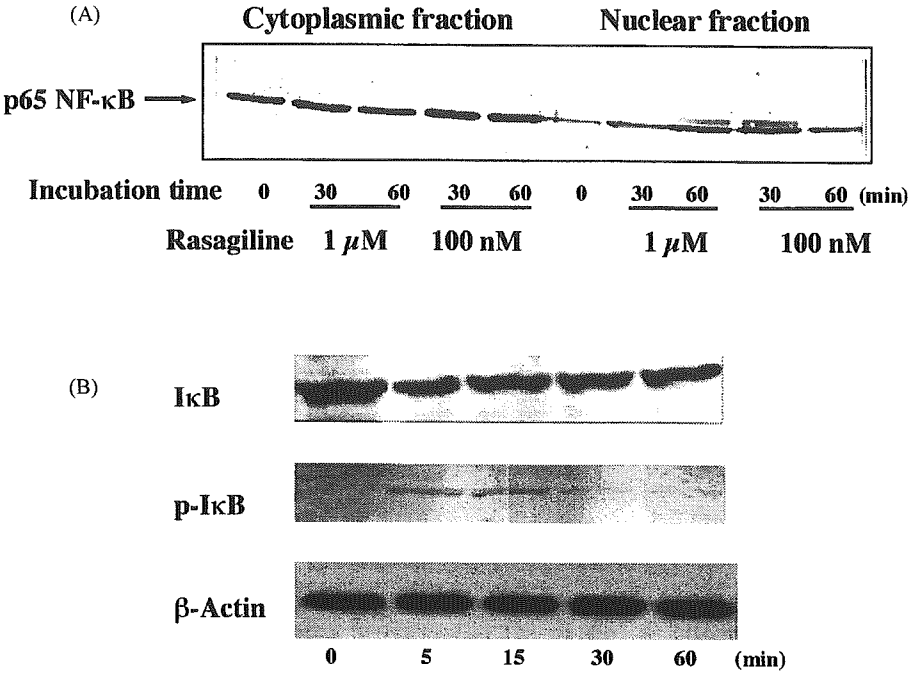

Fig. 2. Rasagiline induced nuclear translocation of p65 NF-κB and phosphorylation of IκB. (A) SH-SY5Y cells were treated with 1 M–100 μM of rasagiline for 30 and 60 min and then the cytoplasmic and nuclear fraction were isolated as described in Section 2. The samples were subjected to Western blotting analysis using anti-p65 NF-κB antibody. (B) SH-SY5Y cells were treated with a proteasome inhibitor, lactacystin, for 4 h and then with 100 nM of rasagiline. The total cell lysate was analyzed by immunoblotting using antibodies against IκB (IκB), phosphorylated IκB (p-κB), and β-actin (β-actin) as control.

were studied on the activation of NF-κB, a common transcription factor for GDNF, BDNF, Bcl-2 and SOD, whose levels were increased by propargylamines. After treatment of SH-SY5Y cells with rasagiline at 1 μM and 100 nM, the p65 subunit of NF-κB in the cytoplasmic and nuclear fraction was analyzed by immunoblotting, as shown in Fig. 2A. The p65 subunit increased significantly in the nuclear fraction after 30 and 60 min incubation with rasagiline. Phosphrylation of IκB was detected in the cells after 5–15 min treatment with 100 nM of rasagiline (Fig. 2B).

The activation of p65 NF-κB was also shown by the increase in its binding activity to oligonucleotide containing the NF-κB consensus-binding site, as shown in Fig. 3A. The binding capacity increased in the cells treated with rasagiline at 1 μM–10 nM for 60 min (Fig. 3B), and the highest binding activity was detected at 100 nM rasagiline. The effects of rasagiline concentration on the binding capacity also followed an inverted U-shaped dose-response curve. The specificity of the binding was examined by competition studies with the wild and mutated oligonucleotide, and the binding was inhibited by the wild, but not by the mutated, oligonucleotide (Fig. 3C).

The role of NF-κB in increasing GDNF was studied further by use of an inhibitor of IκB kinase, sulfasalazine. Sulfasalazine at 100 μM suppressed the increase in GDNF protein level by rasagiline to the basal level, as shown in Fig. 4. Sulfasalazine inhibited also rasagiline- induced increase in the binding capacity of NF-κB to the oligonucleotide containing the binding site, as shown in Fig. 3D.

4. Discussion

In this paper, rasagiline was proved to increase the mRNA and protein levels of GDNF, in addition to those of anti-apoptotic bcl-2 and bcl-xL as previously reported (Akao et al., 2002b). Selegiline and desmethylselegiline, propargylamines structurally related to rasagiline, were reported to increase mRNA level of neuroprotective proteins, bcl-2, SOD, glutathione peroxidase and GDNF in PC12 cells (Tatton et al., 2002). The results reported in this paper clearly show that the activation of NF-κB by rasagiline mediates the increase in the transcription of pro-survival genes. In our previous papers, rasagiline protected SH-SY5Y cells from apoptosis induced by a neurotoxin, *N*M(*R*)Sal, through the stabilization of mitochondrial membrane potential ($\Delta\Psi$m) (Akao et al., 2002a; Maruyama et al., 2000), and the increased expression of Bcl-2 and Bcl-xL (Akao et al., 2002b). It may be interesting enough, since Bcl-2 was confirmed to prevent apoptosis in SH-SY5Y cells (Maruyama et al., 2001a) and also permeability transition in isolated mitochondria induced by the neurotoxin (Akao et al., 2002a). In neurodegenerative disorders, such as PD and Alzheimer's diseases, particular neurons deteriorate in slow and continuous process, where not only apoptogenic,

