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N-Propargylamine protects SH-SY5Y cells from apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol, through stabilization of mitochondrial membrane and induction of anti-apoptotic Bcl-2

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Summary. Propargylamine derivatives, rasagiline and (−)deprenyl, are anti-Parkinson agents and protect neurons from cell death as shown by *in vivo* and *in vitro* experiments. The studies on the chemical structure-activity relationship proved that the propargyl moiety is essentially required for the neuroprotective function. In this paper, neuroprotective activity of free *N*-propargylamine was studied using SH-SY5Y cells expressing only type A monoamine oxidase (MAO) against apoptosis induced by an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol. *N*-Propargylamine prevented apoptosis, whereas *N*-methylpropargylamine and propiolaldehyde did not. *N*-Propargylamine stabilized mitochondrial membrane potential and induced anti-apoptotic Bcl-2 at 1 μM–10 nM. *N*-Propargylamine inhibited MAO-A in competition to substrate with the apparent K_i value of 28 μM, which was significantly higher than the concentration required for neuroprotection. It indicates that MAO inhibition is not prerequisite for the protective function of *N*-propargylamine. The anti-apoptotic function of *N*-propargylamine is discussed in terms of neuroprotection by propargylamines in neurodegenerative diseases, including Parkinson's disease.

Keywords: Propargylamine, neuroprotection, apoptosis, mitochondrial membrane potential, Bcl-2, monoamine oxidase inhibitor.

Abbreviations

DMEM Dulbecco's modified Eagle's medium; *ERK* extracellular signal-regulated kinase; *FACS* fluorescence-augmented flow cytometry; *FCS* fetal calf serum; *2-HMP* *N*-(2-heptyl)-*N*-methylpropargylamine; *MAO*

monoamine oxidase; *MAO-A* and *MAO-B* type A and B MAO; *MAPK* mitogen-activated protein kinase; *NM(R)Sal N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline]; *PBS* phosphate-buffered saline; *PD* Parkinson's disease; *PI* propidium iodide; *PKC* protein kinase C; *PT* permeability transition.

Neuroprotection to delay or halt progressive degeneration of specified neurons is now proposed as a causal therapeutic strategy for Parkinson's disease (PD), Alzheimer's disease, and amyotrophic lateral sclerosis. In these disorders, apoptosis has been suggested to contribute to neuronal death, but this remains to be accepted in general. The well-regulated and slowly advancing process of apoptosis is proposed as a target of neuroprotection (Naoi and Maruyama, 2001; Vila and Przedborski, 2003). Various insults, including oxidative stress, metabolic compromise, excitotoxicity and endogenous and exogenous neurotoxins, are known to induce apoptosis in neurons. Apoptotic signaling is a multi-step pathway initiated by opening a mitochondrial mega-channel, called permeability transition (PT) pore, followed by decline in membrane potential ($\Delta\Psi$m), release of cytochrome c and other apoptosis-inducing factors, activation of caspases and final fragmentation of nuclear DNA. Mitochondrial PT and following apoptotic cascade are regulated by Bcl-2 protein family, and Bcl-2 and Bcl-xL prevent, and BAX and BAD promote cell death (Tsujimoto and Shimizu, 2000).

Recently, various candidates of neuroprotective agents have been proposed and some are now subjected for clinical trials (Ravina et al., 2003). A series of propargylamine derivatives, (−)deprenyl [selegiline, *N*-(phenylisopropyl)-*N*-methyl-propargylamine] (Riederer and Lachenmayer, 2003) and rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Parkinson Study Group, 2002), were reported to have beneficial symptomatic effects in patients with PD. More recently clinical controlled study with rasagiline in Parkinsonian subjects was successfully carried out pointing to its possible action to modify or slow down the disease progress (Parkinson Study Group, 2004). The patients treated with rasagiline for 12 months showed less functional decline than those whose treatment was delayed for 6 months. *In vivo* and *in vitro* experiments confirmed that (−)deprenyl (Tatton et al., 1994; Maruyama and Naoi, 1999), rasagiline (Finberg et al., 1998; Maruyama et al., 2000, 2001a, 2002; Youdim et al., 2001b), and *N*-(2-heptyl)-*N*-methylpropargylamine [2-HMP] (Yu et al., 1994; Maruyama et al., 2001b) protect neuronal cells against apoptosis as induced by various insults. Significant insights into the anti-apoptotic function of propargylamine derivatives have been documented, and mitochondria emerge as a key organelle playing a regulatory role in apoptosis. Our previous studies have shown that the neuroprotection by propargylamine derivatives, specially rasagiline, is attributed to (1) the stabilization of $\Delta\Psi$m and prevention of PT (Maruyama et al., 2001a, b; Naoi et al., 2002a), (2) the induction of anti-apoptotic Bcl-2 family regulating PT (Akao et al., 2002a, b), (3) the release of glial cell line-derived neurotrophic factor (GDNF), a neurotrophic factor selective to dopamine neurons (Maruyama et al., 2004), and (4) activation of anti-oxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 2000).

Most of the neuroprotective propargylamines, such as (−)deprenyl (Knoll et al., 1978) and rasagiline (Youdim et al., 2001a), are inhibitors of type B monoamine oxidase [monoamine, oxygen oxidoreductase (deaminating); EC 1.4.3.4; MAO-B], suggesting the involvement of MAO in the neuroprotective potency. (−)Deprenyl and 2-HMP (Yu et al., 1992) possess a methyl group at the nitrogen position of the propargylamine moiety and this gives them the potent MAO inhibitory activity (Yu et al., 1993). However, the desmethyl

metabolites of (−)deprenyl and 2-HMP, and (*S*)-enantiomer of rasagiline do not inhibit MAO-B, but protect neurons (Mytilineou et al., 1997; Maruyama et al., 2001c). These results suggest that the neuroprotective function of propargylamines may not depend on the MAO-inhibitory activity. On the other hand, the structure activity relationship of rasagiline analogues has indicated that *N*-propargylamine itself may stabilize $\Delta\Psi m$ and prevent apoptotic process (Maruyama et al., 2003). Indeed, free *N*-propargylamine was reported to protect PC12 cells from cell death induced by serum withdrawal (Weinreb et al., 2004) similar to rasagiline.

This paper describes the effects of *N*-propargylamine on apoptosis induced by a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *N*M(*R*)Sal] (Maruyama et al., 1996, 2001b; Naoi et al., 2002b) in human dopaminergic neuroblastoma SH-SY5Y cells. Cell death induced by *N*M(*R*)Sal in SH-SY5Y cells has been confirmed to be apoptotic, not necrotic (Naoi et al., 2003), suggesting that this model may be adequate to examine the anti-apoptotic potency of *N*-propargylamine. The regulation of apoptotic signaling, namely stabilization of mitochondrial PT and expression of anti-apoptotic *bcl-2*, and effects on MAO activity by *N*-propargylamine were examined in SH-SY5Y cells, where only MAO-A activity is expressed. In addition, the structure-activity relationship in concern to anti-apoptotic function and MAO inhibiting potency were examined among *N*-propargylamine-related compounds, *N*-methylpropargylamine and propiolaldehyde.

