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Mitochondrial permeability transition mediates apoptosis induced by *N*-methyl(*R*)salsolinol, an endogenous neurotoxin, and is inhibited by Bcl-2 and rasagiline, *N*-propargyl-1(*R*)-aminoindan

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Abstract

The role of mitochondrial permeability transition (PT) in apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal], was studied by use of dopaminergic neuroblastoma SH-SY5Y cells. *N*M(*R*)Sal reduced mitochondrial membrane potential, ΔΨm, in the early phase of apoptosis, which was not suppressed by a pan-caspase inhibitor, but was antagonized by Bcl-2 and cyclosporin A, suggesting the involvement of the PT in *N*M(*R*)Sal-induced loss of ΔΨm. *N*M(*R*)Sal-induced apoptosis was completely inhibited not only by Bcl-2 and a pan-caspase inhibitor, but also by cyclosporin A, suggesting the essential role of the PT in *N*M(*R*)Sal-induced apoptosis. In mitochondria isolated from rat liver, *N*M(*R*)Sal induced swelling and reduced ΔΨm, which was inhibited by cyclosporin A and Bcl-2 overexpression. These results indicate that *N*M(*R*)Sal induced the PT by direct action on the mitochondria. Rasagiline, *N*-propargyl-1(*R*)-aminoindan, which is a now under a clinical trial for Parkinson's disease, suppressed the ΔΨm reduction, release of cytochrome c, and apoptosis induced by *N*M(*R*)Sal in SH-SY5Y cells. Rasagiline also inhibited the *N*M(*R*)Sal-induced loss of ΔΨm and swelling in the isolated mitochondria, proving that rasagiline directly targets the mitochondria also. Altogether, mitochondrial PT plays a key role both in *N*M(*R*)Sal-induced cell death and the neuroprotective effect of rasagiline.

Keywords: apoptosis, Bcl-2, mitochondrial permeability transition, *N*-methyl(*R*)salsolinol, Parkinson's disease, rasagiline.

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Apoptosis is a mode of cell death that plays an integral part in a variety of biological events and is also implicated in neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's diseases. Therefore, it has been proposed to be a target for protection of declining neurons (Thompson 1995). Endogenous and xenobiotic neurotoxins have been reported to induce apoptosis in neurons in neurodegenerative disorders. Among them, *N*-methyl(*R*)salsolinol [1(*R*),2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; hereafter called *N*M(*R*)Sal] has been suggested to be involved in the pathogenesis of Parkinson's disease (Naoi *et al*. 2001). *N*M(*R*)Sal induces apoptosis in human dopaminergic SH-SY5Y cells (Maruyama *et al*.

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Abbreviations used: ANT, adenine nucleotide translocator; CsA, cyclosporin A; Cyt *c*, cytochrome *c*; DCF, 2,7-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; ΔΨm, mitochondrial membrane potential; FACS, fluorescence-augmented cell sorter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; H_2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; MAO-B, type B monoamine oxidase; ΔΨm, mitochondrial membrane potential; *N*M(*R*)Sal, *N*-methyl(*R*)salsolinol; NPT II, neomycin phosphotransferase II; PBS, phosphate-buffered saline; PI, propidium iodide; PT, permeability transition; SOD, superoxide dismutase; VDAC, voltage dependent anion channel; Z-VAD-FMK, *N*-benzyloxylcarbonyl-Val-Ala-Asp-fluromethylketone.

1997a), which is accompanied by a decline in the mitochondrial membrane potential ($\Delta\Psi m$) (Maruyama *et al*. 2001b), the activation of caspase 3 (Akao *et al*. 1999), the nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1. 2. 1.12, GAPDH; Maruyama *et al*. 2001a], and the oligonucleosomal fragmentation of nuclear DNA. (–)Deprenyl (selegiline), a propargylamine inhibitor of type B monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4; MAO-B], is now used as a monotherapy or an adjunct of L-DOPA therapy in Parkinson's disease. A series of (–)deprenyl and structurally related propargylamines were found to protect neuronal cells, as confirmed by *in vivo* and *in vitro* experiments (Yu *et al*. 1995; Maruyama *et al*. 1998; Youdim *et al*. 2001). We previously showed that the rasagiline [*N*-propargyl-1(*R*)-aminoindan; Maruyama *et al*. 2000, 2001a] and *R*-(2-heptyl)-*N*-methyl-propargylamine (Maruyama *et al*. 2001b) protected SH-SY5Y cells from apoptosis induced by *N*M(*R*)Sal. Rasagiline is now under clinical trial as a monotherapy (Kieburtz and Parkinson Study Group 2001) and as an adjunct to L-DOPA (Rabey *et al*. 2000) in Parkinson's disease.

Mitochondria play a crucial role in many forms of cell death in mammalian cells by releasing several apoptotic factors including cytochrome *c* (Cyt *c*) into the cytoplasm, which activate death-driving caspases. The release of these apoptogenic factors from mitochondria depends on an increase in the permeability of the outer mitochondrial membrane in the early phase of apoptosis. A major apoptosis regulator, the Bcl-2 family of proteins (Adams and Cory 1998; Tsujimoto and Shimizu 2000) consists of antiapoptotic members, Bcl-2, Bcl-xL, and pro-apoptotic members, Bax, Bak and BH3-only proteins, and directly regulates the change in outer mitochondrial membrane permeability by modulating the permeability transition (PT) pore complex (Tsujimoto and Shimizu 2000). In the PT pore, it has been shown that the voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT; Kroemer *et al*. 1998) are the functional targets of the Bcl-2 family of proteins (Shimizu *et al*. 1999, 2001).

The involvement of Bcl-2 in neuroprotection by rasagiline was suggested by the fact that, in our findings, Bcl-2 overexpression and rasagiline prevented the nuclear translocation of GAPDH and following apoptosis induced by *N*M(*R*)Sal in a similar way (Maruyama *et al*. 2001a). It was actually confirmed by our recent results that rasagiline increases the expression of Bcl-2 in SH-SY5Y cells (Akao *et al*. 2002). This paper reports that the PT mediated apoptosis induced by *N*M(*R*)Sal in SH-SY5Y cells and that *N*M(*R*)Sal is able to induce the PT also in isolated mitochondria. In addition, Bcl-2 overexpression and rasagiline can suppress the PT in the cells and in isolated mitochondria. The role of mitochondria in apoptosis and the antiapoptotic properties of rasagiline are discussed.