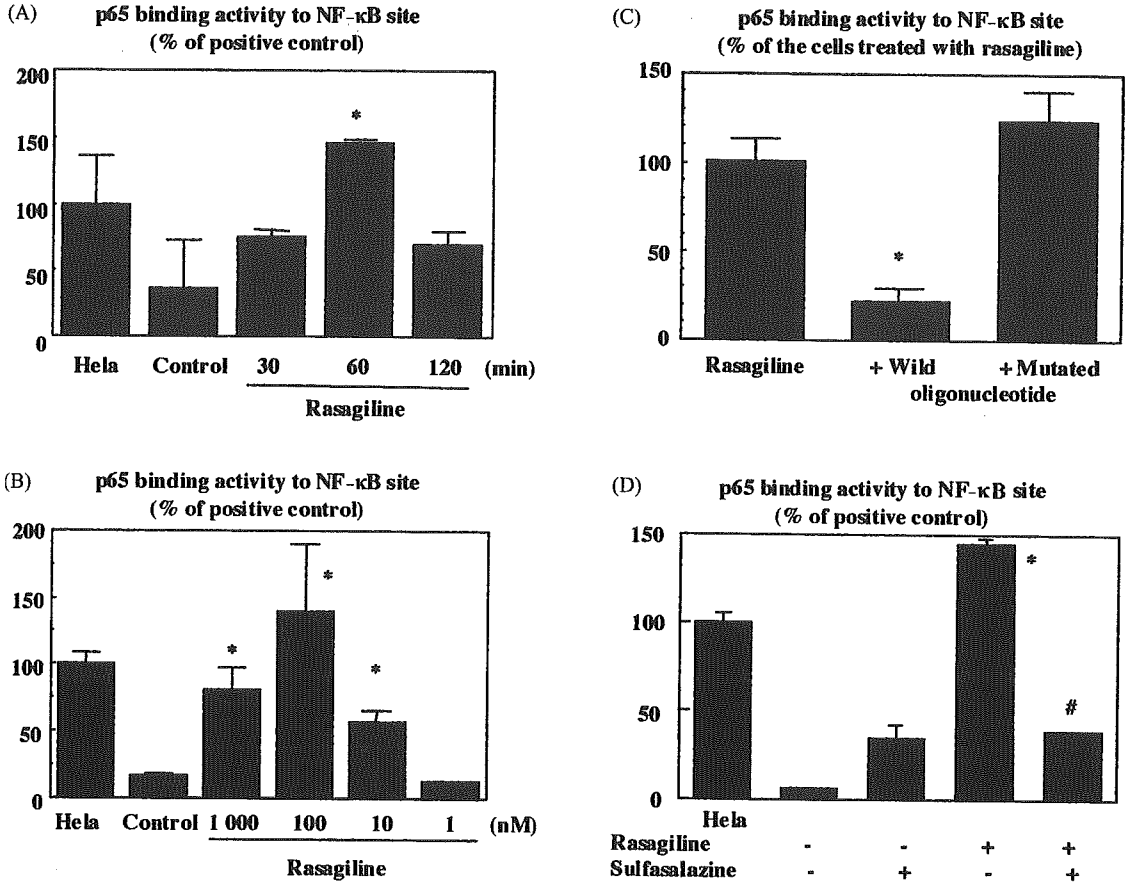

Fig. 3. Increase in NF-κB binding activity by rasagiline and the suppression by NF-κB oligonucleotide and sulfasalazine The lysate of the Hela cells treated with TNF-α: (Hela) was used as a positive control and the amounts of activated NF-κB were expressed as percentage of Hela (A, B, D) or the lysate of the cells treated with 100 nM of rasagiline alone for 60 min (C). (A) The effects of the incubation time were examined in SH-SY5Y cells treated with 100 nM of rasagiline for 30, 60 and 120 min. The activation of NF-κB in the cell lysate was quantified as the p65 binding activity to the NF-κB binding oligonucleotide by use of the NF-κB p65 transcription assay kit as described in Section 2. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (B) The effects of rasagiline concentration were examined in SH-SY5Y cells treated with 1 μM–1 nM of rasagiline for 60 min and the amounts of activated NF-κB was measured. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the control (C) is statistically significant ($P < 0.01$) by ANOVA. (C) The specificity of NF-κB binding to the binding site was examined. SH-SY5Y cells were treated with 100 nM of rasagiline for 60 mm, and the p65 binding activity was measured in the presence or absence of wild (+Wild) or the mutated (+Mutated) oligonucleotide containing the NF-κB binding site. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the binding activity of the cell lysate without oligonucleotide is statistically significant ($P < 0.01$) by ANOVA. (D) Effects of an inhibitor of IκB kinase, sulfasalazine, were examined in SH-SY5Y cells treated with 100 μM of sulfasalazine for 30 min, then, 100 nM of rasagiline. Activated NF-κB in the cells was quantified by the NF-κB p65 transcription assay kit. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (#) The difference from the cells treated with rasagiline alone is statistically significant ($P < 0.01$) by ANOVA.

but also pro-survival factors should be activated. NF-κB may be a common regulator collecting the information of upstream signal events to decide the survival and death of the cells (Grilli and Memo, 1999). Increased levels of NF-κB were detected in the brain of Alzheimer's disease (Kaltschmidt et al., 1997) and PD (Hunot et al., 1997). In apoptosis induced by excitotoxicity and oxidative stress, NF-κB was activated (Qin et al., 1998), and the cytotoxicity was mediated by the activation of pro-apoptotic members of Bcl-2 family (Shou et al., 2002). On the contrary, NF-κB was reported to be involved in cytoprotection against apoptosis induced by oxidative stress and excitotoxic insults (Goodman and Mattson, 1996). These controversial results suggest that the cell types, the conditions of cytotoxic stimuli and the concomitant activation of other transcription factors may determine whether activated NF-κB promotes cell survival or induces cell death.

The molecular mechanisms underlying the activation of NF-κB is now on the way for elucidation (Karin and Ben-Nariah, 2000) NF-κB is activated by IκB phosphorylation by kinase complex (IκB kinase, IKK) composed with IKK-α, -β and -γ. When the IKK complex is phosphorylated, IκBα and IκBβ were cleaved at two serine residues in the N-terminal and dissociated, resulting in the activation of NF-κB. Sulfasalazine, an inhibitor of IKK-α and -β (Weber et al., 2000), inhibited the NF-κB activation and the

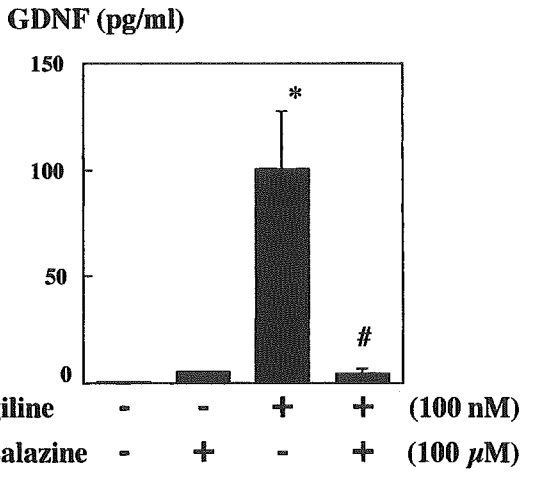

Fig. 4. Effects of sulfasalazine on the increase in GDNF protein by rasagiline. SH-SY5Y cells were incubated with or without 100 μM of sulfasalazine for 30 min, and then treated with 100 nM of rasagiline for 3 h. GDNF protein level was measured by the EIA. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from control is statistically significant ($P < 0.01$) by ANOVA. (#) The difference from the cells treated with rasagiline alone is statistically significant ($P<0.01$) by ANOVA.

induction of GDNF protein, simultaneously. These results suggest that rasagiline may activate NF-κB through the IKK pathway to induce GDNF. It requires further studies to clarify whether rasagiline may affect directly the IKK components or indirectly through activation of upstream kinases in the cells. Recently, rasagiline and TV3324, a rasagiline analogue with a carbamyl moiety, were reported to activate protein kinase C and Erk1/2 mitogen-activated protein (MAP) kinase (Yogev-Falach et al., 2002). These results suggest that the propargylamines may target protein regulating signal transduction in MAP kinase system.

The potency of rasagiline to activate NF-κB and increase GDNF did not linearly depend on the concentration, but followed an inverted U-shaped dose-response curve. We observed also that rasagiline showed such an inverted U-shaped relationship in the anti-apoptotic function (Maruyama et al., 2001b) and in the induction of Bcl-2 (Akao et al., 2002b). It was reported that TNF-α, reactive oxygen species (H_2O_2) and also β-amyloid activated NF-κB according to an inverted U-shaped dose-response curve. At the low concentration, TNF-α was neuroprotective in correlation with NF-κB activation, whereas at the high concentration it was neurotoxic (Kaltschmidt et al., 1999). The mechanism behind inverted U-shape type of the concentration-activity relationship of NF-κB remains to be fully elucidated.

GDNF is now proposed as an agent to rescue declining dopamine neurons in PD as shown by the effectiveness in animal and cellular models (Wang et al., 2002). Lentivirally-delivered GDNF was reported to rescue dying dopamine neurons in a monkey model of PD prepared with 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (Palfi et al., 2002). Infusion of GDNF protein by an implanted intracerebroventricular catheter failed to improve the symptoms in Parkinsonian patients, (Nutt et al., 2003). However, recently it was reported that the direct infusion of GDNF into the putamen of Parkinsonian patients improved clinical symptoms and fluorodopa uptake to the dopamine terminal (Gill et al., 2003). These results indicate that GDNF supplement therapy requires further technical improvement for the administration. If rasagiline, which is now under phase III clinical trials for Parkinsonian patients (Parkinson Study Group, 2002), can also increase endogenous GDNF in the human brain, as shown here in SH-SY5Y cells, it may be a more practical therapy to prevent cell death of dopamine neurons.