Materials and methods

Materials

*N*M(*R*)Sal was prepared according to Teitel et al. (1972). Aminoindan was kindly supplied by TEVA Pharmaceutical Industries (Netanya, Israel). *N*-Propargylamine, kynuramine and 4-quinolinol were purchased from Sigma (St. Louis, MO, USA); *N*-methylpropargylamine from Aldrich (Milwaukee, WI, USA) and propiolaldehyde from Wako (Osaka, Japan); propidium iodide (PI), Mito-Tracker Orange and Green from Molecular Probes (Eugene, OR, USA), Dulbecco's modified Eagle's medium (DMEM) and other drugs 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 from SH-SY5Y cells according to Desagher et al. (1999).

Inhibition of MAO-A activity by N-propargylamine

MAO activity in mitochondria prepared from SH-SY5Y cells was measured fluorometrically according to the method reported by Kraml (1965) by use of kynuramine as a substrate. Kinetics for MAO activities and the inhibition by *N*-propargylamine were studied with 8 different concentrations of kynuramine. The values of the apparent Michaelis constant, K_m, and the apparent inhibitor constant, K_i, were calculated from the double-reciprocal plot of the reaction velocity against the substrate concentration. Protein concentration was determined according to Bradford (1976).

To determine the reversibility of the inhibition by *N*-propargylamine, MAO samples were incubated with 100 μM *N*-propargylamine for 30 min at 37°C, and then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, at 4°C overnight. The MAO activities were quantified before and after the dialysis.

Assessment of apoptosis induced by NM(R)Sal

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Becton Dickinson, San Jose, CA, USA). The cells cultured in a 6-well poly-L-lysine-coated culture flask were incubated in DMEM with or without 1 μM–1 nM *N*-propargylamine at 37°C for 30 min, then with 250 μM *N*M(*R*)Sal for 24 hr. The cells were treated with trypsin, gathered, washed with phosphate-buffered saline (PBS). To determine apoptotic cells, the cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at room temperature for 5 min in the dark, washed and suspended in PBS, then subjected to FACS analysis. The fluorescence intensity at 560–640 nm (FL-2 channel) was detected for PI with excitation at 488 nm. To differentiate singlet from doublet cells, FL-2 (PI)-A (Area) and FL-2 (PI)-W (Width) parameters were used. Cells with a lower DNA content showing less PI staining than G1

were defined to be apoptotic cells (subG1 peak) (Eckert et al., 2001).

Measurement of $\Delta\Psi m$ in mitochondria isolated from SH-SY5Y cells

$\Delta\Psi m$ decline in isolated mitochondria by *N*M(*R*)Sal was quantitatively measured by FACS using MitoTracker Orange and Green. The mitochondria were suspended in DMEM and incubated with 10 μM *N*-propargylamine for 30 min at 37°C, then with 250 μM *N*M(*R*)Sal for 3 h. After stained with 100 nM MitoTracker Orange and Green for 30 min at 37°C, the mitochondria 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.

Determination of Bcl-2 protein levels in the cells treated with N-propargylamine

SH-SY5Y cells treated with 1 μM–1 pM *N*-propargylamine 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 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 at 4°C with anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-tubulin

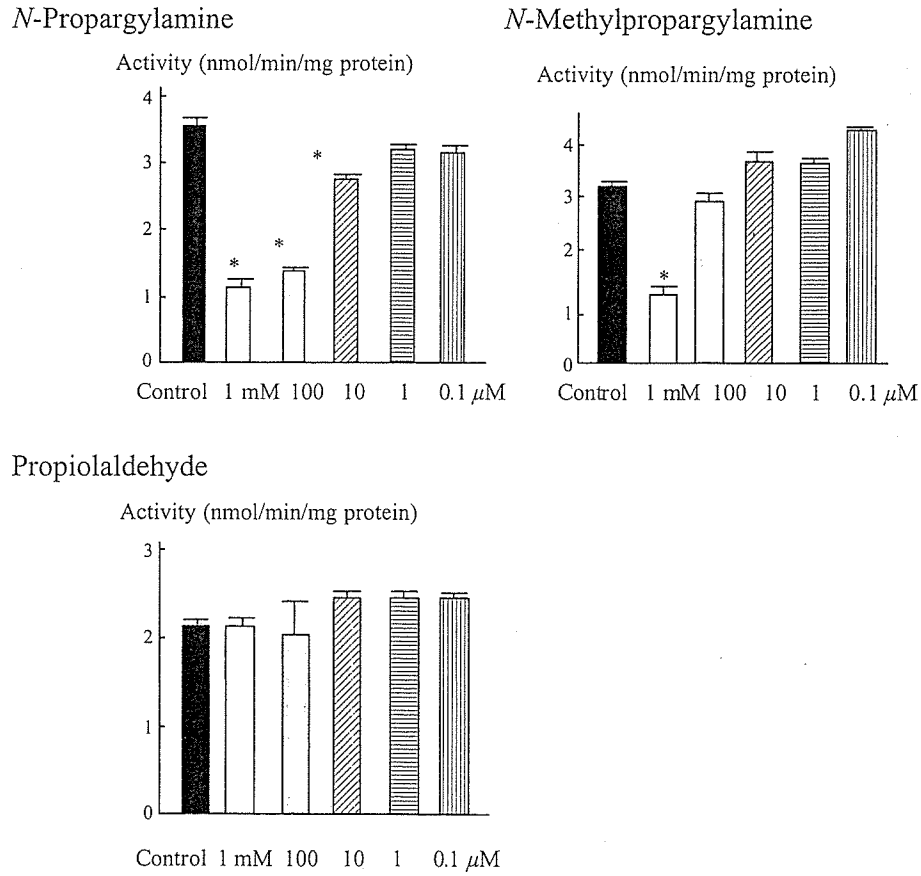


Fig. 1. The effects of *N*-propargylamine analogues on MAO-A activity. Mitochondria prepared from SH-SY5Y cells were used as MAO samples and incubated with 1 mM–0.1 μM *N*-propargylamine analogues and 100 μM kynuramine as a substrate at 37°C for 60 min, and the amounts of produced 4-hydroxyquinol were measured fluorometrically. The column and bar represent the mean and SD of 2 triplicate experiments. $P < 0.01$ from control

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).

Measurement of bcl-2 mRNA level in the cells treated with N-propargylamine

SH-SY5Y cells were cultured in the presence of various concentrations (1 μM–1 pM) of *N*-propargylamine for 24 h, and mRNA levels of *bcl-2* were quantitatively assessed by RT-PCR method (Akao et al., 2002a, b). 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 under the used conditions was confirmed by the real-time PCR method. PCR products were analyzed by electrophoresis on 3% agarose gels, and β-*actin* mRNA was used as an internal standard.