Materials and methods

Materials

*N*M(*R*)Sal was synthesized according to Teitel *et al*. (1972). Rasagiline was prepared as reported previously (Youdim *et al*. 1995) and kindly donated by Teva Pharmaceutical (Netanya, Israel). Rhodamine 123, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazoylcarbocyanine iodide), Hoechst 33342, propidium iodide (PI), and 2,7-dichlorodihydofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Cyclosporin A (CsA) came from Sigma (St Louis, MO, USA), *N*-benzyloxyl-carbonyl-Val-Ala-Asp-fluromethylketone (Z-VAD-FMK), a pan-caspase inhibitor, from Enzyme Systems Products (Dublin, CA, USA), and ATP bioluminofluorescence assay kit and complete protease inhibitor cocktail kit from Roche Diagnostics (Mannheim, Germany). Monoclonal anti-Cyt *c* antibody was purchased from R&D Systems (Minneapolis, MN, USA). 2,7-Dichlorofluorescein (DCF), and other drugs were purchased from Nacarai tesque (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% Nakashibetsu newborn calf serum (Mitsubishi Kasei, Tokyo, Japan) in an atmosphere of 95% air−5% CO_2.

Preparation of SH-SY5Y cells overexpressing Bcl-2

To establish cells overexpressing Bcl-2, we used a pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA, USA). To construct pcDNA3-bcl-2, the full-length human *bcl-2* gene pB4 (Tsujimoto and Croce 1986) was digested with *Eco*RI and then inserted into the *Eco*RI-cleaved pcDNA3 vector. SH-SY5Y cells were transfected with pcDNA3 or pcDNA3-bcl-2 by the lipofection technique (Gibco BRL, Life Technologies, Rockville, MD, USA). Selection was started 3 days later in the culture medium containing G418 (100 μg/mL; Gibco BRL, Life Technologies). G418-resistant clones were isolated by limiting dilution and Bcl-2 expression was examined by RT-PCR and western blot analyses. We isolated two clones of Bcl-2-overexpressing cells named as SH-Bcl-2 A and SH-Bcl-2B, and a control clone carrying the vector alone, named as SH-Neo.

RT-PCR for *bcl-2*

Total RNA was extracted from control SH-SY5Y cells and *bcl-2*-transfected cells by the phenol/guanidium thiocyanate method, followed by DNase I treatment. cDNA generated after reverse transcription of the total RNA (2 μg) was subjected to PCR amplification of the *bcl-2* and *neomycin phosphotransferase II* (*NPT II*). PCR primers used were as follow: for *bcl-2*, 5′-TGCACCT-GACGCCCTTCAC-3′ (sense), and 5′-AGACAGCCAGGAGAAA-TCAAACAG-3′ (antisense); for *NPT II,* 5′-ATGGGATCGGCC-ATTGAACA (sense) and 5′-TGATCCCCTCAGAAGAACTC-3′ (antisense). These primers can specifically amplify the 293-bp and 821-bp DNA fragments of *bcl-2* and *NPT II* cDNAs, respectively. *β-Actin* cDNA was used for an internal standard. PCR products were analyzed by electrophoresis on 2% agarose gels.

Western blot analysis of Bcl-2

Parental SH-SY5Y cells, *bcl-2*-transfected cells (SH-Bcl-2 cells) and those having only *NPT-II* (SH-Neo cells) were washed with phosphate-buffered saline (PBS) and suspended in lysis buffer [2 × PBS, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)]. The lysed protein (5 μg) was separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) using a 12% polyacrylamide gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Du Pont, Boston, MA, USA). After blockage with 5% non-fat milk in PBS containing 0.1% Tween-20, the membrane was incubated overnight 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). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) at room temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Assessment of apoptosis in SH-SY5Y cells

Apoptosis was determined from morphological observation of the cells treated with *N*M(*R*)Sal after staining with Hoechst 33342 and PI, as reported previously (Maruyama *et al*. 2001a). The cells with condensed and fragmented nuclei stained with 10 μM Hoechst 33342 were assessed to be apoptotic, and the dead cells stained by 50 μM PI, but with normal nuclear features, were determined to be necrotic. Approximately 200 cells were counted in four different fields, and four independent experiments were performed. Nucleosomal DNA fragmentation was examined by agarose gel electrophoresis (Akao *et al*. 1999).

Assay for change in ΔΨm

The decline in ΔΨm was determined by the reduction of Rhodamine 123 fluorescence trapped in SH-SY5Y cells (Pastorino *et al*. 1996). The cells cultured in a 6-well poly-L-lysine-coated tissue flask were incubated with 5 μM Rhodamine 123 in Dulbecco's modified Eagle's medium (DMEM). The cells were treated with 250–100 μM *N*M(*R*)Sal in DMEM for 1 h, washed with PBS, and gathered by treatment with trypsin and centrifugation. The cells were suspended in PBS and the fluorescence intensity at 535 nm was measured with excitation at 505 nm. To examine effects of CsA, Z-VAD-FMK, and rasagiline, the cells were pre-incubated for 30 min with 10–0.1 μM CsA, 10 μM Z-VAD-FMK, or 10 μM–100 nM rasagiline, respectively, and then treated with *N*M(*R*)Sal, as described above.

The ΔΨm collapse was also measured using JC-1, as reported (Maruyama *et al*. 2001a,b). SH-SY5Y cells cultured in a 6-well poly-L-lysine-coated tissue flask were washed with DMEM, incubated with JC-1 solution (40 μg/mL), then with 200–100 μM *N*M(*R*)Sal for 30 or 60 min. After washed with PBS, the cells were trypsinized, suspended in PBS, and subjected to fluorescence-augmented cell sorter (FACS). In hyperpolarized environments, JC-1 aggregates as polymers, J-aggregates showing red fluorescence, whereas in hypopolized environments JC-1 monomer shows green fluorescence. The ratio of the fluorescence of J-aggregates to that of the monomers was used as an indicator of intact ΔΨm. Flow cytometry was performed over 20 000 events by three-color flow cytometry with a modified FACSCalibor cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). The laser emission at 534–606 nm and 515 nm, were used for detection of red and green fluorescence, respectively, with excitation at 488 nm.