In conclusion, rasagiline and related propargylamines are possible neuroprotective agents and a part of their pharmacological action may be due to the induction of pro-survival genes through NF-κB activation. The activation of transcription factors related to anti-apoptotic proteins, GDNF and bcl-2 (Maruyama et al., in preparation), by propargylamines might enable us to suppress the neuronal apoptosis in neurodegenerative disorders in general. Clinical trials with propargylamines are waiting for the final evaluation of the efficacy to protect specified neurons from degeneration.

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Neuroprotective function of *R*-(−)-1-(benzofuran-2-yl)-2-propylaminopentane, [*R*-(−)-BPAP], against apoptosis induced by *N*-methyl(*R*)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells

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Abstract

R-(−)-1-(Benzofuran-2-yl)-2-propylaminopentane HCl [*R*-(−)-BPAP] is one of "catecholaminergic and serotonergic enhancers", which were proposed to improve symptoms through increase in impulse-evoked release of monoamine neurotransmitters for Parkinson's disease. It was reported that (−)-BPAP up-regulated the synthesis of neurotrophic factors in mouse astrocytes, suggesting the neuroprotective potency of (−)-BPAP. In this paper, the neuroprotective function of (−)-BPAP and the related compounds was examined against apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal], a possible pathogenic toxin in Parkinson's disease, in human dopaminergic neuroblastoma SH-SY5Y cells. The anti-apoptotic activity was confirmed with some of (−)-BPAP analogues, and the mechanism was found to be due to the direct stabilization of mitochondrial membrane potential and the induction of anti-apoptotic Bcl-2. The studies on structure-activity relationship demonstrated that the potency to stabilize the mitochondrial membrane potential depended on the absolute stereo-chemical structure of BPAP derivatives. The compounds with dextrorotation prevented the mitochondrial permeability transition, whereas those with levorotation did not. The presence of a propargyl or

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propyl group at the amino residue of *R*-(−)-1-(benzofuran-2-yl)-2-propylamine increased potency to stabilize the membrane potential and prevent apoptosis. *R*-FPFS-1169 and *R*-FPFS-1180 had more potent to induce Bcl-2 and prevent apoptosis than the corresponding *S*-enantiomers. These results are discussed with the possible application of BPAP derivatives as neuroprotective agents in Parkinson's disease and other neurodegenerative disorders.

Keywords: *R*-(−)-1-(Benzylfuran-2-yl)-2-propylaminopentane [*R*-(−)-BPAP]; Apoptosis; Bcl-2; Mitochondrial membrane potential; Parkinson's disease; Neuroprotection

Introduction

For neurodegenerative diseases, Parkinson's disease (PD) and Alzheimer's disease (AD), hitherto available therapy intends to replace or increase deficient neurotransmitters, using the metabolic precursors, such as L-DOPA, or the inhibitors of metabolizing enzymes, monoamine oxidase, catechol-O-methyltransferase and cholinesterase. As another strategy of the therapy, some compounds including (−)-enantiomers of deprenyl, amphetamine and methamphetamine, and β-phenylethanolamine, were proposed to enhance the impulse-evoked release of catecholamine and serotonin (Knoll et al., 1996). Among catecholaminergic-serotonergic enhancers, (−)-1-phenyl-2-propylaminopentane [(−)-PPAP] and *R*-(−)-(benzofuran-2-yl)-2-propylaminopentane [*R*-(−)-BPAP, the development number; FPFS-1169] are the most promising agents (Knoll et al., 1999). They do not inhibit type B monoamine oxidase and are not metabolized to amphetamine, in contrast to (−)-deprenyl.

Recently neuroprotection to halt progressive cell death of neurons has been proposed as a future therapy for neurodegenerative disorders. In these disorders, such as PD and AD, apoptosis contributes to neuronal death in most cases (Tatton, 2000) and the well regulated and relatively slow apoptotic process was proposed as a target of neuroprotection (Thompson, 1995; Naoi and Maruyama, 2001). Apoptosis is induced in neurons by various insults; oxidative stress, metabolic compromise, excitotoxicity and neurotoxins. Apoptotic signaling is a multi-step pathway induced by opening a mitochondrial mega-channel called permeability transition (PT) pore, followed by decline in membrane potential, $\Delta\Psi m$, release of apoptosis-inducing factors, activation of caspases and fragmentation of nuclear DNA. Mitochondrial PT pore is regulated by Bcl-2 protein family, preventively by Bcl-2 and Bcl-xL and promotively by BAX and BAD.

After the discovery of (−)-deprenyl as an agent preventing cell death in the animal and cellular models of neurodegenerative disorders (Finnegan et al., 1990; Heikkila et al., 1984), a series of propargylamines with β-phenylethylamine [(−)-deprenyl], cyclic benzylamine [*N*-propargyl-1(*R*)-aminoindan, rasagiline] and aliphatic structure [*N*-(2-heptyl)-*N*-methylpropargylamine] were confirmed to protect neurons against apoptosis induced by various insults (Maruyama and Naoi, 1999; Maruyama et al., 2000, 2001a, 2001b, 2002). However, the effectiveness of these compounds as neuroprotective agents has not been fully confirmed in clinical studies, maybe because of difficulty to evaluate neuroprotective effects in patients. Nevertheless, significant insights into the anti-apoptotic function of propargylamines have been well achieved, and mitochondria emerge as a key organelle playing a role in apoptosis. The regulation of mitochondrial PT was found to be critical for decision of cell survival and death (Naoi et al., 2002). Our studies show that the neuroprotection of propargylamines is ascribed to (1) the stabilization of mitochondria membrane potential, $\Delta\Psi m$, and prevention of PT (Maruyama et al.,

2001a, 2001b), (2) the induction of anti-apoptotic Bcl-2 family regulating PT (Akao et al., 2002a, 2002b) and (3) of neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) selective to dopamine neurons (Maruyama et al., 2004), and (4) of anti-oxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 2000). *R*-(−)-BPAP was found to increase the biosynthesis and secretion of neurotrophins; such as GDNF, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), in mouse asctrocytes, suggesting its neuroprotective potency (Ohta et al., 2002; Shimazu et al., 2003).

This paper describes that (−)-BPAP and related benzofuran derivatives suppressed apoptosis induced by a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol [*R*(1),*N*-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *N*M(*R*)Sal] (Maruyama et al., 1996, 2001b; Naoi et al., 1998) in human dopaminergic neuroblastoma SH-SY5Y cells. The structure-activity relationship and the mechanism underlying the anti-apoptotic function were studied. The results are discussed in relation to the possible application of (−)-BPAP analogues to the neuroprotective therapy for PD and other neurodegenerative diseases.

Materials and methods

Materials

(−)-BPAP and other related benzofuran-2-yl derivatives were synthesized by Fujimoto Pharmaceutical Corp. (Osaka, Japan), and *N*M(*R*)Sal according to Teitel et al. (1972). Rhodamine 123, YO-PRO and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR, USA), Dulbecco's modified Eagle's medium (DMEM) and other drugs from Nacarai tesque (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in an atmosphere of 95% air − 5% CO_2.

Assessment of apoptosis induced by NM(R)Sal and the protection by BPAP derivatives

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with FACScan and CellQuest software (Becton Dickinson, San Jose, CA, USA). Cells cultured in a 6-well poly-L-lysine-coated culture flask were incubated with or without 1 μM − 1 nM (−)-BPAP analogues at 37°C for 30 min, and then for 24 h with 250 μM *N*M(*R*)Sal in Cosmedium-001 culture medium supplemented with fetal calf serum. The cells were treated with trypsin, gathered, washed with the culture medium and twice with phosphate-buffered saline (PBS). The cells were incubated with 100 nM YO-PRO and 1.5 μM PI solution in an ice-bath for 30 min, washed and suspended in PBS, then subjected to FACS analysis.

