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Involvement of type A monoamine oxidase in neurodegeneration: regulation of mitochondrial signaling leading to cell death or neuroprotection

M. Naoi[1], **W. Maruyama**[2], **Y. Akao**[1], **H. Yi**[1], **Y. Yamaoka**[1]

[1] Department of Neurosciences, Gifu International Institute of Biotechnology, Kakamigahara, Gifu, and
[2] Department of Geriatric Medicine, National Institute for Geriatrics and Gerontology, Obu, Aichi, Japan

Summary In neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, apoptosis is a common type of cell death, and mitochondria emerge as the major organelle to initiate death cascade. Monoamine oxidase (MAO) in the mitochondrial outer membrane produces hydrogen peroxide by oxidation of monoamine substrates, and induces oxidative stress resulting in neuronal degeneration. On the other hand, a series of inhibitors of type B MAO (MAO-B) protect neurons from cell death. These results suggest that MAO may be involved in the cell death process initiated in mitochondria. However, the direct involvement of MAO in the apoptotic signaling has been scarcely reported. In this paper, we present our recent results on the role of MAO in activating and regulating cell death processing in mitochondria. Type A MAO (MAO-A) was found to bind an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol, and induce apoptosis in dopaminergic SH-SY5Y cells containing only MAO-A. To examine the intervention of MAO-B in apoptotic process, human MAO-B cDNA was transfected to SH-SY5Y cells, but the sensitivity to *N*-methyl(*R*)salsolinol was not affected, even though the activity and protein of MAO-B were expressed markedly. MAO-B oxidized dopamine with production of hydrogen peroxide, whereas in control cells expressing only MAO-A, dopamine autoxidation produced superoxide and dopamine-quinone, and induced mitochondrial permeability transition and apoptosis. Rasagiline and other MAO-B inhibitors prevent the activation of apoptotic cascade and induce prosurvival genes, such as bcl-2 and glial cell line-derived neurotrophic factor, in MAO-A-containing cells. These results demonstrate a novel function of MAO-A in the induction and regulation of apoptosis. Future studies will clarify more detailed mechanism behind regulation of mitochondrial death signaling by MAO-A, and bring out new strategies to cure or ameliorate the decline of neurons in neurodegenerative disorders.

Abbreviations: *β-PEA* β-phenylethylamine, *DiOC$_6$(3)* 3,3′-dihexyloxacarbocyanide iodine, *DMEM* Dulbecco's modified Eagle's medium, *$\Delta\Psi m$* mitochondrial membrane potential, *FACS* fluorescence-augmented flow cytometry, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *GDNF* glial cell line-derived neurotrophic factor, *HE* hydroethidine, *HPLC-ECD* high-performance liquid chromatography with electrochemical detection, *5-HT* 5-hydroxytryptamine, serotonin, *MAO-A and MAO-B* type A and B monoamine oxidase, *NMRSal and NMSSal* *N*-methyl(*R*)salsolinol and *N*-methyl(*S*)salsolinol, *mPT* mitochondrial permeability transition, *PBS* phosphate-buffered saline, *PD* Parkinson's disease, *PI* propidium iodide, *RNAi* RNA interference, *ROS* reactive oxygen species, *siRNA* small interfering RNA

Correspondence: M. Naoi, Department of Neurosciences, Gifu International Institute of Biotechnology, 1-1 Naka-fudogaoka, Kakamigahara, Gifu 504-0838, Japan
e-mail: mnaoi@giib.or.jp

In neurodegenerative disorders, including Parkinson's disease (PD) and Alzheimer's disease, selective neurons degenerate in specified brain regions in either apoptotic or necrotic process. In PD, the degeneration of dopamine neurons is observed mainly in the substantia nigra. Understanding of the intracellular mechanism of neurodegeneration has been advanced markedly and in the intrinsic pathway to apoptosis mitochondria initiate death signaling. Oxidative and nitrosactive stress, mitochondrial dysfunction, neurotoxins, excitotoxicity, accumulation of misfolded protein and reduced activity of the ubiquitin-proteasome system activate the death cascade (Götz et al., 1990; Andersen, 2004; Bossy-Wetzel et al., 2004; Naoi et al., 2005). The detailed mechanism underlying the cell death in PD has been studied using animal and cellular models, and we found that dopamine-derived *N*-methyl(*R*)-salsolinol [*N*M*R*Sal, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] is an endogenous neurotoxin and causes cell death in dopamine neurons (Naoi et al., 2002a, b, 2004). After continuous infusion in the rat striatum, *N*M*R*Sal induces apoptotic cell death in dopamine neurons in the substantia nigra (Naoi et al., 1996). In human dopaminergic neuroblastoma SH-SY5Y cells, *N*M*R*Sal induced apoptosis by sequential activation of death cascade; decline in mitochondrial membrane potential, $\Delta\Psi$m, opening of mitochondrial permeability transition (mPT) pore, release of cytochrome c, activation of caspase 3, nuclear translocation of glyceraldehydes-3-phosphate

dehydrogenase [GAPDH, D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12], and fragmentation of nuclear DNA (Maruyama et al., 1997, 2001a, b; Akao et al., 2002a). Analyses of clinical samples from parkinsonian patients indicate that *N*M*R*Sal might be involved in the pathogenesis of PD (Maruyama et al., 1996; Naoi et al., 1998).

The mPT is an increase in the permeability of the inner mitochondrial membrane to solutes, by opening of mPT pore, a large proteinaceous pore spanning the outer and inner membrane of mitochondria (Crompton, 1999; Green and Kroemer, 2004). The mPT pore forms a functional micro-compartment with voltage-dependent anion channel in the outer membrane, adenine nucleotide translocator in the inner membrane, and hexokinase at the contact site, but the exact composition has not yet been fully clarified. The (*R*)-enantiomer of *N*-methylsalsolinol (*N*MSal), but not the (*S*)-enantiomer, induces swelling in mitochondrial matrix (Akao et al., 2002a) and $\Delta\Psi$m reduction in SH-SY5Y cells (Maruyama et al., 2001b). The enantio-selective cytotoxicity of *N*M*R*Sal suggests the occurrence of the specified binding site recognizing *N*M*R*Sal in mitochondrial membrane. *N*M*R*Sal was found to inhibit type A, but not type B, monoamine oxidase [MAO-A and MAO-B, monoamine: oxygen oxidoreducatse (deaminating), EC 1.4.3.4]. The inhibition was competitive to the substrate, and the value of the inhibitor constant, K_i, was estimated to be $59.9 \pm 5.4\,\mu M$ (mean $\pm$ SD) (Yi et al., 2006b). This suggests that MAO-A may bind *N*M*R*Sal at or near the substrate-binding site. However, it has never been reported whether MAO is involved directly in apoptotic cascade, or MAO itself is a component of mPT pore.