Statistics

Experiments were repeated at least 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.

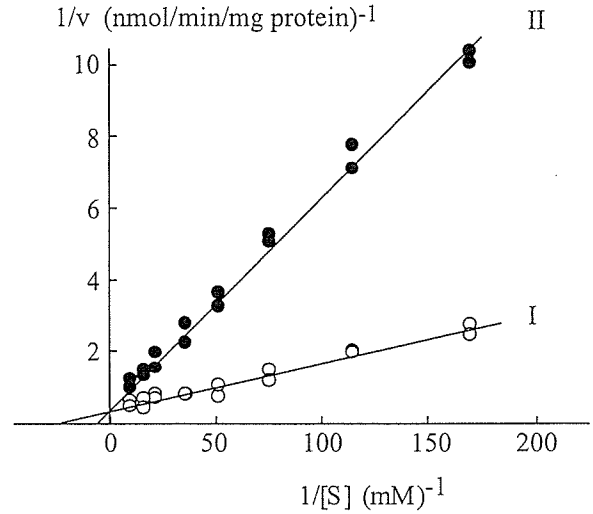


Fig. 2. The effects of *N*-propargylamine on the enzymatic activity of MAO-A. Mitochondria were prepared from SH-SY5Y cells, and MAO activity was measured fluorometrically with 8 different substrate concentrations in the absence (**I**) or presence of 100 μM *N*-propargylamine (**II**). The double reciprocal plots of the reaction velocity against the substrate concentration were used to calculate apparent K_m, K_i and V_{max} values, according the Lineweaver and Burk

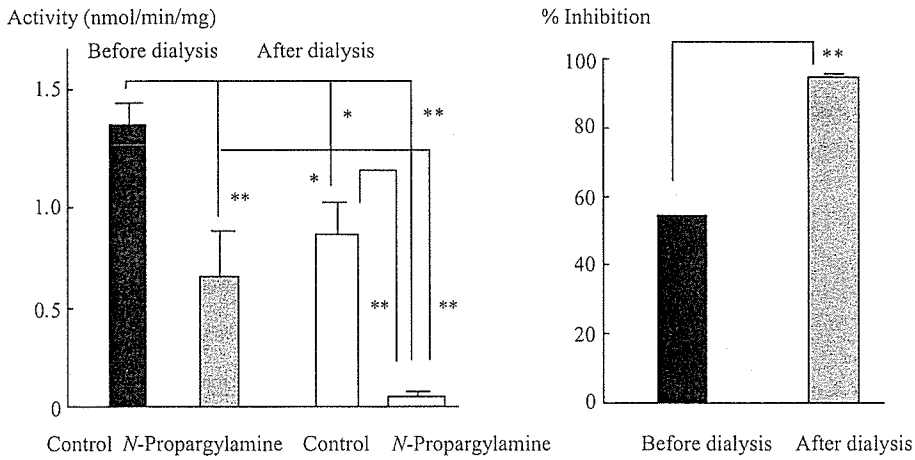
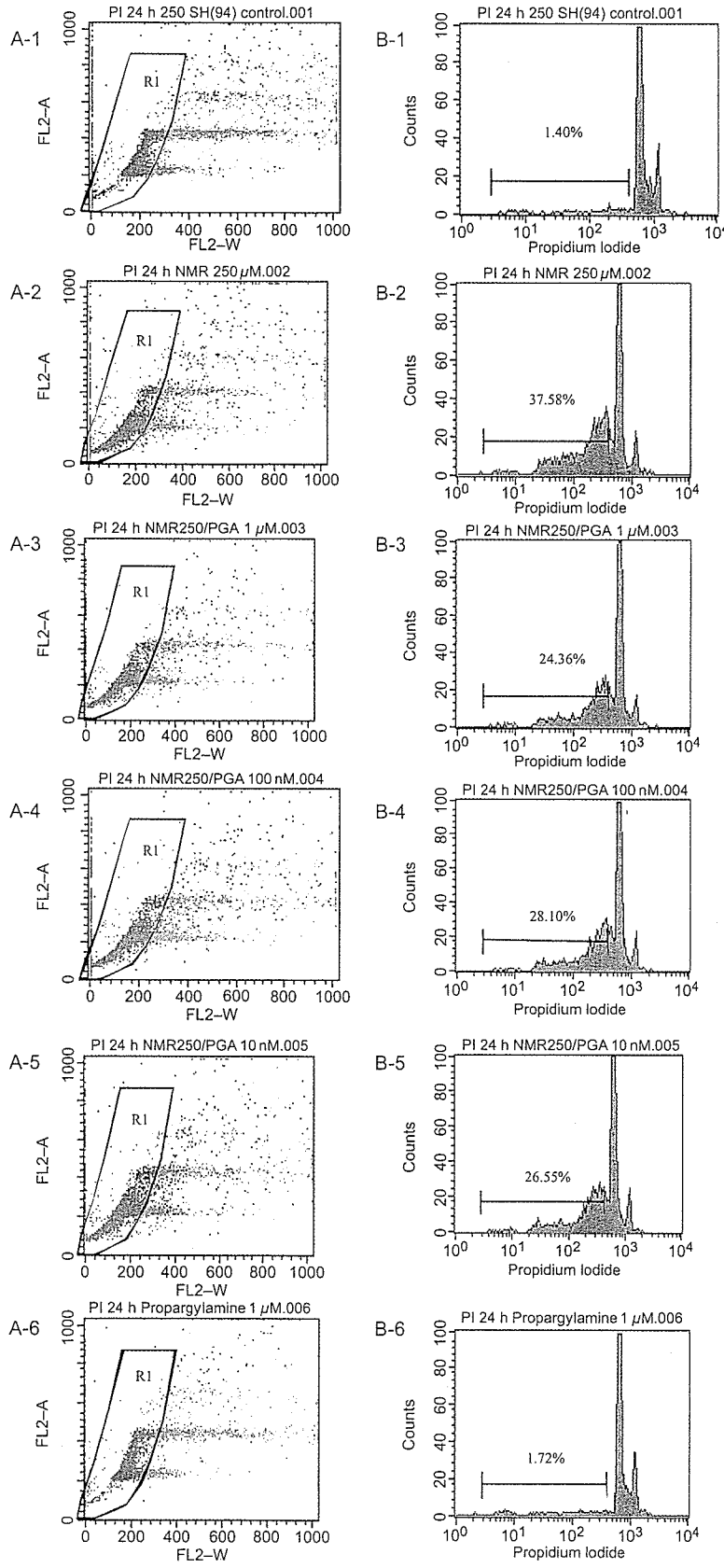


Fig. 3. Reversibility of MAO inhibition by *N*-propargylamine. MAO samples were treated with 100 μM *N*-propargylamine for 30 min at 37°C, then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, at 4°C overnight. The MAO activity was measure with 100 μM kynuramine. The inhibition of MAO activity by *N*-propargylamine before and after dialysis was expressed as % inhibition by comparison of the reduced activity with the activity of control. $^*p<0.05$; $^{**}p<0.01$