Quantitative assay of reactive oxygen and nitrogen species with H_2DCFDA

To measure the synthesis of reactive oxygen and nitrogen species, such as hydrogen peroxide and peroxynitrite in the cells treated with *N*M(*R*)Sal, the oxidation of H_2DCFDA into DCF was quantitatively determined (Crow 1997). The cells were suspended in PBS and incubated with 250–50 μM *N*M(*R*)Sal in the presence of 5 μM H_2DCFDA for 1–3 h at 37°C. The increase in DCF fluorescence was followed at 520 nm with excitation at 504 nm.

Quantitation of ATP levels in the cells treated with *N*M(*R*)Sal

To examine the effects of *N*M(*R*)Sal on mitochondrial ATP synthesis, SH-SY5Y cells suspended in PBS were incubated with 500–50 μM *N*M(*R*)Sal for 1 h at 37°C. The ATP concentration was measured with ATP HS-II assay kit in a luminofluorometer, Lumat LB 9501/16 (Berthold, Bad Wildbad, Germany).

Detection of cytosolic Cyt *c* release after treatment with *N*M(*R*)Sal

To measure Cyt *c* release into cytoplasm, SH-SY5Y cells suspended in DMEM were incubated with 250–100 μM *N*M(*R*)Sal for 6–16 h at 37°C, and gathered cells were lysed in the extraction buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES–KOH buffer, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM dithiothreitol and complete protease inhibitor cocktail). The lysates (6 μg protein) were subjected to western blot analysis and Cyt *c* was detected with anti-Cyt *c* antibody, as in the case for Bcl-2 detection. To examine the effect of rasagiline, the cells were treated with 1 μM–100 nM rasagiline for 30 min, and then with *N*M(*R*)Sal.

Assay for loss of ΔΨm and swelling in isolated mitochondria

Mitochondria were prepared from male Donryu rat liver in the isolation medium [0.3 M mannitol, 10 mM potassium–HEPES buffer, pH 7.4, 0.2 mM EDTA, 0.1% fatty acid-free bovine serum albumin (BSA)], as previously described (Shimizu *et al*. 1998). The mitochondria were suspended in the isolation medium without EDTA (MT-1 medium), and ΔΨm was assessed by measurement of ΔΨm-dependent uptake of Rhodamine 123 into the mitochondria (Narita *et al*. 1998). Isolated mitochondria were suspended to be 1 mg/mL of the MT-1 medium plus 0.5 mM potassium phosphate and 4.2 mM potassium succinate to energize the mitochondria, and incubated at 25°C in the presence of 10 μM Rhodamine 123. The reduction of Rhodamine fluorescence at 535 nm was measured with excitation at 505 nm under continuous stirring. The effect of CsA and rasagiline on the mitochondrial PT pore was examined. The mitochondria were incubated with *N*M(*R*)Sal after pre-treatment with CsA or rasagiline for 30 min.

Mitochondrial swelling was monitored at 0.1 mg protein/mL by the decrease of 90° light scatter at 520 nm under the same reaction conditions for the measurement of Rhodamine fluorescence (Narita *et al*. 1998).

Hepatic bcl-2 transgenic mice and isolation of mitochondria

Transgenic mice expressing human Bcl-2 in their liver were kindly provided by Lacronique *et al*. (1996), and the mitochondria were isolated from Bcl-2 transgenic mice and non-transgenic littermates, as described (Shimizu *et al*. 1998).

Statistics

Experiments were repeated 4–8 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

Inhibition of *N*M(*R*)Sal-induced apoptosis by Bcl-2

Figure 1(a) shows that *N*M(*R*)Sal induced apoptosis in SH-SY5Y cells in a time-dependent way, as determined from the morphological changes after staining with Hoechst 33342 and PI. In all conditions examined, *N*M(*R*)Sal induced necrosis in negligible number of cells (less than 2% of the total). Treatment with 500 μM *N*M(*R*)Sal increased apoptotic cell death in a time-dependent way from 18 to 36 h-incubation (Fig. 1a), and at 24 h approximately 30% of control cells exhibited typical apoptotic nuclei with chromatin condensation and fragmentation, as assessed by staining with Hoechst 33342 (Fig. 1b).

To test whether Bcl-2 inhibits *N*M(*R*)Sal-induced apoptosis, we generated Bcl-2-expressing derivatives of SH-SY5Y cells (SH-Bcl-2 A and SH-Bcl-2B, as described in Materials and methods; Fig. 2a). The western blot analysis indicated that SH-Bcl-2 A and SH-Bcl-2B cells produced significantly larger amounts of human Bcl-2 protein than parent or SH-Neo cells (control cells having a vector alone; Fig. 2a). Because the amounts of Bcl-2 in those clones were approximately the same, SH-Bcl-2 A cells were mainly used in subsequent experiments. After incubation with *N*M(*R*)Sal, nuclear DNA fragmentation was suppressed in SH-Bcl-2 A cells, whereas in SH-Neo cells typical ladder formation was observed (Fig. 2b). The results were consistent with those obtained by morphological observation; in SH-Bcl-2 A cells apoptosis was induced in only 3.71 ± 3.13% cells (Fig. 1b), but in SH-Neo cells about 30% cells were assessed to be apoptotic.