MAO is localized in the outer membrane of mitochondria and catalyses the oxidative deamination of neuroactive, vasoactive and xenobiotic amines generating hydrogen peroxide and aldehydes. The two MAO isoenzymes, MAO-A and MAO-B, share 70% amino acid sequence identity and are encoded by two closely linked genes in the X chromosome (Bach et al., 1988; Shih et al., 1999). These two isomers have distinct specificities for the substrates and inhibitors (Tipton et al., 2004). MAO-A has substrate preference for 5-hydroxytryptamine (5-HT, serotonin) and norepinephrine, and very high sensitivity to an irreversible inhibitor, clorgyline [*N*-methl-*N*-propargyl-3(2,4-dichlorophenoxy)-propylamine], whereas MAO-B oxidizes β-phenylethylamine (β-PEA) and benzylamine and is inhibited by low concentrations of (−)deprenyl [*N*, α-dimethyl-*N*-2-propynylbenzene-ethanlamine] and rasagiline [*N*-propargl-1(*R*)- aminoindan] (Youdim et al., 2001). In human brain MAO-A is expressed in catecholamine neurons, whereas serotonergic neurons and astrocytes contain MAO-B (Westlund et al., 1988). The studies of MAO-A and MAO-B knockout mice clearly proved that these two MAO isoenzymes have distinct functions in monoamine metabolism and play important roles in neurological and psychiatric disorders, including depression and PD (Cases et al., 1995; Lim et al., 1994; Shih et al., 1999). In human brain MAO-B levels increase 2–3 folds in an age-dependent way, resulting in increased oxidative stress, which may induce vulnerability of the brain in age-dependent neurodegenerative disorders.

A series of MAO-B inhibitors with a propargyl moiety, rasagiline and (−)deprenyl, protect neurons from cell death induced by various insults (Maruyama et al., 2001a; Youdim et al., 2005a, b). Rasagiline is now the most potent in neuro-rescue or -protective function, as shown in animal and cellular models of PD, Alzheimer's disease and brain ischemia, and the neuroprotective effect has been also suggested in clinical trials (Parkinson Study Group, 2004). The anti-apoptotic function is due to the direct stabilization of mPT pore (Maruyama et al., 2001a, 2001b) and induction of prosurvival genes, such as antiapoptotic Bcl-2 and Bcl-xL (Akao et al., 2002a, b) and glial cell line-derived neurotrophic factor (GDNF) (Maruyama et al., 2004). However, the neuroprotective function may not necessarily depend on the inhibition of MAO-B activity, as suggested by the facts that the neuroprotective potency is observed with propargylamines without MAO-inhibition (Maruyama et al., 2001c; Yi et al., 2006a), and at the concentration quite lower than those for MAO inhibition (Akao et al., 2002a; Maruyama et al., 2001a, 2004).

In this paper, the role of MAO in the apoptotic cascade was studied by use of *N*M*R*Sal in SH-SY5Y cells containing only MAO-A (wild SH), in relation to the *N*M*R*Sal binding, $\Delta\Psi$M reduction and apoptosis. To confirm the role of MAO-A in apoptotic cascade, the effects of RNA interference (RNAi) targeting MAO was examined by use of small interfering RNA (siRNA) to silence MAO-A in the cells. In addition, the involvement of MAO-B in apoptosis by *N*M*R*Sal was examined in SH-SY5Y cells transfected with cDNA of human MAO-B (MAO-B-SH). The role of MAO-A and -B in inducing anti-apoptotic genes by rasagiline, a MAO-B inhibitor, was studied by use of these SH cells, and also Caco-2 human colon adenocarcioma cells expressing only MAO-B (Wong et al., 2003). The role of MAO isoenzymes is discussed in relation to the regulation of apoptotic signaling in mitochondria, and their possible involvements in neurodegenerative disorders including PD.

Materials and methods

Materials

*N*MR*S*al was synthesized according to Teitel et al. (1972). Kynuramine, 4-quinolinol, dihydroethidine (HE) and dopamine were purchased from Sigma (St. Louis, MO, USA); propidium iodide (PI), MitoTracker Orange and Green, and 3,3′-dihexyloxacarbocyanide iodine [$DiOC_6(3)$] from Molecular Probes (Eugene, OR, USA); 5-hydroxytryptamine (5-HT, serotonin) from Merck (Darmstadt, Germany). Clorgyline, a MAO-A inhibitor, and rasagiline and (−)deprenyl (selegiline), MAO-B inhibitors, were kindly donated by May and Baker (Dagenham, U. K.), TEVA (Netanya, Israel), and Dr. Knoll (Semmellweis University, Budapest, Hungary), respectively. Dulbecco's modified Eagle's medium (DMEM), β-PEA and other drugs were purchased from Nacalai tesque (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan) supplemented by 5% fetal calf serum (FCS) in an atmosphere of 95% air-5% CO_2. Mitochondria were prepared according to Desagher et al. (1999). Caco-2 cells were cultured in DMEM supplemented with 10% FCS and 1% nonessential amino acids.

RNAi of MAO-A in SH-SY5Y cells

To reduce MAO-A expression in mitochondria, siRNA targeting MAO-A mRNA (Sc-35874) was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The siRNAs were transfected into the cells to be 20–35 nM in the final concentration by use of cationic liposomes TransIT-TKO (Mirus Bio, Madison, WI, USA). The transfection efficiency was evaluated by the transfection of the cells with a duplex siRNA-FITC. Non-specific control duplex (57% GC content; Dharmacon, Lafayette, CO, USA) was used as control for non-specific effects. The effects of RNAi targeting MAO-A on the protein amount and activity of MAO and the binding of *N*MR*S*al were determined at 36 h after the transfection. MAO protein was detected by Western blot analyses, using antibody recognizing both MAO-A and -B prepared according to Gargalidis-Moudanos et al. (1997). The polyclonal antisera were isolated from rabbits immunized with the peptide TNGGQ ERKFVGGSGQ, corresponding to amino acids 210–227 in MAO-A and 202–217 in MAO-B, and purified on an affinity column conjugated with the antigen peptide. Bound antibodies were detected using enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

DNA transfection of MAO-B gene in SH-SY5Y cells

To establish transfectants expressing human MAO-B, a pIRES1neo eukaryotic expression vector (Invitrogen, San Diego, CA, USA) was used (Yi et al., 2006b). pIRES1-neo-MAO-B was constructed by including the full-length human MAO-B gene in pECE vector (Lan et al., 1989) and digested with HindIII and inserted into the pIRESneo vector. SH-SY5Y cells were transfected with pIRES1neo or pIRES1neo-MAO-B by using cationic liposomes (Lipofect-AMINE). Selection was started 2 days after the transfection using the culture medium containing geneticin (GIBCO BRL). Individual clones were isolated and characterized by RT-PCR, as described previously (Akao et al., 2002a). Stable clones overexpressing MAO-B protein (MAO-B-SH) were obtained by limiting dilution and confirmed by RT-PCR.

Assay for MAO-A and MAO-B activity

MAO activity in mitochondria was measured fluorometrically by use of kynuramine as a substrate, according to Kraml (1965). Mitochondria prepared from control SH-SY5Y (wild SH) cells were used as a MAO-A sample, and those from MAO-B-SH cells were pre-treated with 1 μM clorgyline at 37°C for 20 min and used as a MAO-B sample. Protein concentration was determined according to Bradford (1976).