A-1
PI 24 h 250 SH(94) control.001
R1
FL2–A
FL2–W
B-1
PI 24 h 250 SH(94) control.001
1.40%
Counts
Propidium Iodide
A-2
PI 24 h NMR 250 μM.002
R1
FL2–A
FL2–W
B-2
PI 24 h NMR 250 μM.002
37.58%
Counts
Propidium Iodide
A-3
PI 24 h NMR250/PGA 1 μM.003
R1
FL2–A
FL2–W
B-3
PI 24 h NMR250/PGA 1 μM.003
24.36%
Counts
Propidium Iodide
A-4
PI 24 h NMR250/PGA 100 nM.004
R1
FL2–A
FL2–W
B-4
PI 24 h NMR250/PGA 100 nM.004
28.10%
Counts
Propidium Iodide
A-5
PI 24 h NMR250/PGA 10 nM.005
R1
FL2–A
FL2–W
B-5
PI 24 h NMR250/PGA 10 nM.005
26.55%
Counts
Propidium Iodide
A-6
PI 24 h Propargylamine 1 μM.006
R1
FL2–A
FL2–W
B-6
PI 24 h Propargylamine 1 μM.006
1.72%
Counts
Propidium Iodide

Results

Inhibition of MAO-A by N-propargylamine

Inhibition of MAO-A activity in mitochondria by *N*-propargylamine is shown in Fig. 1. *N*-Propargylamine reduced MAO activity significantly until at 10 μM, whereas *N*-methylpropargylamine inhibited the activity only at 1 mM, and propiolaldehyde did not even at 1 mM. Kinetic analyses indicate that *N*-propargylamine inhibited MAO-A in competition to the substrate (Fig. 2). The apparent K_i value of *N*-propargylamine was estimated to be 28.0 μM, whereas the values of the apparent K_m and maximal velocity, V_{max}, were 45.5 μM and 2.87 nmol/min/mg protein, respectively. The dialysis experiments showed that the inhibition of MAO by *N*-propargylamine was irreversible (Fig. 3), which is similar to those reported often for (−)deprenyl, rasagiline and other propargylamine derivatives. The activity of MAO-A treated with or without *N*-propargylamine was reduced further by the dialysis procedure, which may be due to the marked un-stability of MAO-A activity.

Anti-apoptotic function of N-propargylamine

Treatment of SH-SY5Y cells with 250 μM *N*M(*R*)Sal resulted in apoptosis of 37.6 ± 3.9% of cells, whereas necrotic cells were virtually not detected. Apoptotic cells were almost negligible in the control or *N*-propargylamine alone-treated cells. Figure 4 shows typical FACS profiles of the cells treated with *N*M(*R*)Sal with or without 1 μM–1 nM *N*-propargylamine. *N*-Propargylamine reduced the number of apoptotic cells to 63–70% of that of *N*M(*R*)Sal-treated cells. The potency of anti-apoptotic function was compared with that of *N*-methylpropargylamine, propiolaldehyde, and rasagiline in a similar manner. Rasagiline was found to be most potent to prevent apoptosis followed by *N*-propargylamine (Fig. 5), but *N*-methylpropargylamine and propiolaldehyde were virtually not effective.

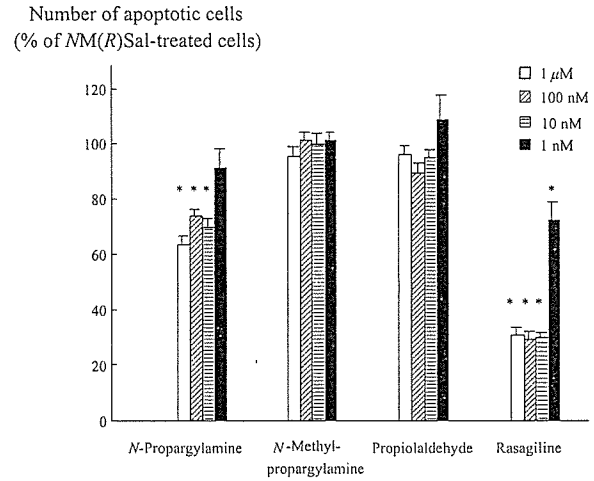


Fig. 5. The anti-apoptotic potency of *N*-propargylamine analogues and rasagiline. SH-SY5Y cells were treated with 1 μM, 100–1 nM propargylamine derivatives, and then with 250 μM *N*M(*R*)Sal overnight. The number of apoptotic cells was determined by FACS as sub G1 peak (Fig. 4) using PI as an indicator. The number of apoptotic cells was expressed as % of apoptotic cells after treated with 250 μM *N*M(*R*)Sal alone. The column and bar represent the mean and SD of three independent experiments measured in triplicate. $^*p < 0.01$ from cells treated with *N*M(*R*)Sal alone

Fig. 4. The effects of *N*-propargylamine against apoptosis induced by *N*M(*R*)Sal. SH-SY5Y cells were treated with 1 μM–10 nM *N*-propargylamine at 37°C for 30 min, then with 250 μM *N*M(*R*)Sal for 24 h. Apoptotic cells were quantitatively determined by FACS, after staining the cells with PI. Three-color flow cytometry was used to analyze the frequency of PI positive cells. **A** The gate to differentiate singlet from doublet cells was determined by FL-2 (PI)-A (Area) and FL-2 (PI)-W (Width) characteristics of PI-stained cells. **B** The frequency of PI-positive cells. The cells in subG1 peak were assessed to be apoptotic, and the number of apoptotic cells was represented as percent of the total, as shown in **B**. #1; control SH-SY5Y cells. #2; cells treated with 250 μM *N*M(*R*)Sal. #3, 4, and 5; cells pretreated with 1 μM, 100 nM, 10 nM *N*-propargylamine, then 250 μM *N*M(*R*)Sal. #6; cells treated with 1 μM *N*-propargylamine

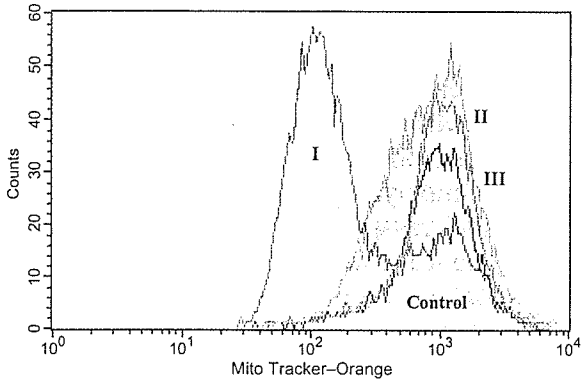


Fig. 6. The effects of *N*-propargylamine on ΔΨm decline induced by *N*M(*R*)Sal in mitochondria prepared from SH-SY5Y cells. Mitochondria were treated for 20 min without (I) or with 1 μM *N*-propargylamine (II), and then with 250 μM *N*M(*R*)Sal for 3 h at 37°C. III: Cells treated with 1 μM *N*-propargylamine alone. Mitochondria were gated by staining with MitoTracker Green, and the ΔΨm was quantitatively measured using MitoTracker Orange fluorescence