Involvement of mitochondrial permeability transition in *N*M(*R*)Sal-induced apoptosis

It was examined whether *N*M(*R*)Sal-induced apoptosis was mediated by mitochondrial permeability transition (PT). As shown in Table 1, ΔΨm as assessed by Rhodamine 123 staining was reduced to 46.9% and 35.5% of control values after a 1-h incubation with 250 μM and 100 μM *N*M(*R*)Sal, respectively. Bcl-2 overexpression completely inhibited the *N*M(*R*)Sal-induced loss of ΔΨm; 104.4 ± 13.6% versus 34.3 ± 8.3% of control in the vector cells. The resting level of ΔΨm in the Bcl-2 overexpressed cells was almost the same as in the vector cells; 116.9 ± 21.9% and 100 ± 5.9 of the control, respectively. Bcl-2 overexpression completely suppressed the decline in the relative fluorescence of JC-1 aggregate representing intact ΔΨm (Fig. 8), as reported

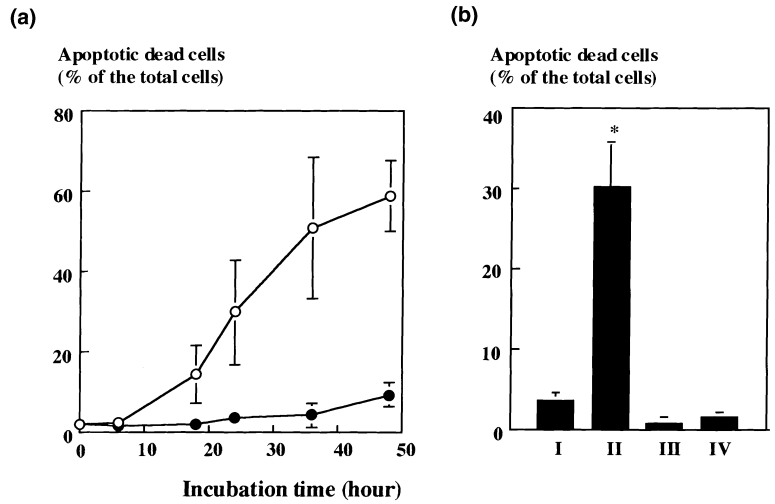

Fig. 1 Apoptosis induced by *N*M(*R*)Sal and the effects of Bcl-2 overexpression. (a) Control SH-SY5Y cells were cultured in the presence (○) or the absence (●) of 500 μM *N*M(*R*)Sal for 6–48 h. The number of apoptotic dead cells was determined by the morphological observation after staining with Hoechst 33342, as described in Materials and methods. Each circle and bar represent the mean and SD of 4 experiments. (b) SH-Bcl-2 and SH-Neo cells were incubated with 500 μM *N*M(*R*)Sal for 24 h, and the number of apoptotic dead cells was determined. I, SH-Neo cells without treatment; II, SH-Neo cells treated with *N*M(*R*)Sal; III, SH-Bcl-2 cells without treatment; IV, SH-Bcl-2 cells treated with *N*M(*R*)Sal. *Difference from the control (I) is statistically significant, $p < 0.01$.

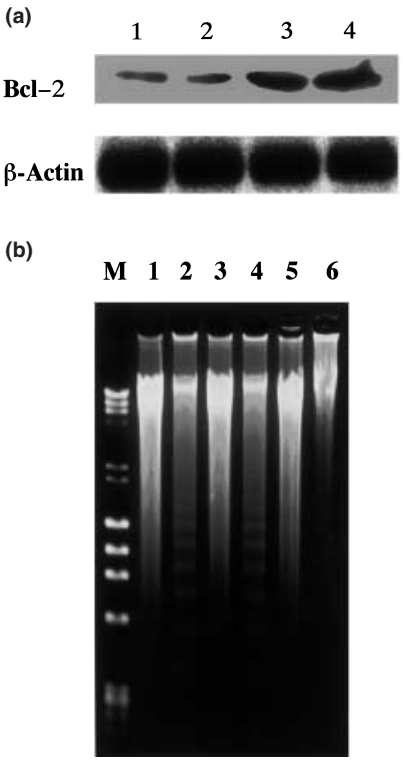

Fig. 2 Establishment of SH-SY5Y overexpressing Bcl-2 and the effects on nucleosomal DNA fragmentation. (a) The amounts of Bcl-2 protein by western blot analysis in SH-SY5Y cells transfected with *bcl-2* gene. Lane 1, Control; lane 2, SH-Neo; lanes 3 and 4, two clones of *bcl-2* transfected cells (SH-Bcl-2A and -B). Cell lysates containing 5 μg protein were subjected to SDS–PAGE in a 12% gel, as described in Materials and methods. (b) Nucleosomal DNA fragmentation by *N*M(*R*)Sal in parental and Bcl-2 overexpressed SH-SY5Y cells. Lane M, markers of molecular weight; lanes 1 and 2, parental cells; lanes 3 and 4, SH-Neo; lanes 5 and 6, SH-Bcl-2A. DNA was extracted from whole cells after incubation without (lanes 1, 3 and 5) or with 500 μM *N*M(*R*)Sal (lanes 2, 4 and 6) for 72 h, and subjected to agarose gel electrophoresis with 2% agarose gel, and stained with ethidium bromide.

(Maruyama *et al*. 2001a). On the other hand, a pan-caspase inhibitor, Z-VAD-FMK, failed to prevent the *N*M(*R*)Sal-induced reduction in ΔΨm (Table 1), but Z-VAD-FMK significantly suppressed the apoptosis (Fig. 3), as reported previously with an inhibitor of caspase 3, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic aldehyde (Akao *et al*. 1999).

To see the involvement of the PT in *N*M(*R*)Sal-induced reduction of ΔΨm, the effect of CsA was examined, which inhibits cyclophilin D, a component of the PT pore (Szabo and Zoratti 1991). As shown in Table 2, 1 and 0.1 μM CsA completely inhibited the *N*M(*R*)Sal-induced reduction in ΔΨm. In the presence of 0.1 μM CsA, the resting ΔΨm level was lower than in control cells, whereas CsA at the concentration higher than 1 μM elevated the level, as reported previously (Kowaltowski *et al*. 2000). In addition, *N*M(*R*)Sal-induced apoptosis was also completely blocked by CsA (Fig. 3), indicating that the PT is directly involved in the apoptosis.

The generation of reactive oxygen and nitrogen species, hydrogen peroxide and peroxynitrite, was examined using H_2DCFDA. In the cells incubated with 250 *N*M(*R*)Sal, the DCF production from H_2DCFDA was almost the same as in control, and 100–50 μM *N*M(*R*)Sal even reduced the production under the conditions used for ΔΨm measurement, as shown in Fig. 4.