Assay for the binding of NMRSal to mitochondria

Mitochondria were suspended in 100 μl of 10 mM Tris-HCl buffer, pH 6.0, and incubated with 10–100 μM *N*MR*S*al for 60 min at 4°C. Then, the cells were washed successively with 1.5 ml of phosphate-buffered saline (PBS) containing 1% bovine serum albumin and twice with PBS alone by centrifugation at 6000 *g* for 10 min. The cells were suspended in 200 μl of 10 mM perchloric acid solution containing 0.1 mM EDTA, mixed, centrifuged, filtered through a Millipore HV filter (pore size 0.45 μm), and applied to high-performance liquid chromatography with electrochemical detection (HLC-ECD), as reported previously (Naoi et al., 1996).

Measurement of $\Delta\Psi m$

The effects of *N*MR*S*al on ΔΨm were quantitatively measured by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Becton Dickinson, San Jose, CA, USA), and MitoTracker Orange and Green, or 3,3′-dihexylpxacarbocyanide iodine [$DiOC_6(3)$] were used as fluorescent indicators (Yi et al., 2006a, b). The cells were cultured in 6-well poly-L-lysine-coated tissue culture flasks, washed with Cosmedium-001 without FCS, and incubated with 100–500 μM *N*MR*S*al or dopamine for 3 h at 37°C. The effects of 5-HT and β-PEA were also examined by addition of 100–500 μM 5-HT and β-PEA. After stained with 100 nM MitoTracker Orange and Green for 30 min at 37°C, or 2.5 nM $DiOC_6(3)$ (Stock solution: 1 μM in ethanol) for 15 min at 37°C. Then, the cells were washed and suspended with PBS and subjected to FACS. The laser emission at 560–640 nm (FL-2) and at shorter than 560 nm (FL-1) with excitation at 488 nm were used for the detection of MitoTracker Orange and Green fluorescence, respectively. $DiO_6(3)$ fluorescence was measured with FL-1.

Assessment of apoptosis induced by NMRSal or dopamine

Apoptosis was quantitatively measured by FACS, as described previously (Yi et al., 2006a). The cells cultured in 6-well poly-L-lysine-coated culture flasks were incubated in DMEM with 100–500 μM *N*MR*S*al or 100 μM dopamine at 37°C for 24 h, and treated with trypsin, gathered, and washed with PBS. The cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at 24°C for 5 min in the dark, washed and suspended in PBS, then subjected to FACS analysis. Cells with a lower DNA content, as shown by PI staining less than G1, were defined to be apoptotic (subG1 peak) (Eckert et al., 2001).

Rasagiline-induced bcl-2 expression in the cells

Wild SH and MAO-B-SH cells and Caco-2 cells were cultured with 10 μM–10 pM rasagiline overnight and Bcl-2 contents in the cells were quantitatively determined by Western blot analysis as reported (Akao et al., 2002b).

Statistics

Experiments were repeated 3 to 4 times in triplicate, 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

Binding of NMRSal to mitochondrial MAO-A and the effects of siRNA for MAO-A

The binding of *N*MR*S*al to mitochondria prepared from control SH-SY5Y (wild SH) cells was kinetically studied

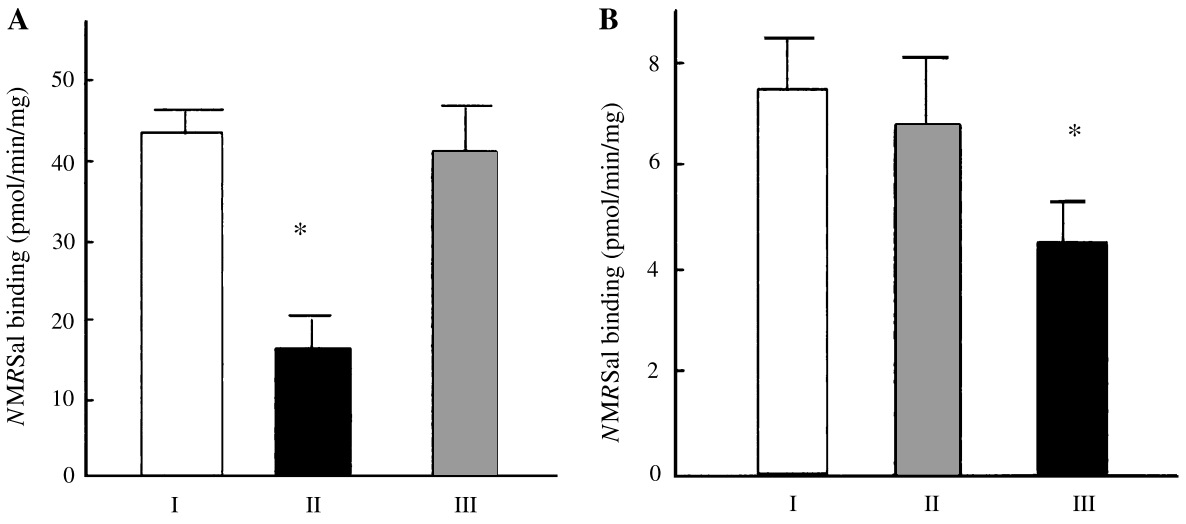

Fig. 1. The effects of MAO-inhibitors and RNAi targeting MAO on *N*M*R*Sal binding to mitochondria. **A** Effects of clorgyline and (−)deprenyl on the *N*M*R*Sal binding to mitochondria. Mitochondria were treated with 1 μM MAO inhibitors for 20 min at 37°C, then incubated with 10 μM *N*M*R*Sal for 1 h at 4°C. The column and bar represent the mean and SD of triplicate measurement of 2 experiments. **B** Effects of MAO-A RNAi. Crude mitochondria were prepared from wild SH (I), negative control (II) and siRNA-treated cells (III), and incubated with *N*M*R*Sal. *N*M*R*Sal binding was quantified by HPLC-ECD. The column and bar represent the mean and SD of triplicate measurements. $^{*}p < 0.05$ from control and negative control cells

and the binding kinetics followed the Michaelis-Menten equation. The values of the apparent Michaelis constant, $K_{m,}$ and the maximal velocity, V_{max}, were obtained to be $80 \pm 15\,\mu M$ and $2.7 \pm 0.5\,nmol/h/mg$ protein, respectively. The involvement of MAO in the binding was examined by use of clorgyline and (−)deprenyl, the selective inhibitor of MAO-A and MAO-B, respectively. As shown in Fig. 1A, clorgyline reduced *N*M*R*Sal binding significantly, but (−)deprenyl did not affect the binding.