Stabilization of ΔΨm by N-propargylamine

*N*M(*R*)Sal at 250 μM induced ΔΨm decline in mitochondria isolated from SH-SY5Y cells, as shown by FACS MitoTracker Orange fluorescence representing ΔΨm (Fig. 6). Pre-

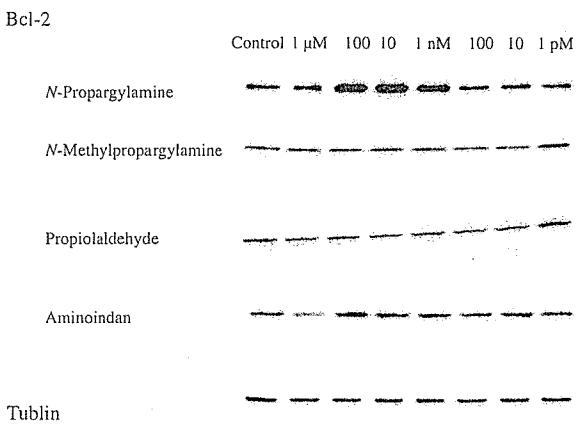


Fig. 7. The effect of *N*-propargylamine analogues and aminoindan on protein levels of Bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine analogues or aminoindan for 24 h, and Bcl-2 protein was quantified by Western blot analysis. Tublin in the cells was used as control

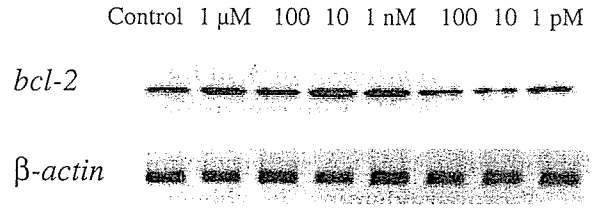


Fig. 8. The effect of *N*-propargylamine on mRNA levels of *bcl*-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine for 24 h, and mRNA levels were measured by RT-PCR method, as written in Materials and methods. β-*Actin* mRNA was used as control

treatment of the cells with 1 μM *N*-propargylamine markedly prevented the decline in ΔΨm, and the protective effect was confirmed at until 10 nM of *N*-propargylamine. On the other hand, *N*-methylpropargylamine and propiolaldehyde did not prevent the decline in ΔΨm (data, not shown).

Induction of anti-apoptotic Bcl-2 by N-propargylamine

Bcl-2 is known to prevent apoptosis and promote survival, through regulating mitochondrial permeability transition. Bcl-2 protein levels in SH-SY5Y cells treated with *N*-propargylamine were estimated by Western blot analysis and increased levels of Bcl-2 protein were observed at the concentrations of *N*-propargylamine employed (Fig. 7). On the other hand, neither *N*-methylpropargylamine nor propiolaldehyde affected Bcl-2 protein levels. Aminoindan, a hydrophobic part of rasagiline, did not increase Bcl-2. The effects of *N*-propargylamine on the mRNA level of *bcl-2* were examined by RT-PCR method. As shown in Fig. 8, *N*-propargylamine significantly increased mRNA level of *bcl-2* at 1 μM–1 nM.

Discussion

N-Propargylamine, *N*-methylpropargylamine and propiolaldehyde are metabolites of a relatively non-selective MAO inhibitor, pargyline [*N*-methyl-*N*-propargylbenzylamine]

(Shirota et al., 1979; DeMaster et al., 1981), even though such metabolites have never been reported for rasagiline at present. The results in this paper clearly demonstrate neuroprotective activity of *N*-propargylamine, whereas other two metabolites did not prevent apoptosis. The results may be relevant with the fact that the propargylamine moiety plays a key role in anti-apoptotic function of rasagiline and other propargylamine derivatives (Maruyama et al., 2003; Yogev-Falach et al., 2003). Rasagiline, the (*R*)-enantiomer of *N*-propargyl-1-aminoindan, has higher anti-apoptotic activity than its (*S*)-enantiomer, TV1022 (Maruyama et al., 2001c), and only the (*R*)-enantiomer of *N*-2HMP shows anti-apoptosis activity (Maruyama et al., 2001a). These results suggest that the stereo-chemical structure of the propargylamine moiety plays a decisive role in the neuroprotective function of complex propargylamines. The dependence of anti-apoptotic activity on the stereo-chemical configuration of propargylamine residue may explain the relative weak anti-apoptotic potency of free *N*-propargylamine as reported in this paper.

In addition, our previous results suggest that there may be a binding site in the outer membrane of mitochondria, which distinguishes the enantiomeric structure of propargylamines and activates the anti-apoptotic and pro-survival cascade (Maruyama et al., 2001b). The binding protein in mitochondria remains to be elucidated, but MAO may be one of the candidates, since rasagline (Youdim et al., 2001a) and (–)deprenyl (Magyar et al., 1998) are potent irreversible inhibitors of MAO-B, as a consequence of the formation of *N*(5)-flavocyanine adduct with the FAD moiety (Nagy and Salach, 1981). *N*-Propargylamine itself irreversibly inhibits MAO-A activity in a competitive way to substrate, as reported in this paper. However, it remains to clarify whether it binds with the FAD moiety covalently, as in the case with phenolic or indane propargylamine derivatives. The parallelism between the MAO-A inhibition and anti-apoptotic function was confirmed with *N*-propargylamine and related compounds, suggesting that MAO-A may be involved in the neuroprotective function of propargylamines. Indeed, our previous work has proved the anti-apoptotic function of rasagiline in SH-SY5Y cells, where only MAO-A is expressed. These results suggest that MAO-A may be involved in neuroprotection and MAO-B inhibition is not required for the anti-apoptotic function of propargylamine derivatives, as described in Introduction. However, the possibility that propargylamines bind MAO at the site other than the substrate-binding site cannot be excluded. In addition, it remains to be clarified how *N*-propargylamine and rasagiline interact PT pore components, such as voltage-dependent anion channel, adenine nucleotide translocator or peripheral benzodiazepine receptors, and stabilize $\Delta\Psi m$.

Rasagiline increases the expression of anti-apoptotic genes, including *bcl*-2 and *bcl-xL*, but not Bax and Bad (Akao et al., 2002a, b), and GDNF (Maruyama et al., 2004). The induction of pro-survival genes by rasagiline is mediated by nuclear NF-κB transcription factor and extracellular signal-regulated protein kinase (ERK) cascade (Maruyama et al., 2004). At the same time, the involvement of mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-kinase (Yogev-Falach et al., 2002, 2003; Bar-Am et al., 2004; Weinreb et al., 2004) was also suggested by the observation that a PKC inhibitor, GF109203X and an ERK inhibitor, PD98059, prevent the neuroprotective activity of *N*-propargylamine and rasagiline (Bar Am et al., 2004; Weinreb et al., 2004). At present, the intracellular mechanisms how *N*-propargylamine and complex propargylamines activate the intracellular signaling and the transcription factors remains to be enigmatic.