The effects of *N*M(*R*)Sal on mitochondrial oxidative phosphorylation were examined by measuring ATP synthesis rate. The intracellular ATP levels did not change in the cells treated with 500–50 μM *N*M(*R*)Sal, 7.73 ± 0.71–9.65 ± 0.27 nmol/mg protein (mean ± SD), from control (8.75 ± 0.24 nmol/mg protein).

PT induction by *N*M(*R*)Sal in isolated mitochondria

To determine whether *N*M(*R*)Sal induced the PT was induced by direct interaction with the mitochondria or through a signaling pathway upstream of the mitochondria, the effects of *N*M(*R*)Sal on ΔΨm were examined in mitochondria isolated from rat liver. After addition of 500, 250 or 100 μM *N*M(*R*)Sal, Rhodamine 123 fluorescence was reduced in a dose-dependent manner (Fig. 5a). On the other hand, the Rhodamine 123 fluorescence remained unchanged even after a 35-min incubation in control mitochondria. Consistent with the observation in the cells (Table 1), the pre-treatment of the mitochondria with 1 μM CsA completely prevented the *N*M(*R*)Sal-induced reduction in ΔΨm (Fig. 5b), indicating that the reduction in ΔΨm was mediated by the PT. The mitochondria isolated from *bcl-2* transgenic mice were not affected by *N*M(*R*)Sal (Fig. 5c).

The PT induction by *N*M(*R*)Sal was also assessed by measuring mitochondrial swelling (Fig. 6). *N*M(*R*)Sal induced the swelling almost immediately, as shown by light scattering (Fig. 6a). Addition of Ca^{2+} also induced the swelling in a dose-dependent way (Fig. 6b), and the intensity of PT induced by 10 μM Ca^{2+} was almost comparable to that by 250 μM *N*M(*R*)Sal. The 25-min delay in the reduction of Rhodamine fluorescence (Fig. 5) may correspond to the time to transport Rhodamine 123 from the medium and accumulate in mitochondria by diffusion secondary to the swelling.

The effects of antioxidants on the ΔΨm decline by *N*M(*R*)Sal were examined in mitochondria isolated from rat liver. Figure 7 shows the quantitative analyses of ΔΨm by measuring Rhodamine uptake, and the presence of catalase, Mn-superoxide dismutase (SOD) or reduced glutathione (GSH) did not prevent the ΔΨm collapse caused by *N*M(*R*)Sal. All these results clearly demonstrate that *N*M(*R*)Sal induced the PT by directly acting on the mitochondria.

Table 1 Effects of *N*M(*R*)Sal, pre-treatment with cyclosporin A, Z-VAD-FMK or rasagiline, and Bcl-2 overexpression on ΔΨm

	Relative fluorescence of Rhodamine 123 % of control
Control cells treated with:	100
*N*M(*R*)Sal (100 μM)	46.9 ± 10.1[a]
*N*M(*R*)Sal (250 μM)	35.5 ± 9.3[a]
Cells pre-treated with:	
Z-VAD-FMK (10 μM) + *N*M(*R*)Sal (100 μM)	66.6 ± 1.9
Z-VAD-FMK (10 μM) + *N*M(*R*)Sal (250 μM)	30.5 ± 8.9
Z-VAD-FMK (10 μM)	115.9 ± 9.1
Rasagiline + *N*M(*R*)Sal (100 μM)	
Rasagiline (10 μM)	136.9 ± 12.7[b]
(1 μM)	103.4 ± 9.5[b]
(100 nM)	124.0 ± 13.6[b]
Bcl-2-overexpressed cells	
SH-Neo control cells treated with	
*N*M(*R*)Sal (250 μM)	34.3 ± 8.3
SH-Bcl-2 A cells treated with	
*N*M(*R*)Sal (250 μM)	104.4 ± 13.6[c]

SH-SY5Y cells were treated with Z-VAD-FMK, or rasagiline for 30 min, and then with *N*M(*R*)Sal for 1 h prior to measurement of Rhodamine fluorescence. Bcl-2-overexpressing cells and vector-expressing cells were treated with 250 μM *N*M(*R*)Sal. The results are expressed as the mean ± SD of three samples from three independent experiments. [a]$p < 0.01$ versus control and [b]versus *N*M(*R*)Sal (100 μM)- and [c]versus *N*M(*R*)Sal (250 μM)- treated cells.

Inhibition of *N*M(*R*)Sal-induced PT, Cyt *c* release, and apoptosis by rasagiline

We previously reported that rasagiline effectively ameliorated *N*M(*R*)Sal-induced apoptosis (Maruyama *et al*. 2001a). To elucidate the mechanism underlying the suppression of apoptosis, we examined the effects of rasagiline on the *N*M(*R*)Sal-triggered reduction in ΔΨm. As shown in Table 1, the pre-treatment of the cells with 10 μM–100 nM rasagiline significantly prevented the reduction in ΔΨm (Table 1), suggesting that rasagiline might have the ability to inhibit the PT. In addition, the effects of rasagiline on ΔΨm reduction were measured by use of JC-1. The red fluorescence of J-aggregate representing the intact ΔΨm reduced after incubation with *N*M(*R*)Sal, whereas in cells treated with 1 μM–10 nM rasagiline, ΔΨm did not change by *N*M(*R*)Sal treatment (Fig. 8).

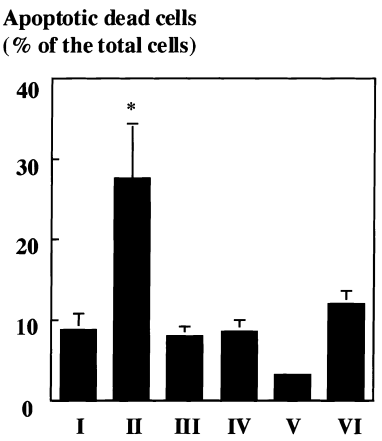

Fig. 3 Effects of CsA and Z-VAD on apoptosis induced by *N*M(*R*)Sal in SH-SY5Y cells. Cells were treated with 500 μM *N*M(*R*)Sal for 24 h with or without pre-treatment with CsA or Z-VAD, then apoptotic cells stained with Hoechst 33342 were detected by morphological observation. Lane I, control cells; lane II, cells treated with *N*M(*R*)Sal; lane III, with 1 μM CsA; lane IV, pre-treated with CsA, then with *N*M(*R*)Sal; lane V, with 100 μM Z-VAD; lane VI, pre-treated with Z-VAD, then with *N*M(*R*)Sal. The column and bar represent the mean and SD. *Difference from the control (I) was statistically significant, $p < 0.01$.

