic signals, and subsequently, PARP is cleaved to the 85-kDa fragment (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Talipexole pretreatment seems to inhibit events leading to apoptosis, such as p53 expression and cleavages of caspase-3 and PARP (Fig. 10). Pramipexole also has similar effects.

PKA activator (diBu-cAMP) or PKC inhibitor (calphostin C) induced a decrease in protein levels of antiapoptotic Bcl-2 and Bcl-x but did not change the levels of proapoptotic Bax, Bak, and Bad (Fig. 11). With this pretreatment, MPP⁺-induced apoptotic changes (cleavages of caspase-3 and PARP) and cell death were enhanced. Recent studies suggest that Bcl-2 protein inhibits ROS production and apoptosis (Hockenbery *et al.*, 1993; Kane *et al.*, 1993) and that Bcl-2 inhibits the loss of mitochondrial membrane potential and activation of caspase-3 (Shimizu *et al.*, 1996). In view of these observations, we consider that (1) talipexole (or pramipexole) has a direct scavenging effect on ROS but only a small protective effect and (2) treatment with talipexole (or pramipexole) induces an increase in Bcl-2, which affords more potent protection against MPP⁺-induced apoptosis. In addition, PKA inhibition or PKC activation may participate with neuroprotection by talipexole (or pramipexole). However, because dopamine receptor antagonists did not inhibit talipexole-induced protection, the detail mechanisms of this neuroprotection are still unclear. Talipexole (or pramipexole) may affect other receptors regulating PKA, PKC, or both, which are not still identified as these binding receptors,

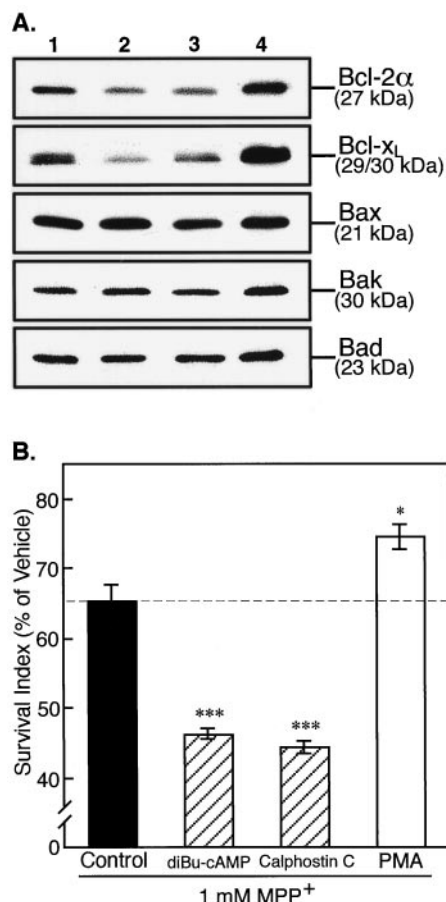


Fig. 11. Enhancement of MPP⁺-induced cell death by reduction of Bcl-2 protein level. **A**, SH-SY5Y cells were treated for 4 days with vehicle (lane 1), 1 mM diBu-cAMP (lane 2), 30 nM calphostin C (lane 3), or 100 nM PMA (lane 4). Samples (10 μg protein/lane) were subjected to immunoblotting with antibodies against Bcl-2, Bcl-x, Bax, Bak, and Bad. **B**, Pretreated cells were further treated with 1 mM MPP⁺ for 2 days, and then cell survival was measured by MTT assay. Each value is the mean ± standard error (%) of three determinations, based on the vehicle control as 100%. The F value in ANOVA was F(3,8) = 84.144 ($p < 0.0001$). In addition, the Bonferroni/Dunn test was performed for *post hoc* comparisons. *, $p < 0.05$; ***, $p < 0.001$ versus Control of MPP⁺ treatment (filled column; Bonferroni/Dunn test).

whereas talipexole (or pramipexole) may be uptaken into neuronal cells through monoamine transporters (dopamine, norepinephrine, and so on) or other mechanisms, and then these drugs directly or indirectly may regulate intracellular events to Bcl-2 expression. Further studies of neuroprotective mechanisms of talipexole (or pramipexole) in detail are necessary before definite conclusions can be drawn.