These results in this paper point out that free *N*-propargylamine itself shows the anti-apoptotic activity, in a similar way as

rasagiline and other propargylamines (see Youdim 2003, for a review). If *N*-propargylamine is identified as a metabolite of rasagiline in humans, it may be involved, at least partially, in neuroprotective function of rasagiline, as shown by recent clinical trial (Parkinson Study Group, 2004). Further studies on the metabolites of rasagiline and other propargylamines in humans will clarify the pharmacodynamics of these neuroprotective and anti-apoptotic agents in PD and other neurodegenerative disorders.

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Type A monoamine oxidase is the target of an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol, leading to apoptosis in SH-SY5Y cells

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Abstract

Mitochondrial monoamine oxidase (MAO) has been considered to be involved in neuronal degeneration either by increased oxidative stress or protection with the inhibitors of type B MAO (MAO-B). In this paper, the role of type A MAO (MAO-A) in apoptosis was studied using human neuroblastoma SH-SY5Y cells, where only MAO-A is expressed. An endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol, an MAO-A inhibitor, reduced membrane potential, $\Delta\Psi m$, in isolated mitochondria, and induced apoptosis in the cells, which 5-hydroxytryptamine, an MAO-A substrate, prevented. In contrast, β-phenylethylamine, an MAO-B substrate, did not suppress the $\Delta\Psi m$ decline by *N*-methyl(*R*)salsolinol. The binding of *N*-methyl(*R*)salsolinol to mitochondria was inhibited by clorgyline, a MOA-A inhibitor, but not by (–)deprenyl, an MAO-B inhibitor. RNA interference targeting MAO-A significantly reduced the binding of *N*-methyl(*R*)salsolinol with simultaneous reduction in the MAO activity. To examine the intervention of MAO-B in the apoptotic process, human MAO-B was transfected to SH-SY5Y cells, but the sensitivity to *N*-methyl(*R*)salsolinol was not affected, even although the activity and protein of MAO increased markedly. These results demonstrate a novel function of MAO-A in the binding of neurotoxins and the induction of apoptosis, which may account for neuronal cell death in neurodegenerative disorders, including Parkinson's disease.

Keywords: apoptosis, dopamine neuron, mitochondria, neurotoxin, Parkinson's disease, RNA interference.

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Apoptosis is a common type of cell death in neurodegenerative disorders, including Parkinson's disease (PD) and Alzheimer's disease. Understanding of the intracellular mechanism of apoptosis has been advanced markedly and mitochondria initiate apoptotic signalling in an intrinsic pathway to cell death (Thompson, 1995). Previously, we found that a dopamine-derived endogenous neurotoxin, *N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *N*M(*R*)Sal] might be involved in the pathogenesis of PD, as shown by analyses of clinical samples (Maruyama *et al.* 1996; Naoi *et al.* 1998). *N*M(*R*)Sal induces apoptotic cell death in dopamine neurons in the substantia nigra of rats (Naoi *et al.* 1996) and human dopaminergic neuroblastoma SH-SY5Y cells (Maruyama *et al.* 1997). Apoptosis induced by *N*M(*R*)Sal is initiated by decline in mitochondrial membrane potential, $\Delta\Psi m$, sequentially followed by release of cytochrome *c*, activation of caspase 3, nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12], and fragmentation of nuclear DNA (Maruyama *et al.* 2001a; Akao *et al.* 2002a,b;

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Abbreviations used: β-PEA, β-phenylethylamine; DMEM, Dulbecco's modified Eagle's medium; $\Delta\Psi m$, mitochondrial membrane potential; FACS, fluorescence-augmented flow cytometry; FCS, fetal calf serum; 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; *N*M(*R*)Sal, and *N*M(*S*)Sal, *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; RNS, reactive nitrogen species; ROS, reactive oxygen species; siRNA, small interfering RNA.

Naoi *et al.* 2002a,b). Mitochondrial permeability transition (mPT) is an increase in the permeability of the inner mitochondrial membrane to solutes, by opening of the mPT pore, a large proteinaceous pore spanning the outer and inner membrane of mitochondria (Crompton 1999). The mPT pore forms a functional microcompartment with voltage-dependent anion channel in the outer membrane, adenine nucleotide translocator in the inner membrane, and hexokinase at the contact site; however, the exact composition has not yet been fully clarified. The (*R*)-enantiomer of *N*-methylsalsolinol (*N*MSal), but not (*S*)-, induces mPT in SH-SY5Y cells (Maruyama *et al.* 2001b) and in isolated mitochondria (Akao *et al.* 2002a), suggesting the occurrence of a selective binding site of *N*M(*R*)Sal in the mitochondrial membrane.

Previously we reported that *N*MSal inhibits monoamine oxidase [MAO, monoamine: oxygen oxidoreducatse (deaminating), EC 1.4.3.4] (Minami *et al.* 1993). 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. In human brain, MAO levels increase 2–3-fold in an age-dependent way, resulting in increased oxidative stress, which may induce vulnerability of the brain to age-dependent neurodegenerative disorders, such as PD. Increased influx of reactive oxygen and nitrogen species (ROS, RNS) in mitochondria inhibits complex I (Ben-Shachar *et al.* 1995), which further increases oxidative stress and activates apoptotic signalling (Cohen *et al.* 1997; Bianchi *et al.* 2003; Shamoto-Nagai *et al.* 2003).

MAO is present in two isoenzymes, type A and type B (MAO-A, MAO-B), which 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 (Lim *et al.* 1994; Cases *et al.* 1995; Shih *et al.* 1999). In contrast, 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.* 2005). It suggests that MAO may be involved in the regulation of apoptotic signalling, even although the neuroprotective function may not necessarily depend on the inhibition of MAO-B activity (Maruyama *et al.* 2001c). However, it has never been reported whether MAO is directly involved in mPT, or MAO itself is a component of the mPT pore.

In this paper, the role of MAO in the apoptotic cascade was studied by use of *N*M(*R*)Sal in the wild type of SH-SY5Y (wild SH) cells containing only MAO-A. To confirm the role of MAO-A in the apoptotic cascade, the effects of RNA interference (RNAi) targeting MAO was examined by use of small interfering RNA (siRNA) to silence MAO-A in SH-SY5Y cells. In addition, the involvement of MAO-B was examined in SH-SY5Y cells transfected with cDNA of human MAO-B (MAO-B-SH). The role of MAO isoenzymes in neuronal cell death is discussed in relation to the activation of apoptotic signalling in neurodegenerative disorders including PD.