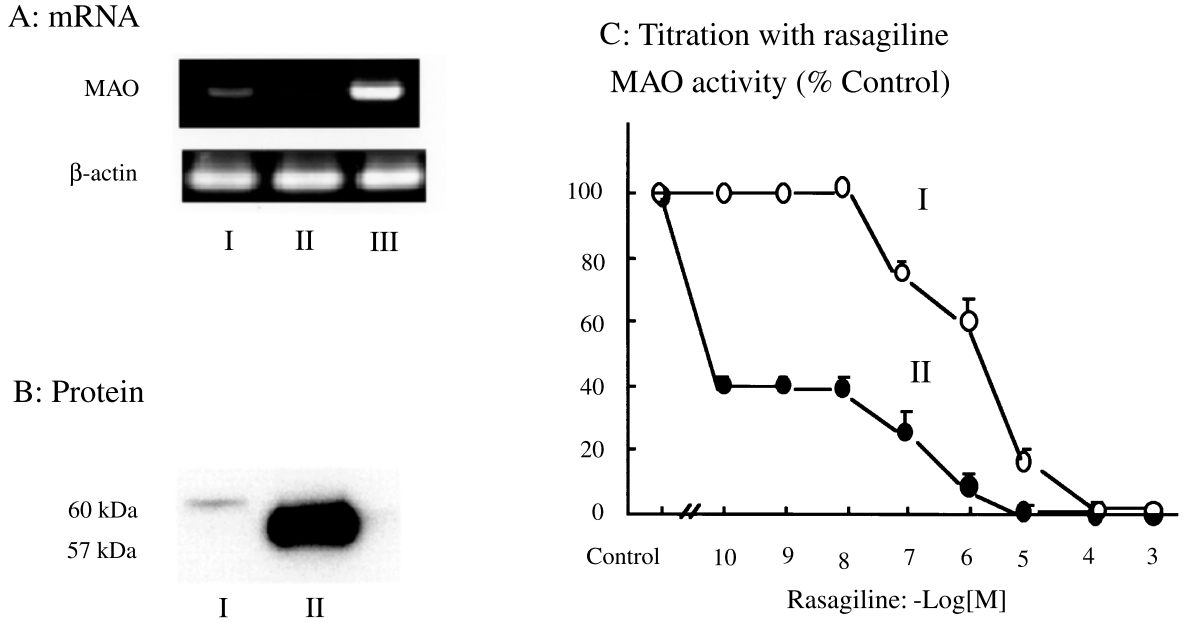

Fig. 2. Establishment of SH-SY5Y cells transfected with human MAO-B. SH-SY5Y cells were transfected with human MAO-B cDNA. **A** mRNA isolated from wild SH cells (I), cells transfected with IRES vector alone (II), and with full length MAO-B cDNA (III). β-Action was used as control. **B** Western blot analyses of MAO protein in mitochondria isolated from wild SH (I) and MAO-B-SH cells (II). MAO protein was detected with the antibody recognizing both MAO-A and -B. **C** Effects of rasagiline, a MAO-B inhibitor, on MAO activity. Mitochondria were prepared from wild SH (I) and MAO-B-SH cells (II), and MAO activities were measured with 100 μM kynuramine as a substrate, after treatment with rasagiline (0.1 nM–1 mM) at 37°C for 20 min. Each point and bar represent the mean and SD of triplicate measurements

In order to confirm whether *N*M*R*Sal binds to MAO-A in mitochondria, MAO-A expression was inactivated using RNAi. In the siRNA-transfected cells, MAO protein with about 60 kDa was significantly reduced, whereas in non-specific siRNA-transfected cells the protein amount was almost the same as in control. The functional effects of RNAi were confirmed by reduction in MAO activity to 0.22 ± 0.02 nmol/min/mg protein in the siRNA treated-cells from 0.34 ± 0.03 nmol/min/mg protein in control. In non-specific siRNA-transfected cells, the MAO activity was the same as in control, 0.34 ± 0.01 nmol/min/mg protein. Figure 1B shows that RNAi targeting MAO-A markedly reduced *N*M*R*Sal binding to 4.47 ± 0.88 pmol/mg protein in siRNA-treated cells from 7.46 ± 0.95 and 6.83 ± 1.40 pmol/mg protein in control and non-specific siRNA-treated cells.

Effects of transfected MAO-B on MMRSal binding

To specify the role of MAO-A and -B in the binding of *N*M*R*Sal and the induction of apoptosis, SH-SY5Y cells transfected with human MAO-B cDNA (MAO-B-SH) were prepared from control cells expressing only MAO-A (wild SH). The expression of mRNA of MAO-B was confirmed in MAO-B-SH cells (Fig. 2A). MAO-A and MAO-B protein in wild SH and MAO-B-SH cells were detected by Western blot analyses and their apparent molecular weights were determined to be approximately 60 and 57 kDa, respectively (Fig. 2B). MAO activity in mitochondria isolated from MAO-B-SH cells increased significantly to be 22.9 ± 0.93 from 2.82 ± 0.18 nmol/min/mg protein in those from wild SH cells. The sensitivity to rasagiline, an irreversible inhibitor of MAO-B, increased by MAO-B transfection, as shown by the inhibitor concentration-ac-