Measurement of changes in $\Delta\Psi m$

Decline in $\Delta\Psi$m induced by *N*M(*R*)Sal was quantified by measuring the reduction of Rhodamine 123 fluorescence pre-loaded in the cells (Patorino et al., 1996), as reported previously (Akao et al., 2002a). To examine the effects of (−)-BPAP analogues, the cells cultured in 6-well poly-L-lysine-coated tissue culture flasks were stained with 5 μM Rhodamine 123 in DMEM for 30 min at 37°C. After washed twice with PBS, the cells were suspended in DMEM, incubated with 1 μM − 1 nM BPAP derivatives for

30 min, then with 250 μM *N*M(*R*)Sal for 1 h. After washed and gathered by treatment with trypsin, the cells were suspended in PBS and the fluorescence at 535 nm was measured with excitation at 505 nm in a Shimadzu spectrofluorophotometer, RF-5000 (Kyoto, Japan).

Measurement of bcl-2 mRNA level in the cells treated with BPAP derivatives

SH-SY5Y cells were cultured in the presence of various concentrations (100 nM-10 pM) of (−)-BPAP analogues for 24 h, and mRNA levels of bcl-2 were quantitatively assessed by RT-PCR method (Akao et al., 2002a, 2002b). The cells were gathered and washed with PBS, and the total RNA was extracted by the phenol/guanidinium thiocyanate method. cDNA was generated by reverse transcription of 2 μg of the total RNA, and the cDNA fragments were amplified using the PCR primers. The linearity of the amount of PCR product to the time of PCR amplification was confirmed under the conditions used in this study. PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin was used as an internal standard. The amounts of mRNA were quantified using NIH imaging software (version 1.62, developed at the U.S. National Institute for Health).

Measurement of Bcl-2 levels in the cells treated with (−)-BPAP derivatives

SH-SY5Y cells treated with 1 μM-1 pM (−)-BPAP analogues for 24 h, and the cells were gathered, washed with PBS and suspended in RIPA buffer [10 mM Tris-HCl buffer, pH 7.5, containing 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl and 1 mM EDTA 2Na]. The lyzed protein (5 μg) was separated by SDS-PAGE using a 10 – 20% gradient polyacrylamide gel (Bio-Rad Lab., Hercules, CA, USA) and electroblotted onto PVDF membranes (Du Pont, Boston, MA, USA). After blockage with 5% nonfat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight

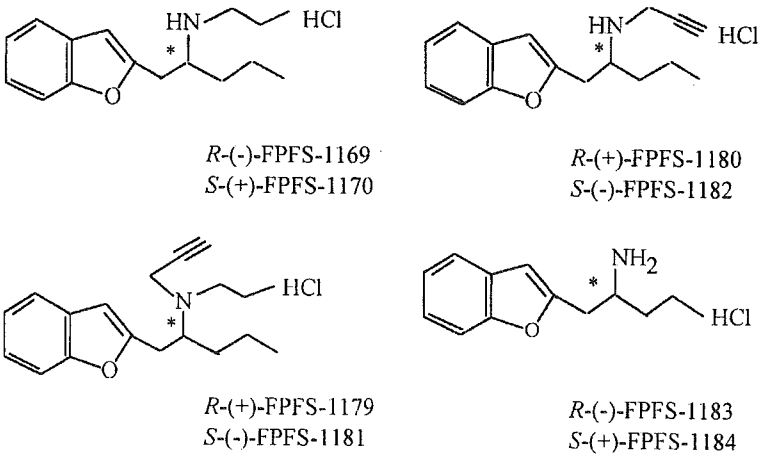

Fig. 1. Chemical structure and abbreviations of used BPAP derivatives. FPFS-1169 and FPFS-1170: *R*-(−)- and *S*-(+)-1-(benzofuran-2-yl)-2-propylamino-pentane hydrochloride, FPFS-1180 and FPFS-1182: *R*-(+)- and *S*-(−)- *N*-(2-propynyl)-1-(benzofuran-2-yl)-2-aminopentane hydrochloride, FPFS-1179 and FPFS-1181: *R*-(+)- and *S*-(−)-*N*-(2-propynyl)-1-(benzofuran-2-yl)-2-propylaminopentane hydrochloride, FPFS-1183 and FPFS-1184: *R*-(−)- and *S*-(+)-1-(benzofuran-2-yl)-2-aminopentane hydrochloride.

Table 1
Anti-apoptotic function of *R*-(−)-BPAP analogues on apoptosis induced by *N*-methyl(*R*)salsolinol

	Number of apoptotic cells ([1]; % of the total, [2];% of apoptotic cells in *N*M(*R*)Sal-treated cells)					
	Control[1]	*N*M(*R*)Sal[1]	1 μM[2]	100 nM[2]	10 nM[2]	1 nM[2]
R-(−)FPFS-1169	3.2	36.8	61.7 ± 11.6*	65.4 ± 7.5*	52.7 ± 14.5*	108.7 ± 1.5
S-(+)-FPFS-1170	5.1	30.9	67.8 ± 9.5*	63.2 ± 1.9*	73.7 ± 4.4*	79.6 ± 11.9
R-(+)-FPFS-1179	3.0	28.9	128.8 ± 16.0	125.2 ± 14.2	120.5 ± 16.7	144.5 ± 30.1
S-(−)-FPFS-1181	5.6	32.0	91.7 ± 6.3	89.1 ± 11.5	112.9 ± 14.2	101.4 ± 13.5
R-(+)-FPFS-1180	4.5	29.6	77.9 ± 6.9*	81.1 ± 7.5*	75.8 ± 7.3*	62.6 ± 10.8*
S-(−)-FPFS-1182	3.41	18.7	92.5 ± 2.3	94.3 ± 6.2	117.9 ± 0.1	97.3 ± 14.5
R-(−)-FPFS-1183	4.7	29.1	79.7 ± 14.2	66.8 ± 8.0*	76.9 ± 18.0	97.3 ± 16.8
S-(+)-FPFS-1184	3.45	34.2	71.3 ± 8.3*	128.8 ± 20.9	114.7 ± 9.1	99.6 ± 15.3

* Statistically different from *N*M(*R*)Sal-treated cells, $p < 0.05$.

at 4 °C with anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-β-actin antibody as control (Sigma, St. Louis, MO, USA). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) at room

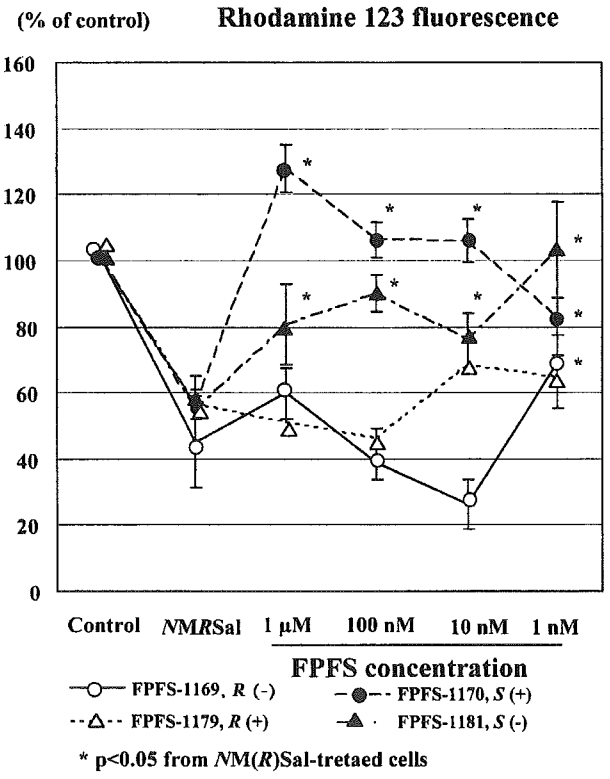

Fig. 2. The effects of BPAP derivatives on ΔΨm decline induced by *N*M(*R*)Sal in SH-SY5Y cells. As described in Materials and methods, the cells were treated with FPFS-1169, FPFS-1170, FPFS-1179 and FPFS-1181, then with *N*M(*R*)Sal, and the fluorescence intensity of Rhodamine 123 was expressed as percentage of control. Each point and bar represent the mean and SD of three experiments. *; Difference from the cells treated with *N*M(*R*)Sal alone was statistically significant, $p < 0.05$.

temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA), and quantified by computer-assisted image analysis with the NIH imaging software.