The Bcl-2 protein level is known to be up-regulated in brains of patients with Parkinson's disease (Mogi *et al.*, 1996), as well as those with Alzheimer's disease (Kitamura *et al.*, 1998b). Such up-regulation of Bcl-2 protein may represent a compensatory response of remaining neurons to protect them from oxidative stress and subsequent apoptosis. Therefore, neurons with lower Bcl-2 expression may be more vulnerable to apoptotic death. Because an increase of Bcl-2 was induced by treatment for ≥ 4 days with talipexole and pramipexole (but not bromocriptine), these drugs may be useful for long term treatment of patients with Parkinson's disease. On the other hand, pretreatment with bromocriptine also inhibited MPP⁺-induced apoptotic cell death (Fig. 2), although it had no effect on the protein levels of Bcl-2 family members under our experimental conditions (Figs. 7B and 8). Recent studies have suggested that bromocriptine directly scavenges superoxide (Yoshikawa *et al.*, 1994) and NO radical (Nishibayashi *et al.*, 1997), inhibits lipid peroxidation (Tanaka *et al.*, 1995), inhibits glutamate uptake into synaptic vesicles (Carlson *et al.*, 1989), and enhances glutamate uptake through human glutamate transporter hGLUT-1 (Yamashita *et al.*, 1995). These effects may contribute to the neuroprotective action of bromocriptine in contrast to talipexole and pramipexole.

In conclusion, MPP⁺ treatment induced delayed ROS production in SH-SY5Y cells in comparison with the immediate production induced by H₂O₂. This was followed by apoptotic cell death. Simultaneous treatment with talipexole markedly inhibited MPP⁺-induced ROS production and slightly inhibited MPP⁺-induced apoptosis. On the other hand, pretreatment with talipexole markedly protected the cells against MPP⁺-induced apoptosis, and this protective effect might not be mediated by dopamine receptors. This pretreatment induced an increase in antiapoptotic Bcl-2 level and inhibited MPP⁺-induced ROS production. Pramipexole pretreatment exhibited similar effects. Although pretreatment with bromocriptine also had a protective effect, there was no change in the level of Bcl-2 family members. On the other hand, pretreatment with diBu-AMP or calphostin C, which caused a decrease in Bcl-2 level, enhanced MPP⁺-induced apoptosis. These results suggest that talipexole directly scavenges ROS, but this effect affords only slight protection; however, pretreatment markedly increases Bcl-2, which results in more potent protection against MPP⁺-induced apoptosis. Pramipexole has similar effects, but bromocriptine may lack the ability to induce Bcl-2 expression.

Acknowledgments

We thank Dr. M. Ino (St. Marianna University School of Medicine, Tokyo, Japan) for the gift of human neuroblastoma cell line SH-SY5Y.

References

Arnt J, Bøgesø KP, Hyttel J, and Meier E (1988) Relative dopamine D₁ and D₂ receptor affinity and efficacy determine whether dopamine agonists induce hyperactivity or oral stereotypy in rats. *Pharmacol Toxicol* **62**:121–130.

Carlson MD, Kish PE, and Ueda T (1989) Glutamate uptake into synaptic vesicles: competitive inhibition by bromocriptine. *J Neurochem* **53**:1889–1894.

Castellani R, Smith MA, Richey PL, and Perry G (1996) Glycooxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. *Brain Res* **737**:195–200.

Davis GC, Williams AC, Markey SP, Ebert MH, Caine ED, Reichert CM, and Kopin IJ (1979) Chronic parkinsonism secondary to intravenous injection of meperidine analogues. *Psychiatr Res* **1**:249–254.

Dole MG, Jasty R, Cooper MJ, Tompson CB, Nuñez G, and Castle VP (1995) Bcl-x₁ is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. *Cancer Res* **55**:2576–2582.

Farooqui SM (1994) Induction of adenylate cyclase sensitive dopamine D₂-receptors in retinoic acid induced differentiated human neuroblastoma SH-SY5Y cells. *Life Sci* **55**:1887–1893.

Greenlund LJS, Deckwerth TL, and Johnson EM Jr (1995) Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* **14**:303–315.

Hockenbery DM, Oltvai ZN, Yin X-M, Millman CL, and Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**:241–251.

Itano Y and Nomura Y (1995) 1-Methyl-4-phenylpyridinium ion (MPP⁺) causes DNA fragmentation and increases the Bcl-2 expression in human neuroblastoma, SH-SY5Y cells, through different mechanisms. *Brain Res* **704**:240–245.

Kane D, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, and Bredehse DE (1993) Bcl-2 inhibition of neuronal death: decreased generation of reactive oxygen species. *Science (Washington DC)* **262**:1274–1277.

Kitamura Y, Kamoshima W, Shimohama S, Nomura Y, and Takahashi T (1998a) Nitric oxide donor-induced p53-sensitive cell death is enhanced by Bcl-2 reduction in human neuroblastoma cells. *Neurochem Int* **32**:93–102.

Kitamura Y, Kohno Y, Nakazawa M, and Nomura Y (1997) Inhibitory effects of talipexole and pramipexole on MPTP-induced dopamine reduction in the striatum of C57BL/6N mice. *Jpn J Pharmacol* **74**:51–57.

Kitamura Y, Shimohama S, Kamoshima W, Ota T, Matsuoka Y, Nomura Y, Smith MA, Perry G, Whitehouse PJ, and Taniguchi T (1998b) Alteration of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1 and CPP32, in Alzheimer's disease. *Brain Res* **780**:260–269.