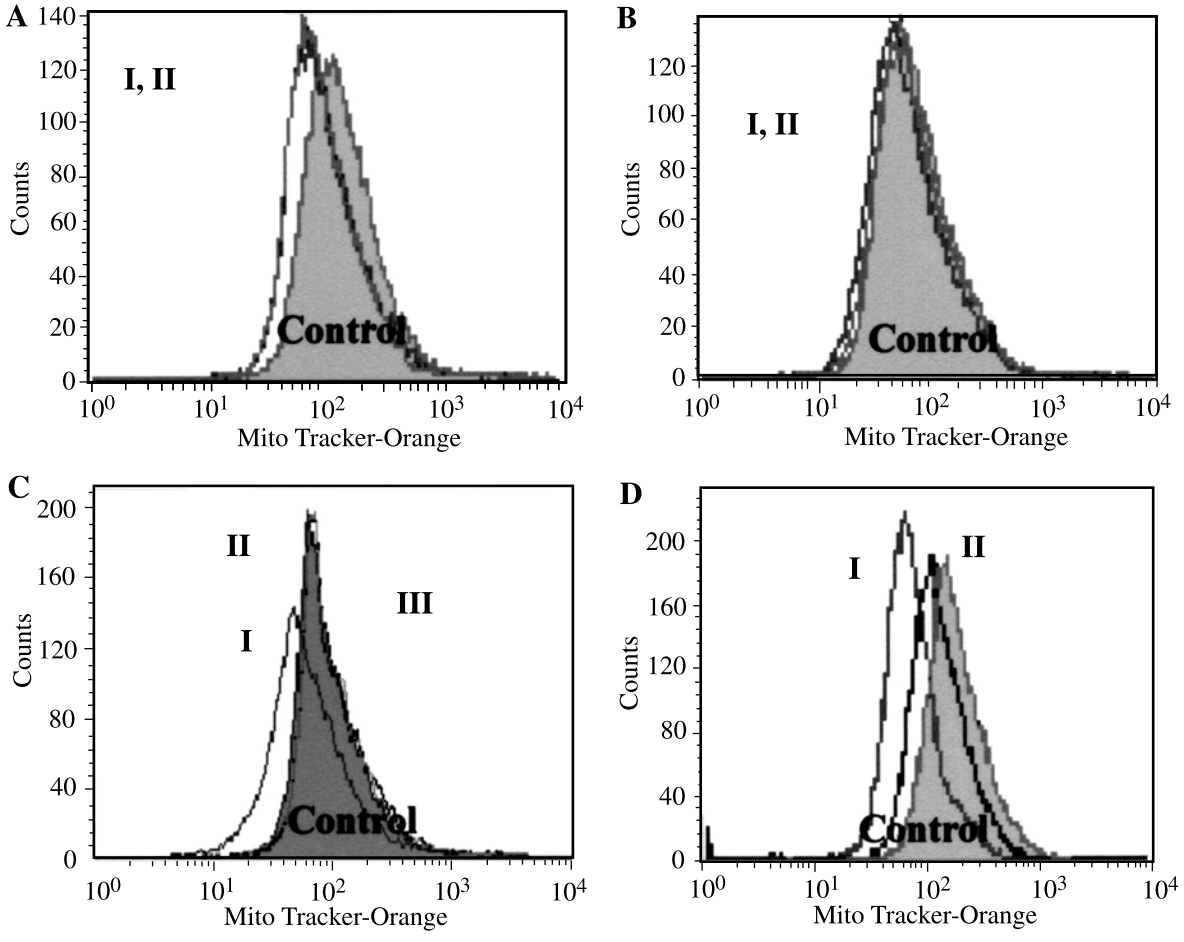

Fig. 3. *N*M*R*Sal reduced $\Delta\Psi$m in isolated mitochondria. **A** and **B**: Mitochondria were prepared from wild SH (A) and MAO-B-SH cells (B) and incubated with 500 μM (I) and 250 μM *N*M*R*Sal (II) at 37°C for 3 h. **C** Mitochondria isolated from wild SH cells were incubated with 100 μM *N*M*R*Sal in the absence (I) and presence of 100 μM 5-HT (II), or treated with 5-HT alone at 37°C for 3 h. **D** Mitochondria prepared from wild SH were incubated with the anti-MAO antibody diluted by 100-folds (I) or 500-folds (II) at 37°C for 3 h. $\Delta\Psi$m was measured by FACS after stained with MitoTracker Orange and Green

tivity studies (Fig. 2C), indicating that increased MAO activity was due to transfected MAO-B.

The binding of *N*M*R*Sal to mitochondria prepared from wild SH and MAO-B-SH cells was examined. The binding velocity of *N*M*R*Sal to mitochondria isolated from wild SH and MAO-B-SH cells were 163.6 ± 52.6 and 150.1 ± 20.9 pmol/min/mg protein, respectively. The transfection of MAO-B did not increase *N*M*R*Sal binding, suggesting that *N*M*R*Sal did not bind to MAO-B, as shown also by the fact *N*M*R*Sal did not inhibit MAO-B activity.

NMRSal induced ΔΨm decline and apoptosis in MAO-A containing cells

Involvement of MAO-A and -B in apoptosis induced by *N*M*R*Sal was examined using mitochondria prepared from wild SH and MAO-B-SH cells. Figure 3A and B show that *N*M*R*Sal reduced ΔΨm in mitochondria containing MAO-A, but did not affect ΔΨm in those prepared from MAO-B-SH cells. 5-HT, a substrate of MAO-A, prevented ΔΨm decline induced by *N*M*R*Sal (Fig. 3C), whereas β-PEA, a MAO-B substrate, did not. In addition, clorgyline, an irreversible inhibitor of MAO-A reduced ΔΨm, which 5-HT prevented. On the other hand, a reversible MAO-A inhibitor moclobemide did not. Figure 3D shows that the antibody against MAO reduced ΔΨm in a doe-dependent way.

The role of MAO-A in apoptosis was shown by competition with 5-HT. *N*M*R*Sal induced apoptosis in wild SH, which 5-HT prevented completely (Fig. 4A). The number of apoptotic cells after *N*M*R*Sal treatment was 36.9% of the total and reduced to 5.3% by addition of 5-HT, which was almost the same as in control cells or cells treated with 5-HT alone; 5.5 and 4.6%. Clorgyline also induced apoptosis in the cells at the concentration higher than 100 nM (Fig. 4B). Clorgyline (50 μM) increased the number of apoptotic cells to 28.7% of the total from 12.4% and 8.4% in control and 5-HT alone (1 mM)-treated cell, and 5-HT reduced the number of clorgyline-induced apoptotic cells to 14.7%.