Statistics

Experiments were repeated 4 to 8 times, and the results were expressed as the mean and SD. Differences were statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's F-test. A p value less than 0.05 was considered to be statistically significant.

Results

Anti-apoptotic function of BPAP derivatives

Fig. 1 shows the chemical structure of (−)-BPAP analogues used in these experiments. Apoptosis was induced in 19–37% of SH-SY5Y cells after treatment with 250 μM *N*M(*R*)Sal, whereas necrotic

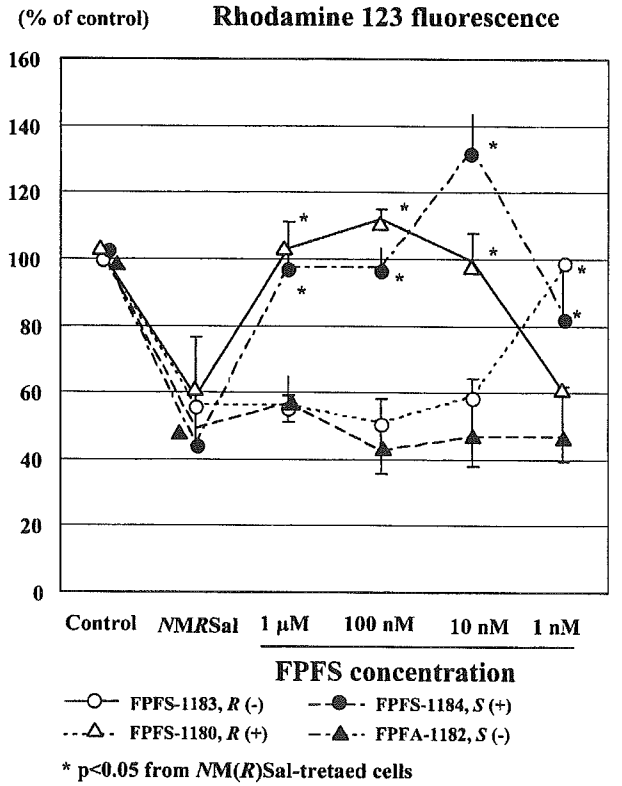

Fig. 3. The effects of BPAP derivatives on $\Delta\Psi$m decline induced by *N*M(*R*)Sal in SH-SY5Y cells. The cells were treated with FPFS-1180, FPFS-1182, FPFS-1183 and FPFS-1184, as described in legend for Fig. 2.

cells were virtually negligible. As summarized in Table 1, *R*-(−)- and *S*-(+)-BPAP (the development number; FPFS-1169 and FPFS-1170) and FPFS-1180 reduced the number of apoptotic cells to 50–60% of that of *N*M(*R*)Sal-treated cells. *R*-(−)1-(Benzofuran-2-yl)-2-aminopentane (FPFS-1183) and the *S*-(+)-enantiomer (FPFS-1184) protected the cells from apoptosis only at 100 nM and 1 μM, respectively.

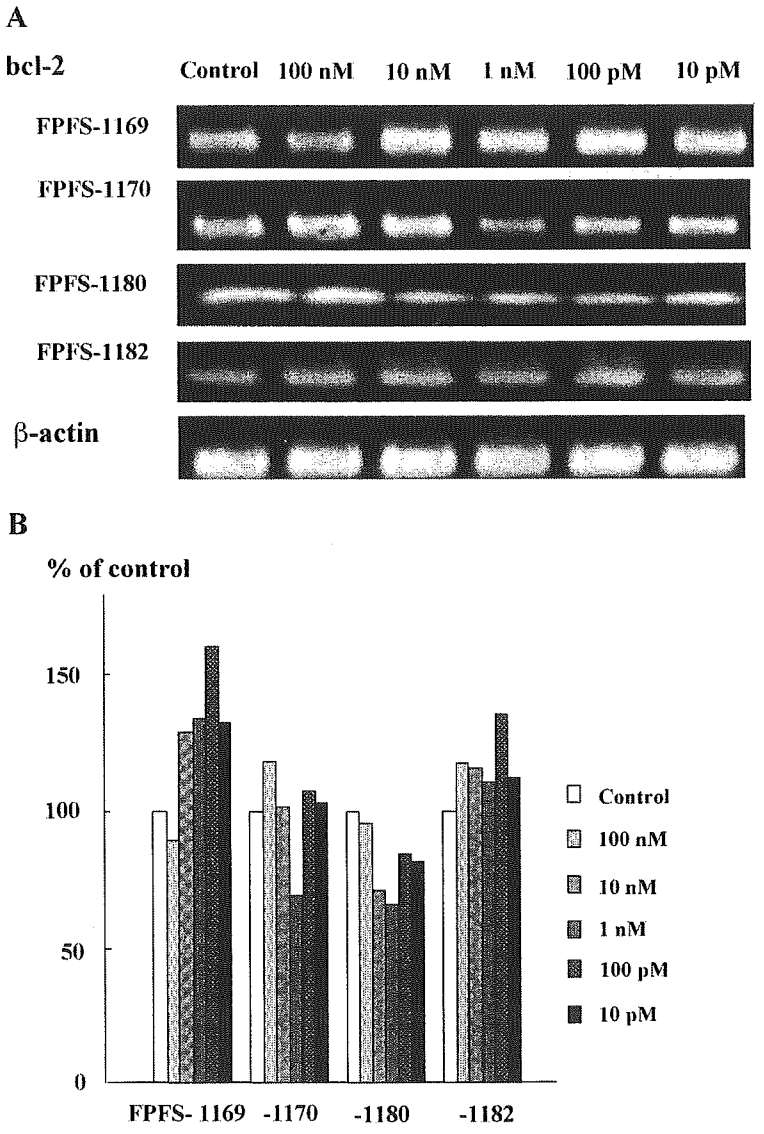

Fig. 4. The effect of BPAP derivatives on mRNA levels of bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 100 nM–10 pM BPAP derivatives for 24 h, and mRNA levels were measured, as written in Materials and methods. β-Actin mRNA was used as control. (A) Gel electrophoresis of bcl-2 and β-actin mRNA. (B) One of the quantitative data of mRNA amounts.

R-(+)-FPFS-1179 and *S*-(−)-FPFS-1181 containing both a propargyl and a propyl group at the 1-amino group did not protect the cells from apoptosis.