As shown in Fig. 9, *N*M(*R*)Sal-treatment induced cytosolic translocation of Cyt *c*. The lease of Cyt *c* into cytoplasm by *N*M(*R*)Sal was in a dose-dependent way, and began to increase at 6 h after the treatment and increased further for 24 h. On the other hand, in SH-Bcl-2A cells, *N*M(*R*)Sal did not increase the cytosolic levels of Cyt *c* (data not shown). The pre-treatment of the cells with 100 nM and 1 μM rasagiline markedly prevented the release of Cyt *c* (Fig. 9).

When isolated mitochondria were treated with rasagiline and then with *N*M(*R*)Sal, the PT was significantly inhibited in a dose-dependent way (Fig. 5d). The reduction of Rhodamine fluorescence was suppressed by rasagiline but, interestingly, rasagiline failed to inhibit the loss of ΔΨm induced by 50 μM Ca^{2+} overload (Fig. 5d). The quantitative analyses of the reduction in Rhodamine fluorescence also confirmed that rasagiline inhibited the *N*M(*R*)Sal-induced decline of ΔΨm, as shown in Fig. 7.

Table 2 Effects of cyclosporin A on ΔΨm decline induced by *N*M(*R*)Sal

	Relative fluorescence of Rhodamine 123 % of control
Control cells treated with:	100
*N*M(*R*)Sal (100 μM)	49.7 ± 8.4[a]
Cells pre-treated with:	
Cyclosporin A (1 μM)	375.0 ± 57.2[a,b,c]
Cyclosporin A (1 μM) + *N*M(*R*)Sal (100 μM)	439.1 ± 41.6[a,b,c]
Cyclosporin A (0.1 μM)	47.2 ± 16.0[b,c]
Cyclosporin A (0.1 μM) + *N*M(*R*)Sal (100 μM)	138.3 ± 27.5[b,c]

SH-SY5Y cells were treated with CsA, for 30 min, and then with 100 μM *N*M(*R*)Sal for 1 h. The Rhodamine fluorescence in the cells was measured, as described in Materials and methods. The results are expressed as the mean ± SD of three samples from three independent experiments. [a]$p < 0.01$ versus control, [b]versus *N*M(*R*)Sal (100 μM)-treated cells, and [c]versus 0.1 μM CsA-treated cells.

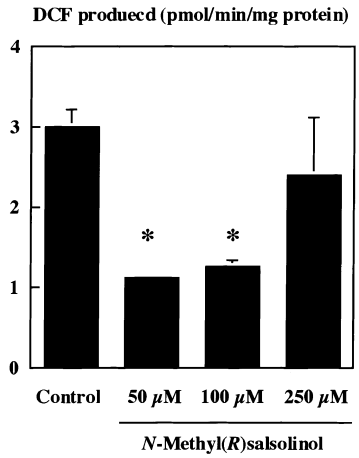

Fig. 4 Effects of *N*M(*R*)Sal on the generation of reactive oxygen and nitrogen species. SH-SY5Ycells were incubated with 50, 100 and 250 μM *N*M(*R*)Sal at 37°C for 1–3 h in the presence of 5 μM H_2DCFDA, and the DCF production was measured fluorometrically. The column and bar represent the mean and SD of three samples of three independent experiments. *$p < 0.01$ from control.

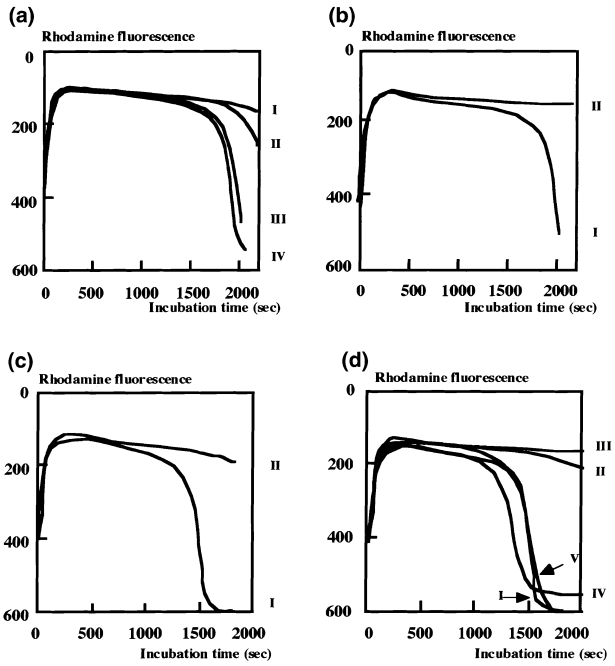

Fig. 5 Induction of the PT by *N*M(*R*)Sal in isolated mitochondria and prevention by Bcl-2, CsA and rasagiline. Mitochondria isolated from liver of rat or *bcl-2*-transgenic mice were suspended to be 1 mg/mL of the MT-1 medium added by 0.5 mM potassium phosphate and 4.2 mM potassium succinate, and ΔΨm was assayed by measuring the Rhodamine 123 fluorescence at 25°C. (a) Mitochondria isolated from rat liver were treated without (I) or with 100 (II), 250 (III) and 500 μM *N*M(*R*)Sal (IV), and the reduction in Rhodamine fluorescence was followed, as described in Materials and methods. The fluorescence intensity was expressed in arbitrary unit. (b) Mitochondria isolated from rat liver were incubated with 500 μM *N*M(*R*)Sal without (I) or with pre-treatment of 1 μM CsA (II). (c) Mitochondria prepared from *bcl-2* transgenic mice (II) and from control mice (I) were treated with 500 μM *N*M(*R*)Sal. (d) Mitochondrial isolated from rat liver were pre-treated for 30 min with 1 μM (I), 10 μM (II), or 25 μM rasagiline(III, V), or without rasagiline (IV), and then treated with 500 μM *N*M(*R*)Sal (I, II and III) or Ca^{2+} (IV, V).