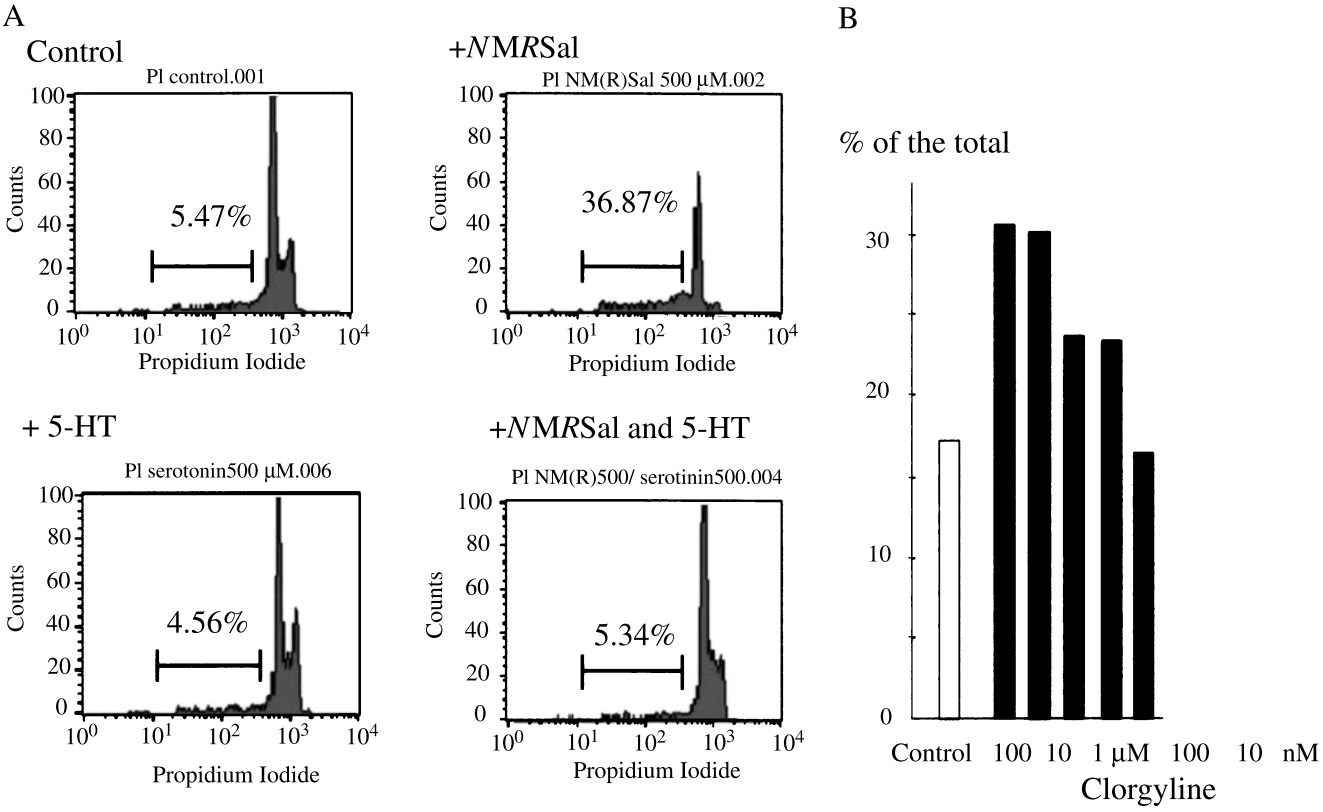

Fig. 4. Effects of 5-HT on *N*M*R*Sal-induced apoptosis and apoptosis by clorgyline in wild SH cells. **A** Control cells were incubated with *N*M*R*Sal (+*N*M*R*Sal) or 500 μM 5-HT (+5-HT) or *N*M*R*Sal and 5-HT (+*N*M*R*Sal and 5-HT) at 37°C overnight. Apoptotic cells were quantified by FACS after staining with PI. The cells with lower DNA content showing less PI staining than G1 were defined to be apoptotic. The number in Fig. 4A represents the number of apoptotic cells in the total (%). **B** Wild SH cells were incubated with 10 μM–10 nM clorgyline at 37°C overnight and apoptotic cells were quantified by FACS-PI method. The column represents the number of apoptotic cells as % of the total

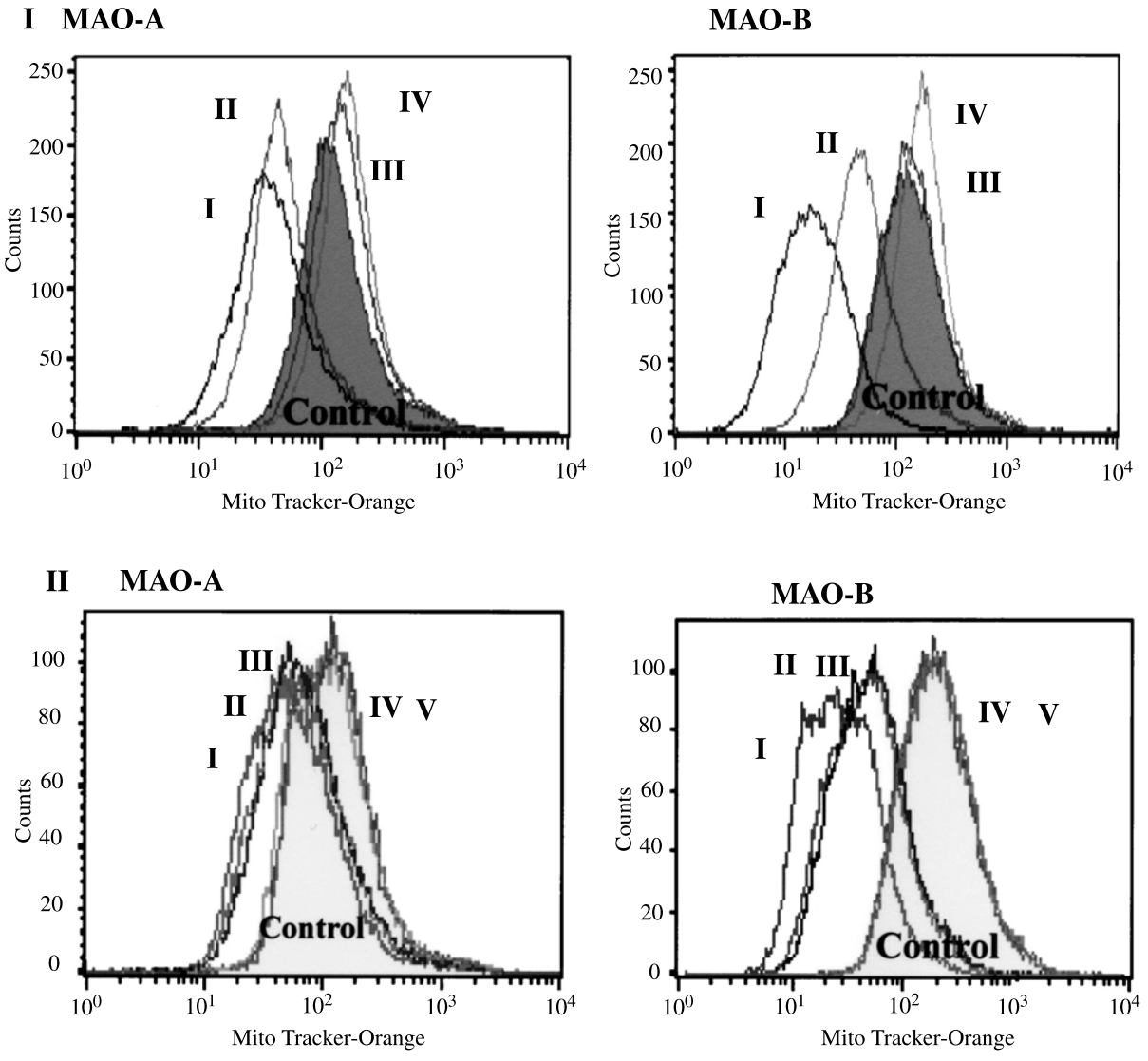

Fig. 5. ΔΨm reduction by dopamine oxidation and effects of MAO inhibitors. Mitochondria were prepared from MAO-A only containing wild SH (**MAO-A**) and MAO-B-SH cells (**MAO-B**). **I** Mitochondria incubated at 37°C for 3 h with 100 µM dopamine (I), 100 µM L-DOPA (II), 1 µM Fe^{2+} (III) and Fe^{3+} (IV). **II** Mitochondria were treated with 100 µM dopamine in the absence (**I**) of 1 µM clorgyline (**II**) or (−)deprenyl (**III**). **IV** and **V**: Mitochondria treated with clorgyline or (−)deprenyl. ΔΨm was measured by FACS with MitoTracker Orange

The role of MAO-A and MAO-B in the cytotoxicity of dopamine oxidation

Dopamine is oxidized either by enzymatic oxidation of MAO to 3,4-dihydrophenylactaldehyde and hydrogen peroxide, or by non-enzymatic autoxidation to dopamine-quinone and superoxide. The role of MAO-A and -B in the dopamine-induced cell death process was studied using mitochondria isolated from wild SH and MAO-B-SH cells. As shown in Fig. 5, I, dopamine and L-DOPA reduced ΔΨm markedly in MAO-A-containing mitochondria, whereas in MAO-B-containing mitochondria dopamine reduced ΔΨm more markedly than by L-DOPA and in MAO-A-containing mitochondria. Clorgyline and (−)deprenyl, inhibitors of MAO-A and MAO-B, did not prevent ΔΨm decline in MAO-A-containing mitochondria, but they partially prevented the ΔΨm decline in MAO-B-containing mitochondria (Fig. 5, II). Using FACS and fluorescent dyes, H_2DCFDA for hydrogen radical, nitric oxide and peroxynitrite (Crow, 1997) and HE for superoxide (Bindokas et al., 1996), ROS produced from dopamine oxidation was confirmed to be superoxide in MAO-A-containing mitochondria, whereas MAO-B produced hydrogen peroxide in addition to superoxide. Reduced glutathione, ascorbic acid and superoxide dismutase prevented ΔΨm decline in

MAO-A and -B-containing-mitochondria, whereas catalase did not. Dopamine oxidation modifies SH residues in mitochondrial complex I with formation of quinoprotein and inhibits the enzymatic activity of mitochondrial oxidative phophorylation (Naoi et al., in preparation).