The stabilization of ΔΨm

*N*M(*R*)Sal induced PT in SH-SY5Y cells, and the fluorescence of pre-loaded Rhodamine 123 reduced. The effects of BPAP derivatives on the stabilization of ΔΨm were studied, as shown in Figs. 2 and 3. After 2 h treatment with 250 μM *N*M(*R*)Sal, the fluorescence intensity reduced to about 50% of

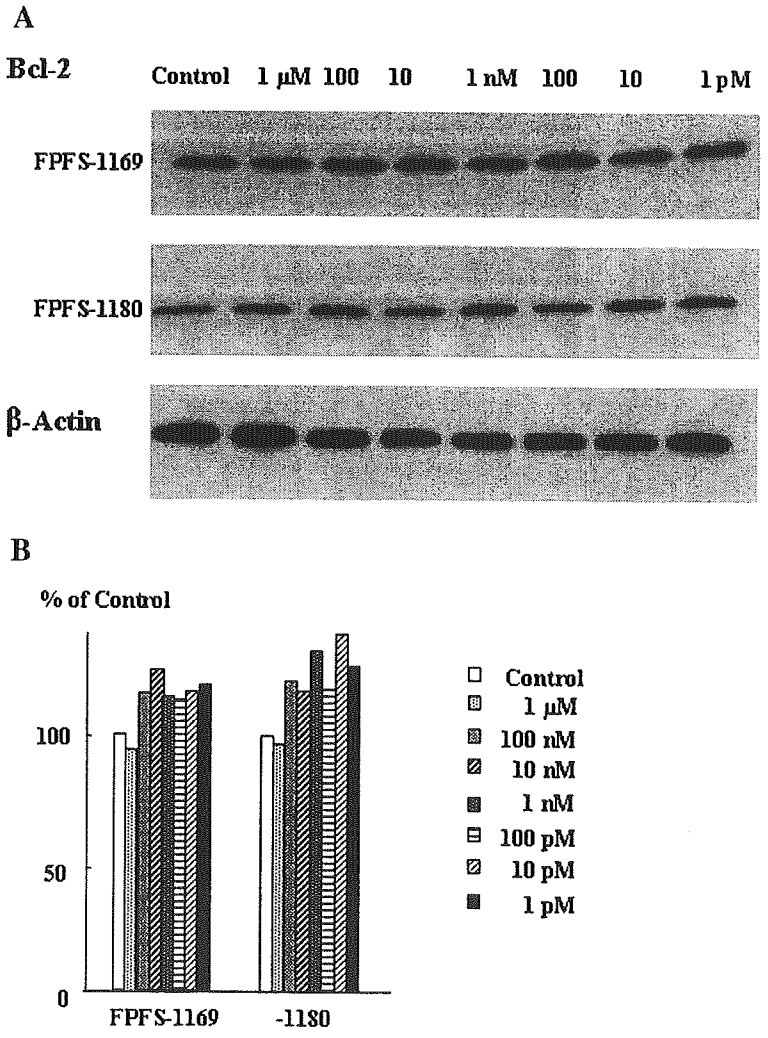

Fig. 5. The effects of BPAP derivatives on the protein level of Bcl-2. SH-SY5Y cells were treated with 1 μM–1 pM BPAP derivatives, and the Bcl-2 protein levels were determined by Western blot analysis. β-Actin was used as an internal control. (A) Western blots of Bcl-2 and β-Actin. (B) One of the quantitative data of Bcl-2 protein amounts.

control, and *S*-(+)-FPFS-1170, *R*-(+)-FPFS-1180 and *S*-(+)-FPFS-1184, the analogues with dextrorotation, prevented the reduction at 1 μM – 10 nM, whereas the corresponding enantiomers with levorotation, *R*-FPFS-1169, *S*-FPFS-1182, *R*-FPFS-1183, did not. Neither *R*-(+)-FPFS-1179 nor *S*-(−)-FPFS-1181 prevented the ΔΨm reduction.

Induction of anti-apoptotic bcl-2 mRNA and protein

The effects of BPAP derivatives on the mRNA level of apoptosis-preventing and survival-promoting bcl-2 were examined by RT-PCR method. As shown in Fig. 4, *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180 increased mRNA level of bcl-2 at 10 nM – 10 pM, whereas *S*-(+)-FPFS-1170 and *S*-(−)-FPFS-1182 did not. Fig. 4B shows one of the quantitative data of blc-2 mRNA in the cells treated with (−)-BPAP analogues.

Bcl-2 protein levels in SH-SY5Y cells treated with BPAP derivatives were examined by Western blot analysis. As shown in Fig. 5, *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180 increased the Bcl-2 levels at 100 nM–1 pM.

Discussion

This paper presents that a series of benzofuran derivatives prevented apoptosis induced by an endogenous neurotoxin, *N*M(*R*)Sal, through the stabilization of PT pore closed state and the induction of bcl-2. As reported previously with another neuroprotective agent, rasagiline, prevention of apoptosis by these compounds was confirmed from reduction of apoptotic cells staining with Hoechst 33342 and from prevention of nucleosomal DNA fragmentation.

The studies on the structure-activity relationship show that benzofuran derivatives with dextrorotation, *S*-FPFS-1170, *R*-FPFS-1180 and *S*-FPFS-1184, prevented the ΔΨm decline, whereas the corresponding derivatives with levorotation, *R*-FPFS-1169, *S*-FPFS-1182 and *R*-FPFS-1183, did not affect the ΔΨm. However, *R*-(−)-FPFS-1169, *S*-(+)-FPFS-1170, and *R*-(+)-FPFS-1180 could protect the cells from apoptosis. Their neuroprotective potency may be due to the induction of anti-apoptotic Bcl-2, as shown here with *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180. Our previous results using rasagiline, and isolated mitochondria and SH-SY5Y cells (Maruyama et al., 2001a, 2001b; Akao et al., 2002a) indicate the presence of a binding site enantio-specific for (*R*)-enantiomers in the outer membrane of mitochondria. The stabilization of PT pore-closed state by benzofuran derivatives depended on the absolute stereo-chemical structure, suggesting the presence of a binding site of BPAP derivatives with dextrorotation. The presence of both a propargyl and a propyl group at the 1-amino group in *R*-(+)-FPFS-1179 and *S*-(−)-FPFS-1181 abolished the potency to suppress ΔΨm decline and apoptosis, suggesting that the presence of these two residues inhibited the binding to the target protein in mitochondria. The induction of bcl-2 mRNA and BCL-2 protein was increased at the lower concentrations (Figs. 4B and 5B), which may be comparable with our previous results with rasagiline. The effects of rasagiline concentration on the potency to induce GDNF showed an inverted U-shape dose-response curve (Maruyama et al., 2004). The mechanism of these results remains to be clarified, but the binding site(s) may have such an affinity to these neuroprotective drugs.

Rasagline (Akao et al., 2002b) and (−)deprenyl (Tatton et al., 2000) induced Bcl-2 and GDNF (Maruyama et al., 2004), a dopamine neuron-specific neurotrophic factor, in cultured cells, and increased

the enzymatic activities of superoxide dismutase and catalase in dopamine neurons of rat brains (Carrillo et al., 2000). Neuroprotective agents were found to induce anti-apoptotic and pro-survival genes though the activation of transcription factors. Rasagiline activates a nuclear transcription factor, NF-κB, which mediates the induction of pro-survival genes encoding Bcl-2, GDNF and anti-oxidant enzymes in general. At present, it remains to be clarified whether BPAP derivatives activate transcription factors, such as NF-κB, to induce anti-apoptotic genes. In addition, the relation of the gene induction to catecholaminergic-serotonergic enhancing effects should be further examined to elucidate the mechanism behind neuroprotection by a series of BPAP derivatives.