Not only the reduction of Rhodamine fluorescence, but also the swelling of isolated mitochondria, was inhibited by rasagiline, as shown in Fig. 7(a). On the other hand, rasagiline did not affect the swelling induced by Ca^{2+} overload (Fig. 6c).

Discussion

Our data provided evidences that the endogenous neurotoxin, *N*M(*R*)Sal, induced apoptosis in SH-SY5Y cells through induction of the mitochondrial PT: (i) *N*M(*R*)Sal induced the loss of ΔΨm in SH-SY5Y cells, which was inhibited by CsA (a PT inhibitor) and Bcl-2 (regulators of PT), but not by a pan-caspase inhibitor; (ii) *N*M(*R*)Sal-induced cytosolic release of Cyt *c* and apoptosis, which was blocked by Bcl-2

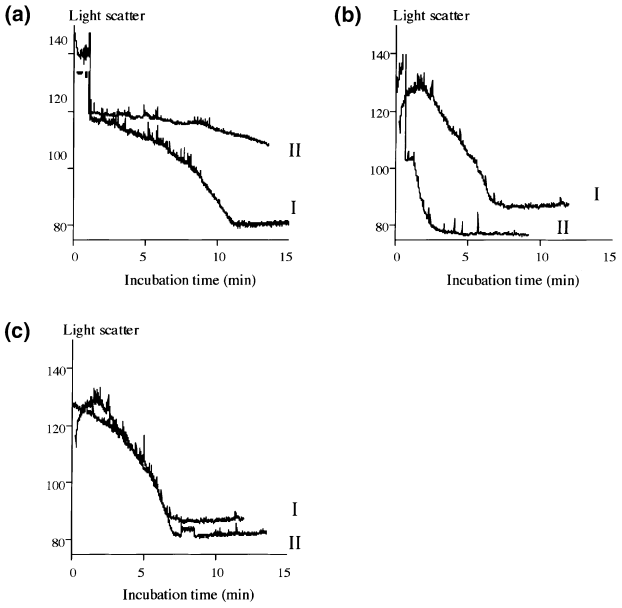

Fig. 6 Mitochondrial swelling induced by *N*M(*R*)Sal and Ca^{2+} in isolated mitochondria, and the effect of rasagiline. The isolated mitochondria (0.1 mg protein/mL) were suspended in the MT-1 added by 0.5 mM potassium phosphate and 4.2 mM potassium succinate, and the increase in 90°C light scatter was measured at 25°C. (a) Mitochondria treated for 30 min without (I) or with 25 μM rasagiline, and then the light scatter was followed after addition of 250 μM *N*M(*R*)Sal; (b) after addition of 10 (I) or 50 μM (II) Ca^{2+}; (c) after 10 μM Ca^{2+} addition without (I) or with 30 min pre-treatment of 25 μM rasagiline (II).

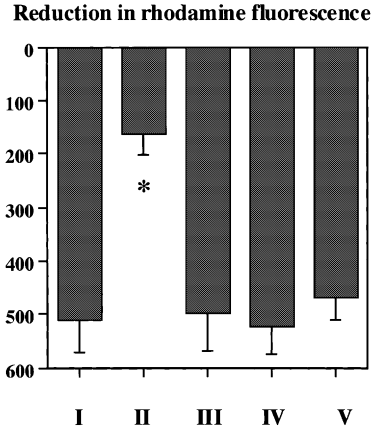

Fig. 7 The effects of antioxidants and rasagiline on ΔΨm decline by *N*M(*R*)Sal in isolated mitochondria. Rat liver mitochondria (1 mg protein/mL) were treated with 250 μM *N*M(*R*)Sal after 30 min treatment with rasagiline (25 μM), catalase (500 unit/mL), Mn-SOD (200 unit/mL) or GSH (10 mM). The ΔΨm reduction was assessed by measuring the Rhodamine fluorescence for 30 min at 25°C, and the reduction was expressed in arbitrary unit. I, cells treated with *N*M(*R*)Sal; II, cells treated with rasagiline, then *N*M(*R*)Sal; III, IV, and V, cells treated with *N*M(*R*)Sal in the presence of catalase, Mn-SOD or GSH, respectively. The column and bar represent the mean and SD of four independent experiments. $^*p < 0.01$ from *N*M(*R*)Sal-treated cells.

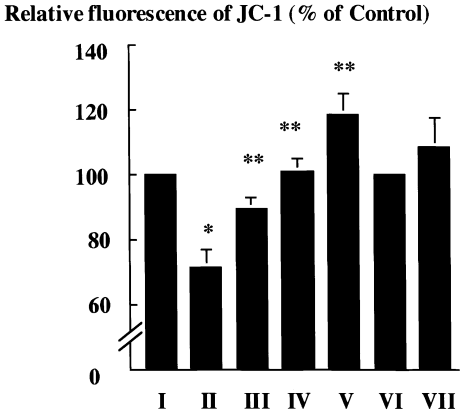

Fig. 8 The effects of rasagiline on ΔΨm detected by use of JC-1. SH-SY5Ycells were stained with JC-1, then treated with 100 μM *N*M(*R*)Sal as described in Materials and methods. Lane I, Control cells; lanes II–IV, control cells were treated with *N*M(*R*)Sal for 1 h at 37°C after 30 min pre-treatment without (II) or with 10 nM (III), 100 nM (IV) or 1 μM rasagiline (V); lanes VI and VII, Bcl-2-overexpressed cells were treated without (VI) or with 100 μM *N*M(*R*)Sal for 1 h (VII). The ratio of red/green fluorescence was expressed as percents of control. The column and bar represent the mean and SD of triplicate measurements of three experiments; $^*p < 0.01$ from control, $^{**}p < 0.01$ from *N*M(*R*)Sal-treated cells.

and CsA; (iii) *N*M(*R*)Sal induced the swelling and loss of ΔΨm in isolated mitochondria, which was also inhibited by CsA and Bcl-2 overexpression. The direct involvement of the PT in apoptotic death of mammalian cells has been well documented in a variety of apoptosis systems. The important role of the PT in apoptosis is also supported by the findings that the PT pore complex, particularly VDAC (Shimizu *et al.* 1999, 2001) and ANT (Kroemer *et al.* 1998), is a direct functional target for Bcl-2 family of proteins.