Involvement of MAO-A in neuroprotection by rasagiline, a MAO-B inhibitor

A series of propargylamine MAO-B inhibitors protect neuronal cells in cellular and animal models of PD and other neurodegenerative disorders. The role of MAO in the neuroprotective function by rasagiline was confirmed in wild SH cells containing only MAO-A. The antiapoptotic, neuroprotective function of rasagiline is ascribed to two mechanisms. One is the stabilization of mitochondrial homeostasis and the prevention of mPT, and the other the induction of anti-apoptotic genes, bcl-2 and GDNF, as shown in Fig. 6. Opening of mPT pore leads to $\Delta\Psi$m loss and swelling of the matrix, which was completely suppressed by rasagiline. Rasagiline prevents the cytochrome c release from mitochondria caused by rupture of the outer membrane due to the swelling, and suppresses the activation of casspase 3 (Maruyama et al., 2001a; Akao et al., 2002a) and the nuclear translocation of GAPDH (Maruyama et al., 2001b). Rasagiline increases the gene expression and protein amounts of bcl-2 (Aako et al., 2002b) and GDNF (Maruyama et al., 2004) in wild SH cells, and also the activity of catalase and superoxide dismutase in rats (Carrillo et al., 2000). The gene induction has the concen-

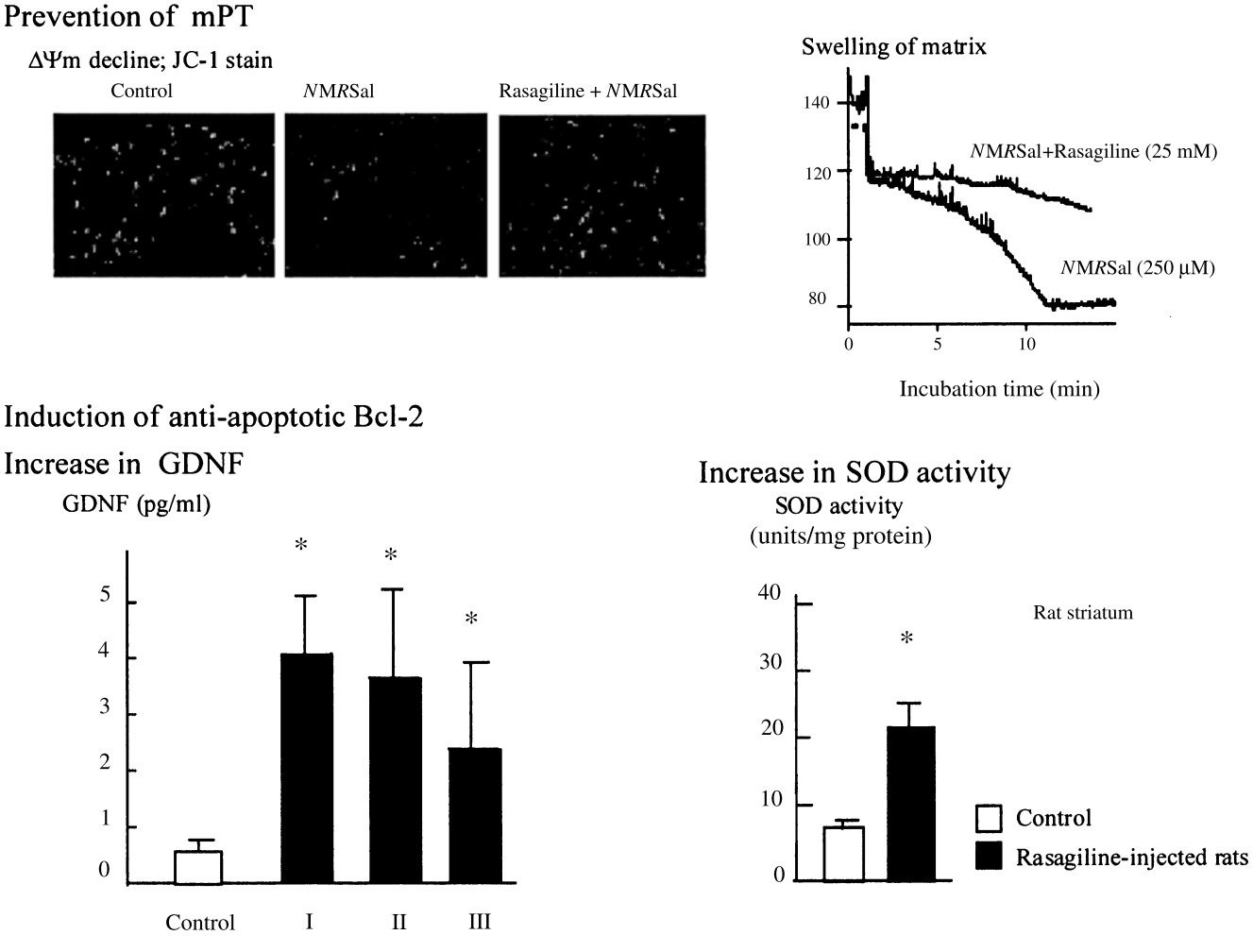

Fig. 6. Intracellular mechanism behind neuroprotective function of rasagiline. Rasagiline stabilizes mPT pore and prevents $\Delta\Psi$m collapse and swelling of mitochondrial matrix. The activation of following death cascade, release of cytochrome c, activation of caspases and nuclear translocation of GAPDH is completely suppressed. Rasagiline increases GDNF in SH-SY5Y cells in a dose-dependent way. Cells were treated with 1 μM (I), 100 (II) and 10 nM (III) rasagiline at 37°C overnight and GDNF amount was assessed by ELISA. In rat brain regions containing dopamine neurons SOD and catalase activities increases significantly after systematic administration of rasagiline for 3 weeks. $^{*}p < 0.01$