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Neuromelanin inhibits enzymatic activity of 26S proteasome in human dopaminergic SH-SY5Y cells

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Summary. Recently, impairment of the ubiquitin-proteasome system is suggested to be responsible for the neuronal death in ageing and Parkinson's disease. The specific degeneration of dopamine neurons containing neuromelanin (NM) suggests that NM itself may be involved in the cellular dysfunction and death, even though the direct link has never been reported. We examined the effects of NM isolated from the human substantia nigra on the proteasome activity in human dopaminergic SH-SY5Y cells. NM reduced the activities of 26S proteasome, as shown *in situ* using a green fluorescent protein homologue targeted to 26S proteasome and also *in vitro* using ubiquitinated lysozyme as a substrate. However, NM did not affect 20S proteasome activity *in vitro*. NM reduced the amount of PA700 regulatory subunit of 26S proteasome, but did not affect that of α- and β-subunits of 20S proteasome. These results suggest that NM may inhibit the ubiquitin-26S proteasome system, and determine the selective vulnerability of dopamine neurons in ageing and related disorders.

Keywords: Dopamine neuron, neuromelanin, 26/20S proteasome, ubiquitin, ubiquitin-proteasome system, proteasome sensor protein.

Abbreviations

AMC 7-Aminomethylcoumarin, *DMSO* dimethyl sulfoxide, *DTT* dithiothreitol, *ECF* enhanced chemofluorescence, *GFP* green fluorescent protein, *Lyso* lysozyme, *MCA* 4-methyl-coumaryl-7-amide, *MEM* minimum essential medium: MPP^+ 1-methl-4-phenylpyrimidium ion, *NM* neuromelanin, *PBS* phosphate-buffered saline, *PD* Parkinson's disease, *PSI* carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [Z-Ile-Glu(Obu^t)-Ala-Leu-H (aldehyde)], *SDS* sodium dodecyl sulfate, *SN* substantia nigra, *UCH-L1* ubiquitin C-terminal hydrolase L1, *UP* ubiquitin-proteasome, *ZsGFP* a green fluorescence protein homologue from *Zoanthus sp.*, *Z-LLE-MCA* carboxy-L-leucyl-L-leucyl-L-glutamyl-MCA, *Z-LLVY-MCA* carbobenzoxy-L-leucyl-L-leucyl-L-valyl-L-tyrosine-MCA.

Neuromelanin (NM) is present in the neurons of the brainstem, especially the substantia nigra (SN) and the locus coeruleus, which are preferentially affected in ageing and Parkinson's disease (PD) (Emborg et al., 1998; Mann and Yates, 1974), suggesting that NM may be involved in the vulnerability of these neurons (Hirsch et al., 1988; Kastner et al., 1992). However, the direct link between NM and degeneration of dopamine neurons has never been proved. The role of NM was discussed either in a preventing or promoting way to the degeneration of nigro-striatal dopamine neurons. NM was reported to increase the vulnerability of SN neurons (Youdim et al., 1994; Offen et al., 1997), and iron(III)-melanin complex was cytotoxic to dopaminergic neurons *in vitro* (Jellinger et al., 1992; Mochizuki et al., 1993; Double et al., 2002). Increased production of reactive oxygen species was proposed to account for the cytotoxicity of NM-metal conjugates (Ben-Shachar et al., 1991; Zareba et al., 1995). More recently NM was reported to increase the secretion of cytokines and nitric oxide from microglia, which might be related to degeneration of dopamine neurons in PD (Wilms et al., 2003). On the other hand, NM has been proposed to be neuroprotective, by scavenging redox active metals (Fe, Cu and Mn), toxic metals (Cd, Hg and Pb) (Youdim et al., 1994; Zecca et al., 1994), pesticides (Lindquist et al., 1987), beta-carbolines (Oestergren et al., 2004), and other toxins, such as 1-methyl-4-phenylpyridinium ion (MPP^+) (D'Amato et al., 1986) and 1,2(*N*)-dimethyl-6,7-dihydroxyisoquinolinium ion, an oxidation product of an endogenous dopaminergic neurotoxin, *N*-methyl-(*R*)salsolinol [1,2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] (Naoi et al., 1994). In addition, NM synthesis reduces accumulation of cytotoxic quinone produced by dopamine oxidation (Sulzer et al., 2000).

The neuronal death in PD was considered to be due to increased oxidative stress, mitochondrial dysfunction, and accumulation of oxidatively modified proteins in the cells. Recently in the familial parkinsonism, mutations in α-synuclein (Polymeropoulus et al., 1997), parkin, an E3 ubiquitin-protein ligase (Kitada et al., 1998; Shimura et al., 2000), and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Leroy et al., 1998) were reported. These results suggest the contribution of the ubiquitin-proteasome (UP) system to neurodegeneration also in sporadic form of PD. Increased number of evidences suggest that impaired

UP system leads to accumulation of oxidatively-modified and/or ubiquitinated protein, and might account for the formation of Lewy body, the pathological hallmark of PD (McNaught et al., 2002a). Actually, proteasome subunits were found to co-localize in Lewy body (Ii et al., 1997), in addition to proteins associated with the UP system, α-synuclein, UCH-L1, and parkin (Gai et al., 2000; Shimura et al., 2001). Recently, aggresome formed in response to the accumulation of abnormal proteins in the cells is suggested to be a precursor of Lewy body in PD (McNaught et al., 2002b). These results suggest again that reduced activity of proteasome system may play a key role in induction of cell death through accumulation of abnormal proteins.

In the UP system, 20S proteasome is composed of 4 rings to make a cylindrical structure made of seven either α or β subunits of 20S. Binding of a regulatory complex called 19S (ATPase, PA700) to both the end of the 20S cylinder produces 26S proteasome. 26S proteasome degrades protein substrates, such as polyubiquitinated proteins and ornithine decarboxylase, in an ATP-dependent process, whereas 20S proteasome degrades oxidized proteins in ubiquitination- and ATP-independent manner.

In this paper, the effects of NM on proteasome were studied in SH-SY5Y cells by use of NM purified from the SN of control human brains, as suggested by one of us (P. R.). To measure the activities of 26S and 20S proteasome, respectively, we used *in vitro* assays for 26S proteasome using ubiquitinated lysozyme (Lyso) and synthesized fluorescent peptides, and *in situ* assays by use of SH-SY5Y cells transfected with a green fluorescence protein homologue from reef coral, *Zoanthus sp.* (ZsGFP) fused to the degradation domain for 26S proteasome with ornithine decarboxylase. The results are discussed in relation to the possible involvement of NM in dysfunction and death of dopamine neurons in ageing and PD through inhibiting the UP system.

Materials and methods

Materials

7-Aminomethylcoumarin (AMC) and substrates for proteasome, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methyl-coumaryl-7-amide (MCA) (Suc-LLVY-MCA, a substrate for chymotrypsin-like activity), benzyloxycarbonyl-L-leucyl-L-leucyl-L-glutamic acid-MCA (Z-LLE-MCA, for peptidyl-glutamyl peptide hydrolase-like activity), and an inhibitor of proteasome, benzyloxycarbonyl-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [PSI, Z-Ile-Glu(Obu^t)-Ala-Leu-H (aldehyde)] were purchased from Peptide Institute (Osaka, Japan). L-Cysteine and Lyso were purchased from Sigma (St. Louis, MO, USA). Anti-Lyso polyclonal antibody was obtained from Chemicon International (Temecula, CA, USA), anti-polyubiquitin monoclonal antibody from NBT (Tokyo, Japan). Antibodies against α- and β-subunit of 20S proteasome, and the proteasome activator, PA700 complex, were purchased from Calbiochem (San Diego, CA, USA). A proteasome sensor vector, pZsProSensor-1, was purchased from BD Biosciences (Palo Alto, CA, USA), and minimum essential medium (MEM) and other reagents were from Nacalai Tesque (Kyoto, Japan).