Ko LJ and Prives C (1996) p53: puzzle and paradigm. *Gene Dev* **10**:1054–1072.

Kroemer G, Zamzami N, and Susin A (1997) Mitochondrial control of apoptosis. *Immunol Today* **18**:44–51.

Lange KW, Rausch W-D, Gsell W, Naumann M, Oestreicher E, and Riederer P (1994) Neuroprotection by dopamine agonists. *J Neural Transm* **43**:183–201.

Langston JW, Ballard P, Tetrad JW, and Irwin I (1983) Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science (Washington DC)* **219**:979–980.

Langston JW and Irwin I (1986) MPTP: current concepts and controversies. *Clin Neuropharmacol* **9**:485–507.

Liu X-H, Kato H, Chen T, Kato K, and Itoyama Y (1995) Bromocriptine protects against delayed neuronal death of hippocampal neurons following cerebral ischemia in the gerbil. *J Neurol Sci* **129**:9–14.

Mierau J, Schneider FJ, Ensinger H, Chio CL, Lajiness ME, and Huff RM (1995) Pramipexole binding and activation of cloned and expressed dopamine D₂, D₃ and D₄ receptors. *Eur J Pharmacol* **290**:29–36.

Mizuno Y, Ikebe S, Hattori N, Nakagawa-Hattori Y, Mochizuki H, Tanaka M, and Ozawa T (1995) Role of mitochondria in the etiology and pathogenesis of Parkinson's disease. *Biochim Biophys Acta* **1271**:265–274.

Mochizuki H, Goto K, Mori H, and Mizuno Y (1996) Histochemical detection of apoptosis in Parkinson's disease. *J Neurol Sci* **137**:120–123.

Mochizuki H, Nakamura N, Nishi K, and Mizuno Y (1994) Apoptosis induced by 1-methyl-4-phenylpyridinium ion (MPP⁺) in ventral mesencephalic-striatal co-culture in rat. *Neurosci Lett* **170**:191–194.

Mogi M, Harada M, Kondo T, Mizuno Y, Narabayashi H, Riederer P, and Nagatsu T (1996) bcl-2 protein is increased in the brain from parkinsonian patients. *Neurosci Lett* **215**:137–139.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL, and Miller DK (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature (Lond)* **376**:37–43.

Nicklas WJ, Youngster SK, Kindt MV, and Heikkilä RE (1987) MPTP, MPP⁺ and mitochondrial function. *Life Sci* **40**:721–729.

Nishibayashi S, Asanuma M, Kohno M, Gómez-Vargas M, and Ogawa N (1996) Scavenging effects of dopamine agonists on nitric oxide radicals. *J Neurochem* **67**:2208–2211.

Ogawa N (1994) Levodopa and dopamine agonists in the treatment of Parkinson's disease: advantages and disadvantages. *Eur Neurol* **34**:20–28.

Ramsay RR and Singer TP (1986) Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J Biol Chem* **261**:7585–7587.

Reed JC, Meister L, Tanaka S, Cuddy M, Yum S, Geyer C, and Pleasure D (1991) Differential expression of bcl-2 protooncogene in neuroblastoma and other human tumor cell lines of neural origin. *Cancer Res* **51**:6529–6538.

Ross RA and Biedler JL (1985) Presence and regulation of tyrosinase activity in of human neuroblastoma cell variants in vitro. *Cancer Res* **45**:1628–1632.

Schapiro AH, Cooper JM, Dexter D, Jenner P, Clark JB, and Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1**:1269.

Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, and Tsujimoto Y (1996) Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* **13**:21–29.

Snyder SH and D'Amato RJ (1986) MPTP: A neurotoxin relevant to the pathophysiology of Parkinson's disease. *Neurology* **36**:250–258.

- Sriram K, Pai KS, Boyd MR, and Ravindranath V (1997) Evidence for generation of oxidative stress in brain by MPTP: in vitro and in vivo studies in mice. *Brain Res* **749**:44–52.
- Tanaka M, Sotomatsu A, Yoshida T, and Hirai S (1995) Inhibitory effects of bromocriptine on phospholipid peroxidation induced by DOPA and iron. *Neurosci Lett* **183**:116–119.
- Tatton NA and Kish SJ (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* **77**:1037–1048.
- Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zheng Z, Beidler DR, Poirier GG, Salvesen GS, and Dixit VM (1995) Yama/CPP32 β , a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**:801–809.
- Yamashita H, Kawakami H, Zhang Y-X, Tanaka K, and Nakamura S (1995) *Lancet* **346**:1305.
- Yoshikawa T, Minamiyama Y, Naito Y, and Kondo M (1994) Antioxidant properties of bromocriptine, a dopamine agonist. *J Neurochem* **62**:1034–1038.

Send reprint requests to: Takashi Taniguchi, Ph.D., Department of Neurobiology, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan. E-mail: taniguti@mb.kyoto-phu.ac.jp