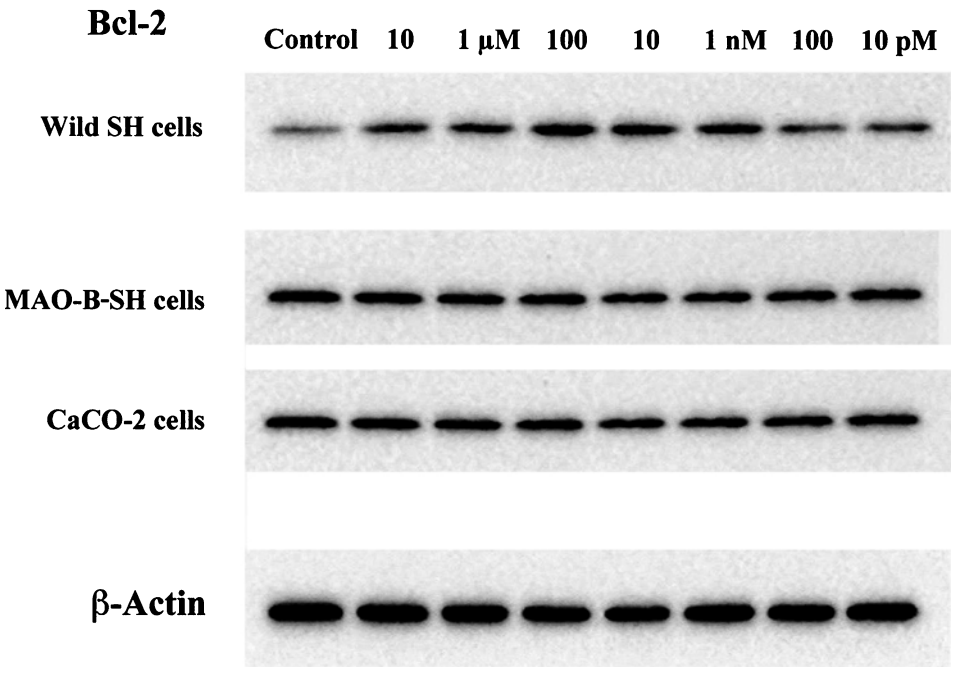

Fig. 7. Induction of Bcl-2 in wild and MAO-B-SH-SY5Y cells, and Caco-2 cells. Only MAO-A containing control and MAO-B transfected cells, and only MAO-B expressing Caco-2 cells were treated with 10 μM-10 pM rasagiline at 37°C overnight and the amount of Bcl-2 was determined by Western blot analysis. β-Action was used as a control

tration optima at two quite different ranges, 100–10 nM and 100–10 pM (Akao et al., 2002b). These concentrations were quite lower than those required for inhibition of MAO-A and -B. The IC_{50} values for inhibition of rat brain MAO activity were reported to be 412 nM and 4.4 nM for MAO-A and -B, respectively (Youdim et al., 2001).

The involvement of MAO-B in the induction of antiapoptotic genes was studied in MAO-B transfected cells and Caco-2 cells expressing only MAO-B. Even though marked expression of MAO-B was confirmed by the increased activity and protein amount, transfected MAO-B did not increase the sensitivity to rasagiline, as shown in Fig. 7. In Caco-2 cells bcl-2 was not induced by rasagiline at the concentrations of 10 μM–10 pM, suggesting that MAO-B may not be involved, or non-neuronal cells may not be responsible to rasagiline. These results suggest that MAO-A may play a major role in the antiapoptotic function of propargylamines, and that MAO-A may have a specified binding site of rasagiline other than that of the substrate and induce antiapoptotic genes. However, these results cannot exclude the possibility that MAO-B itself is involved in regulating apoptotic cascade in other types of cells. In addition, it remains to clarify how the signaling from mitochondria activates the transcription factors, such as NF-κB, which mediates the induction of Bcl-2 and GDNF by rasagiline (Maruyama et al., 2004).

Discussion

This paper reports for the first time the direct involvement of MAO-A in apoptosis. All the hitherto papers discussed the role of MAO in neuronal degeneration mainly in relation to the enzymatic oxidation of monoamines and the induction of oxidative stress (Cohen et al., 1997). In addition, the role of MAO-B in PD was augmented by the fact that MAO-B oxidizes a protoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into toxic 1-methyl-4-phenylpyridinium ion (MPP^+) (Heikkila et al., 1985). In concern to the role of MAO-A in apoptosis, higher MAO-A levels were expressed in apoptosis induced by depletion of nerve growth factor in PC12 cells, and increased ROS generation was considered to potentiate apoptosis (De Zutter and Davis, 2001). On the other hand, Malorni et al. (1998) reported that clorgyline and pargyline, inhibitors of MAO-A and MAO-A and -B, protected human melanoma M14 cells from apoptosis induced by serum withdrawal. These MAO inhibitors prevented the mPT induced by tyramine, a substrate for MAO-A and -B, in mitochondria isolated from rat liver (Marcocci et al., 2002). The protective function of MAO-A inhibitors was considered to be due to maintaining mitochondrial homeostasis by a direct effect on mPT pore in addition to inhibiting monoamine oxidation, but the detailed mechanisms were not presented in their paper.

Our results point out a novel direct involvement of MAO-A in mitochondrial apoptotic mechanism in addition to the enzymatic generation of ROS. RNAi targeting MAO-A reduced *N*M*R*Sal binding to mitochondria, in almost the same degree as the reduction of MAO protein amount and enzymatic activity. Kinetic studies on the inhibition of MAO-A activity suggest *N*M*R*Sal binding to the substrate binding site in MAO, as shown by competition with 5-HT, a MAO-A substrate, but not β-PEA, a MAO-B substrate. The binding of *N*M*R*Sal to MAO initiates the activation of apoptotic signaling. It is supported further by the fact that overexpression of MAO-B in SH-SY5Y cells did not increase the sensitivity to cytotoxic *N*M*R*Sal, and that *N*M*R*Sal binding to mitochondria inhibited by clorgyline, but not (−)deprenyl. The binding of *N*M*R*Sal to the active site of MAO-A may induce the conformational changes in MAO and the opening of mPT pore. The decline in $\Delta\Psi$m by anti-MAO antibody suggests the interaction of MAO with mPT pore. However, at present it requires further studies to clarify the mechanism behind the interaction of MAO with other components of mPT pore.

MAO-B is commonly considered to play a major role in the cell death of PD, since in human basal ganglia MAO-B is more abundant than MAO-A and accounts for about 80% of the total MAO activity (O'Carrol et al., 1983). MAO-B in glia cells, but not neurons, may play a major role in the enzymatic oxidation of dopamine and ROS production (Damier et al., 1996). However, in MAO-A only containing cells, superoxide and dopamine quinone produced by dopamine autoxidation induce mPT and apoptosis as well as in MAO-B overexpressed cells. These results suggest again that MAO-A may determine the cell death and survival in neurons. However, we should examine further using *in vivo* and *in vitro* models of neurodegeneration to establish the role of MAO-A and -B in regulation of death cascade and induction of antiapoptotic genes for neuroprotection by rasagiline analogues.

The results in this paper point out the direct involvement of MAO-A in apoptotic mechanism induced by a dopaminergic neurotoxin, *N*M*R*Sal, and similar, but less marked, effects on $\Delta\Psi$m were observed also with MPP^+. Selective MAO-A inhibitors, *N*M*R*Sal and MPP^+, might activate mitochondrial apoptotic signaling through binding to MAO-A, and induce cell death in MAO-A containing neurons. RNAi effectively reduced MAO in this cell model, suggesting that RNAi can be applied to prepare animal and cellular models by silencing MAO-A gene, and future studies by neurochemical and behavioral analyses may bring new insights on the function of MAO-A in neurodegeneratve and psychiatric disorders, such as bipolar emotional disorders.

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