Materials and methods

Materials

*N*M(*R*)Sal was synthesized according to Teitel *et al.* (1972). Kynuramine and 4-quinolinol were purchased from Sigma (St Louis, MO, USA); propidium iodide (PI), MitoTracker Orange and Green from Molecular Probes (Eugene, OR, USA); 5-hydroxytryptamine (5-HT, serotonin) from Merck (Darmstadt, Germany). Clorgyline, an MAO-A inhibitor, and rasagiline and (–)deprenyl (selegiline), MAO-B inhibitors, were kindly donated by May and Baker (Dagenham, UK), 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).

RNAi of MAO-A in SH-SY5Y cells

To reduce MAO in mitochondria, siRNA targeting MAO-A mRNA (Sc-35874) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). To silence MAO-A, SH-SY5Y cells were seeded in a 6-well plate at a concentration of 2×10^5/well (40–50% confluence) 1 day before the transfection. 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) according to the manufacturer's Lipofection protocol. The transfection efficiency was evaluated by the transfection of the cells with a duplex siRNA-FITC. The expression of interferon-induced OAS-1 mRNA was studied by RT–PCR using 5′-CGATGTGCTGCCTGCCTTTGATGC-3′ (sense) and 5′-GTCTCCACCACCCAAGTTTCCTGT-3′ (antisense) as primers. 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*M(*R*)Sal 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 TNGGQERKFVGGSGQ, 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 (MAO-B-SH), we used a pIRES1neo eukaryotic expression vector (Invitrogen, Carlsbad, CA, USA). For construction of pIRES1-neo-MAO-B, the full-length human MAO-B gene included in pECE vector (Lan *et al.* 1989) was digested with *Hin*dIII and then inserted into the pIRES1neo vector. SH-SY5Y cells were transfected with pIRES1neo or pIRES1neo-MAO-B by using cationic liposomes (Lipofectamine) according to the manufacturer's Lipofection protocol (Gibco BRL, Rockville, MD, USA). Selection was started 2 days after the transfection using the culture medium containing 0.7 mg/mL geneticin (Gibco BRL). Individual clones were isolated and characterized by RT–PCR, as described previously (Akao *et al.* 2002a). In brief, the total cellular RNA of the transfected and original cells was isolated by the phenol/guanidium thiocyanate method with Dnase I treatment. By reverse transcription of 2 μg of total RNA, cDNAs were obtained, and the respective cDNA region was amplified by PCR. PCR primers were as follow: for MAO-B (sense) 5′-GGACCAACCCAGAATCG-TAT-3′ and (antisense) 5′-CAACTGGAGCTTCTTCTCCA-3′. This primer can specifically amplify the 791-bp DNA fragments of MAO-B. β-Actin cDNA was used for an internal standard. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). PCR products were analyzed by electrophoresis on 2% agarose gels. 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 an MAO-A sample, and those from MAO-B-SH cells were pretreated with 1 μM clorgyline at 37°C for 20 min and used as an MAO-B sample. Kinetics for MAO-A and MAO–B activities and the effects of the (*R*)- and (*S*)-enantiomer of *N*MSal were studied with eight graded concentrations of kynuramine. Values of the apparent Michaelis constant, K_m, and the inhibition constant, K_i, were calculated by double-reciprocal plot of the reaction velocity against the substrate concentrations. Protein concentration was determined according to Bradford (1976).

Assay for the binding of *N*M(*R*)Sal to mitochondria

Mitochondria were prepared and suspended in 100 μL of 10 mM Tris-HCl buffer, pH 6.0, and incubated with 10–100 μM *N*M(*R*)Sal 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*M(*R*)Sal on $\Delta\Psi m$ 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 were used as fluorescent indicators. 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*M(*R*)Sal 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 staining with 100 nM MitoTracker Orange and Green for 30 min at 37°C, 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.

Assessment of apoptosis induced by *N*M(*R*)Sal

Apoptosis was quantitatively measured by FACS. The cells cultured in 6-well poly-L-lysine-coated culture flasks were incubated in DMEM with 100–500 μM *N*M(*R*)Sal 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. To differentiate singlet cells from doublet ones, FL-2A (area) and FL-2-W (width) parameters of PI fluorescence pulse (FL-2 at 560–640 nm, excited at 488 nm) were used. 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). The effects of 5-HT on cell death were also examined after being cultured with *N*M(*R*)Sal or clorgyline for 24 h in the presence of 100 μM-1 mM 5-HT.

Statistics

Experiments were repeated 3–4 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 *N*M(*R*)Sal to mitochondria and the effects of MAO-A gene silencing by siRNA in SH-SY5Y cells

The binding of *N*M(*R*)Sal to mitochondria prepared from wild type of SH-SY5Y (wild SH) cells was kinetically studied and the binding kinetics followed to the Michaelis–Menten equation, as shown in Fig. 1(a). The value of the