Oxidative stress and metabolic compromise, such as mitochondria dysfunction, have been proposed to account for cell death induced by salsolinol, another catechol isoquinoline (Storch *et al.* 2000; Kim *et al.* 2001). However, in the case of *N*M(*R*)Sal, the direct involvement of these factors in the PT induction was excluded. Figure 7 shows that, in isolated mitochondria antioxidants, catalase, Mn-SOD and GSH, did not prevent the ΔΨm decline by *N*M(*R*)Sal. In addition, *N*M(*R*)Sal at 250 μM did not increase, but at 100 and 50 μM even reduced the level of DCF produced by hydrogen peroxide and peroxynitrite. It coincides with our previous observation that *N*M(*R*)Sal scavenged hydroxyl radical at lower concentrations (Maruyama *et al.* 1995). The mitochondria ATP synthesis was not affected by *N*M(*R*)Sal at 500–50 μM, which is supported by our previous results that *N*M(*R*)Sal is a very weak inhibitor of mitochondrial Complex I and II with the IC_{50} value of 1.9 mM and 610 μM, respectively (Morikawa *et al.* 1998). All these results

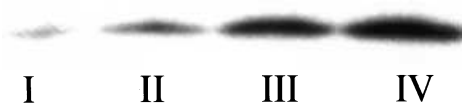

Fig. 9 Effects of rasagiline on cytosolic Cyt *c* release induced by *N*M(*R*)Sal. SH-SY5Y cells, cultured in 6-well poly-L-lysine coated flasks with DMEM, were treated with rasagiline for 30 min, then with *N*M(*R*)Sal for 16 h. The cells were lysed and subjected to western blot analysis. Lane I, control cells; lanes II and III, cells treated with 100 nM and1 μM rasagiline, then treated with 250 μM *N*M(*R*)Sal; lane IV, cells treated with 250 μM *N*M(*R*)Sal.

indicate that the PT caused by *N*M(*R*)Sal is ascribed to the direct interaction with mitochondria, and not to oxidative stress or mitochondrial dysfunction.

It is of interest to note that the (*S*)-enantiomer of *N*MSal was ineffective in inducing cell death in SH-SY5Y cells (Maruyama *et al*. 2001b) as well as in reducing $\Delta\Psi$m in the isolated mitochondria (data not shown). These results suggest that *N*M(*R*)Sal probably interacts in a stereo-specific manner with some protein(s) on the mitochondria to trigger the PT. This is also supported by the observation that the redox potential of (*R*)-enantiomer of *N*MSal was the same as that of the (*S*)-enantiomer (Deng *et al*. 1997), suggesting that the enantio-selective toxicity of the (*R*)-enantiomer was not due to the difference in potency to generate reactive oxygen and nitrogen species.

To relate the results reported here to the cell death in Parkinson's disease, the concentrations of *N*M(*R*)Sal in the nigra-striatum of human brains should be considered. The *N*M(*R*)Sal concentrations in the substantia nigra and the striatum from control human brains were determined to be about 100 nM (Maruyama *et al*. 1997b). However, in the dopamine neurons the concentration should be much higher, as the isoquinoline is synthesized from dopamine *in situ* and the analyzed brain samples contained cells other than dopamine neurons. In addition, about 3% and only 0.05% of *N*M(*R*)Sal added to the medium were taken in SH-SY5Y cells and the mitochondria, respectively (Maruyama *et al*. 1997a). The concentrations of *N*M(*R*)Sal in the cells and in mitochondria were expected to be in several micrometers and 100–10 nM order, respectively, suggesting that the *N*M(*R*)Sal concentrations used here may be reasonably expected in the nigra-striatum of human brains.

In the present study we have also shown that rasagiline inhibited the *N*M(*R*)Sal-induced PT in the cells as well as in the isolated mitochondria, which provides the first evidence that this antiapoptotic and anti-Parkinson's disease drug directly regulates the apoptotic signaling pathway in mitochondria. Cytosolic translocation of Cyt *c* is an amplifying factor for the caspase cascade, and *N*M(*R*)Sal increased Cyt *c* in cytoplasm in a dose- and time-dependent way. Rasagiline suppressed the release of Cyt *c* as shown in this paper, and prevented the following activation of caspase 3 (Maruyama *et al*. 2000), nuclear translocation of GAPDH and nucleosomal DNA fragmentation (Maruyama *et al*. 2001a). Our studies on the relationship between the chemical structure and antiapoptotic activity of propargylamines reveal that the (*R*)-enantiomers have more potent antiapoptotic activity than the (*S*)-enantiomers (Maruyama *et al*. 2002). It should be emphasized that SH-SY5Y cells do not express MAO-B (Maruyama *et al*. 1997a), indicating that the binding site of rasagiline should be a mitochondrial protein other than MAO-B. However, rasagiline was unable to suppress the Ca^{2+}-induced PT, and the drug does not seem to be a general inhibitor for the PT, such as Bcl-2, bonkrekic acid and CsA, all of which target respective components of the PT pore complex. The characterization of the target protein of rasagiline requires further experiments.

In neurodegenerative disorders and aging, neuronal cell death has been considered to be impossible or extremely difficult to halt or prevent. In Parkinson's disease, (–)deprenyl used clinically as an adjunct of L-DOPA therapy was found to affect symptoms, even though not the cause of the disease. One of the reasons why (–)deprenyl has failed to show neuroprotective effects in clinical trials may be due to its rapid metabolism into amphetamine derivatives. Recently, Abu-Raya *et al*. (2002) clearly showed that, in PC12 cells, L-amphetamine, a major metabolite of (–)deprenyl, interferes with the neuroprotective property of (–)deprenyl. In contrast, rasagiline, a cyclic benzylamine, does not produce amphetamine, but rather aminoindan, which does not interfere with neuroprotective actions of (–)deprenyl or rasagiline. Our data demonstrate that rasagiline is a novel drug to regulate directly the mitochondrial apoptotic machinery. Further studies will make it possible to determine whether rasagiline or structurally related compounds prevent, or at least, delay the progress of neurodegeneration in Parkinson's disease and other age-related disorders.

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