Preparation of NM

NM was isolated from the SN, dissected from brains of neurologically normal adult individuals within 40 h *post mortem* at −15°C, as described previously (Dzierzega-Lecznar et al., 2004; Gerlach et al., 1995; Wakamatsu et al., 2003). Proteinase K-treated NM is essentially free of

adhering proteins (Dzierzega-Lecznar et al., 2004), to remove iron NM was further incubated in EDTA (Double et al., 2003). The identity and purity of this NM sample has been assessed by elemental analysis, amino acid analysis and electron paramagnetic resonance spectroscopy as previously reported (Zecca et al., 1996, 2000). NM was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration. NM suspension was sonicated for 20 min and then shaken gently for 4 days for rehydration at room temperature under protection from light.

Establishment of SH-SY5Y cells expressing a proteasome sensor vector

Transfectant with a proteasome sensor vector was established using a pZsProSensor-1 eukaryotic expression vector, designed to express ZsGFP fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome. ZsGreen fluorescence increases in the cells expressing the proteasome sensor vector, depending on its intracellular accumulation due to reduction of 26S proteasome activity. SH-SY5Y cells were transfected with pZsProSensor-1 by the lipofection technique according to the manufacturer's protocol (Gibco, Life Technologies, Invitrogen, Calsbad, CA, USA). Selection was started 3 days after the transfection using the Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented with 5% newborn calf serum containing 100 μg/ml of G418 (Gibco, Life Technologies) in 5% CO_2 −95% atmosphere at 37°C. G418-resistant clones were isolated by limiting dilution. To confirm the expression of the proteasome sensor vector, the cells were cultured in the presence or the absence of 100 μM PSI for 24 h. The living cells were harvested, gathered by centrifugation, washed with phosphate-buffered saline (PBS), and then, the cells were suspended with PBS to be 5×10^5 cell/ml. The intensity of the fluorescence of ZsGreen was measured by a spectrofluorophotometer, RF-5300 (Shimadzu, Kyoto, Japan) with excitation at 493 nm and emission at 505 nm.

Measurement of in situ 26S proteasome activity using ZsGFP-expressed cells

The *in situ* activity of 26S proteasome was estimated in SH-SY5Y cells transfected with the proteasome sensor vector (PSV-SH-SY5Y cells). PSV-SH-SY5Y cells were cultured in 6-well poly-L-lysine coated flasks as described above. NM suspension was mixed with the culture medium to be 0.05–1 μg/ml and incubated at 37°C for 2 h. The culture medium was changed with the medium containing L-Cyst-DMSO solution without (control) or with NM and the cells were cultured for 1 to 3 days. Accumulation of NM and ZsGFP in PSV-SH-SY5Y cells after treatment with NM was observed morphologically using a fluorescence microscope (Olympus BX60; Olympus, Tokyo, Japan) as reported previously (Maruyama et al., 2001). The fluorescence of ZsGFP in the living cells was measured as described above. The protein amount was measured according to Bradford (1976) and the fluorescence intensity of the cells was expressed as arbitrary fluorescence unit/mg protein. The cytotoxicity of NM was examined by morphological observation of the cells.

Analysis of in vitro 26S proteasome activity using polyubiquitinated Lyso

To estimate the direct effects of NM on *in vitro* activity of 26S proteasome, a substrate selective for 26S proteasome, ubiquitin-conjugated Lyso, was prepared according to Shringarpure et al. (2003). Authentic Lyso dissolved in distilled water (1 mg/ml) was heated at 100°C for 5 min and then, cooled immediately. Heat-denatured Lyso was conjugated with ubiquitin using Ubiquitin protein conjugating kit (Calbiochem, San Diego, CA, USA) according to the manufacture's instruction. In brief, Lyso was incubated with the reaction mixture containing full compliment of purified conjugation enzymes (E1, E2 and E3s), 150 μM of ubiquitin solution for 4 h. The sample was stored at −20°C until analysis.

Enzyme samples were prepared from SH-SY5Y cells as reported previously (Shamoto-Nagai et al., 2003). Cells were washed twice and suspended in 50 mM Tris-HCl buffer, pH 8.0, homogenized and centrifuged at 14,000 g for 60 min. Glycerol was added to the supernatant to be the

final concentration of 20%. The sample was concentrated by approximate 3 folds using a 300 kDa cut off membrane filter (Pall, Ann Arbor, Michigan, USA) (Rodgers and Dean, 2003). All the procedure was carried out at 4°C.

The *in vitro* activity of ubiquitin-26S proteasome system was determined by measuring degradation of ubiquitin-conjugated Lyso. The enzyme sample (100 μg) was incubated with NM in L-Cyst-DMSO solution or L-Cyst-DMSO solution as control in at 37°C for 30 min, then with mixture of ubiquitinated Lyso at 37°C for 4 h. The reaction was terminated by adding the 1/4 volume of Laemmli's sample buffer (100 mM Tris-HCl containing 4% SDS, 12% β-mercaptoethanol and 20% glycerol). The protein was subjected to SDS-PAGE using 12.5% polyacrylamide gel (WAKO, Tokyo, Japan) and immunoblotting was performed as reported previously (Shamoto-Nagai et al., 2003) using anti-Lyso or anti-polyubiquitin antibodies and enhanced chemofluorescence (ECF) Western blotting kit (Amersham Biosciences, Piscataway, NJ, USA). The fluorescence intensity at 540 nm was quantified with excitation at 488 nm using a Fluoroimager 595 (Amersham Biosciences, Piscataway, NJ, USA). The amounts of ubiquitinated Lyso and free Lyso were quantified using antibody against polyubiquitin and Lyso, respectively, and NIH Image software.

Proteasome activity in NM-treated cells

SH-SY5Y cells were cultured in 6-well poly-L-lysine coated flasks after addition of the L-Cyst-DMSO solution with or without NM (0.05–1 μg/ml in the final concentration). After 1 or 3 days, the cells were mechanically harvested and the enzyme sample was prepared, as described above. The activities of proteasome were measured using synthetic substrates (Shamoto-Nagai et al., 2003). The enzyme sample was incubated with 50 μM of Z-LLVY-MCA or Z-LLE-MCA for 30 min at 37°C, and the fluorescence intensity of AMC cleaved from the substrate was measured in a RF-5300 spectrofluorophotometer with excitation at 380 nm and emission at 440 nm.

Assay for the in vitro activity of 20S proteasome

The activity of 20S proteasome was measured *in vitro* as reported previously (Okada et al., 1999; Shamoto-Nagai et al., 2003). Enzyme preparation (50 μg protein) was treated with L-Cyst-DMSO in the presence or absence of NM (0.75–7.5 μg/ml) in the reaction mixture [1 mM dithiothreitol (DTT), 0.5 mM EDTA 2Na, 2 mM ATP in 50 mM Tris-HCl buffer, pH 8.0] at 37°C for 30 min, and then the substrate, Z-LLVY-MCA or Z-LLE-MCA, was added to be 50 μM in the final concentration. After further incubation for 30 min at 37°C, the reaction was terminated by adding the same volume of 100 mM Tris-HCl buffer, pH 8.0, containing 1% SDS. AMC fluorescence was measured at 440 nm with excitation at 380 nm, as described above. The activity of the proteasome was expressed as pmoles of AMC produced per min per mg protein.

Western blot analyses of proteasome subunits in the cells incubated with NM

After treatment with NM for 1 or 3 days, the cells were gathered, washed with PBS, and lyzed in the RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Fifty μg of protein was subjected to SDS-polyacrylamide electrophoresis using 12.5% polyacrylamide gel (Bio Craft, Tokyo, Japan), and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Proteasome subunits were visualized using polyclonal antibody against α and β subunits of 20S proteasome and PA700 complex as described previously (Shamoto-Nagai et al., 2003).

Statistics

Experiments were repeated at least 3 times. The data was expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.