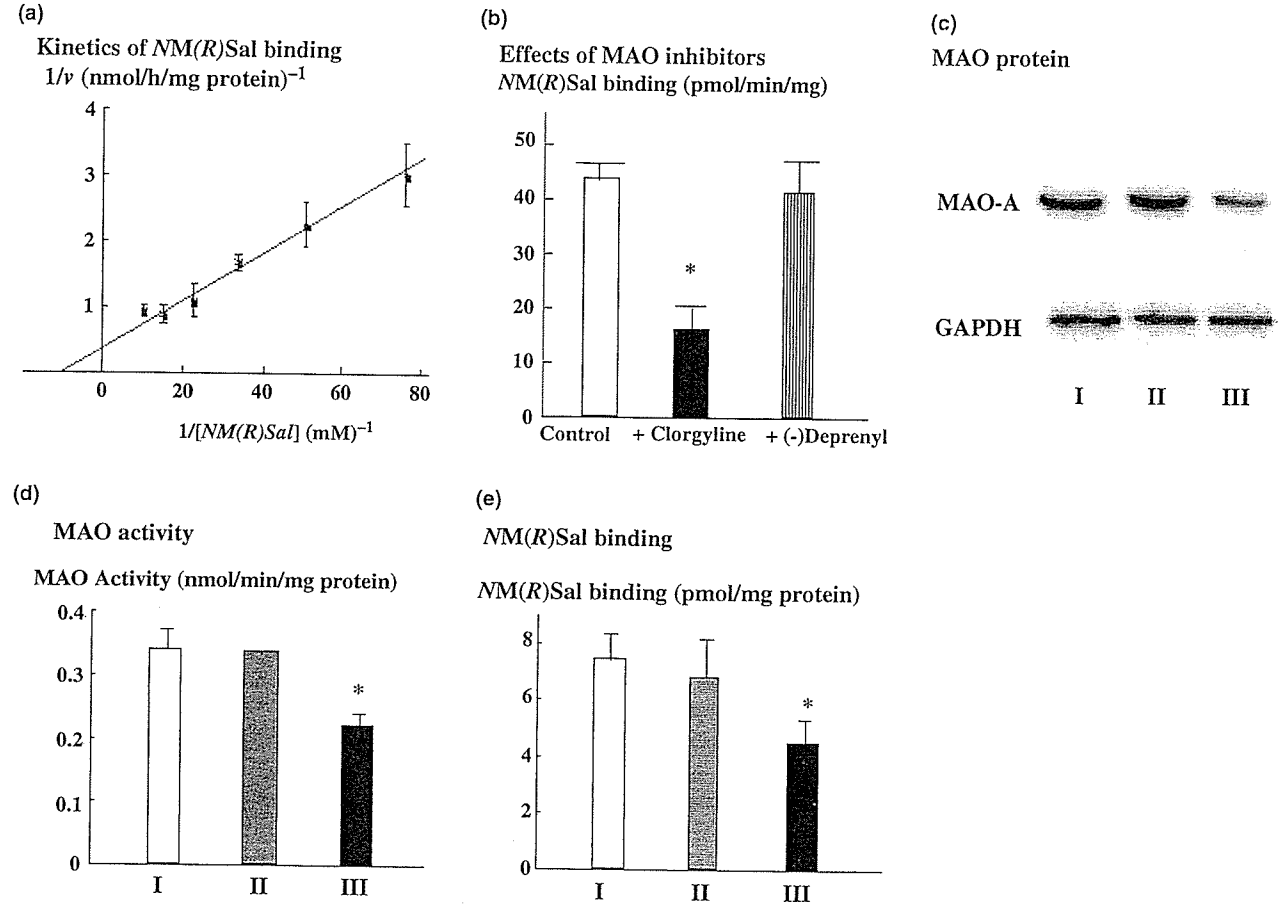


Fig. 1 Binding of NM(*R*)Sal to mitochondria and the effects of RNAi targeting MAO in SH-SY5Y cells. (a) NM(*R*)Sal binding to mitochondria prepared from wild SH cells. NM(*R*)Sal was quantified by HPLC-ECD, as described in Materials and methods. The reciprocal of binding velocity was plotted against that of NM(*R*)Sal concentration. The spot and bar represent the mean and SD of triplicate measurements. (b) Effects of clorgyline and (–)deprenyl on the NM(*R*)Sal binding to mitochondria. Mitochondria were treated with 1 μM MAO inhibitors for 20 min at 37°C, then incubated with 10 μM NM(*R*)Sal for 1 h at 4°C. The column and bar represent the mean and SD of triplicate measurement of two experiments. (c, d, e) SH-SY5Y cells were transfected with siRNA, and mitochondria were prepared from (I) control (II) non-specific siRNA transfected cells, and (III) cells transfected with siRNA targeting MAO-A. (c) MAO protein detected by western blot analysis. GAPDH was used as control. (d) MAO activity measured fluorometrically by use of 100 μM kynuramine as a substrate. (e) NM(*R*)Sal binding 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.

apparent K_m and V_{max} were obtained as 80 ± 15 μM and 2.7 ± 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. As shown in Fig. 1(b), clorgyline reduced NM(*R*)Sal binding significantly, but (–)deprenyl did not affect the binding.

In order to confirm whether NM(*R*)Sal binds to MAO-A in mitochondria, MAO-A expression was inactivated using RNAi. The transfection efficiency in the SH-SY5Y cell was more than 90%, as determined from the control siRNA-fluorescence (data not shown). Western blot analysis of MAO in the siRNA-transfected cells showed that MAO-A 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 (Fig. 1c). 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 (Fig. 1d). In non-specific siRNA-transfected cells, the MAO activity was the same as in control, 0.34 ± 0.01 nmol/min/mg protein. Figure 1(e) shows that RNAi targeting MAO-A markedly reduced NM(*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.

Transfection of human MAO-B DNA into SH-SY5Y cells

To specify the role of MAO-A and MAO-B in the binding of NM(*R*)Sal and the induction of apoptosis, SH-SY5Y

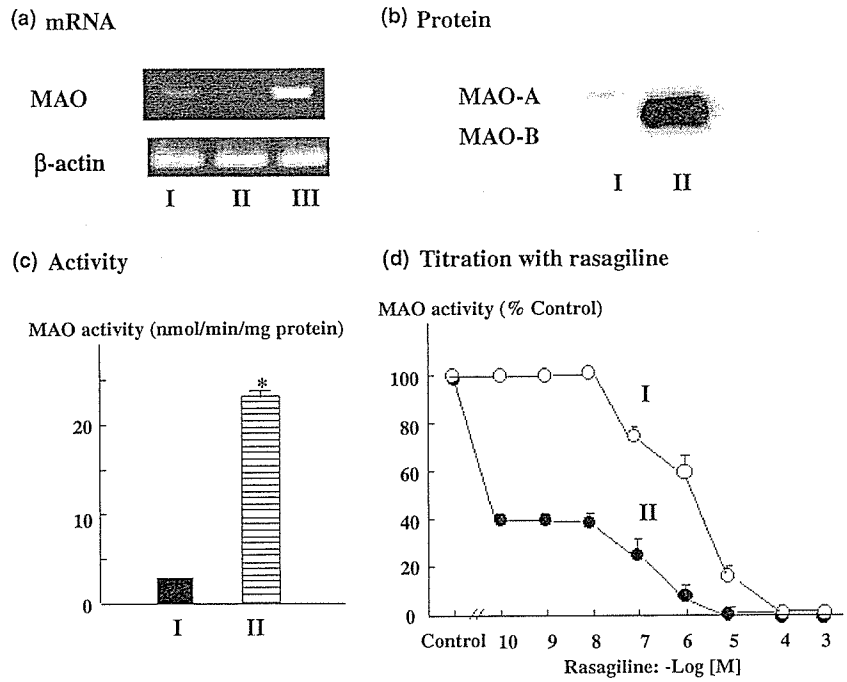


Fig. 2 Establishment of SH-SY5Y cells transfected with human MAO-B. SH-SY5Y cells were transfected with MAO-B as described in Materials and methods. (a) mRNA isolated from wild SH cells (I), cells transfected with IRES vector alone (II), and with full-length MAO-B cDNA (III). β-Actin was used as control. (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 MAO-B. The molecular weight of MAO-A and MAO-B were assessed to be 60 and 57 kDa, respectively. (c) MAO enzyme activity in mitochondria from wild SH (I) and from MAO-B-SH cells (II). The column and bar represent the mean and SD. $p < 0.01$. (d) Effects of rasagiline, an 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.

cells transfected with human MAO-B DNA (MAO-B-SH) were prepared from wild type of cells expressing only MAO-A (wild SH). Figure 2(a) shows the expression of mRNA in MAO-B-SH cells. 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). When activity was measured with 100 μM kynuramine as a substrate, MAO activity in mitochondria isolated from MAO-B-SH cells increased significantly from 2.82 ± 0.18 nmol/min/mg protein to 22.9 ± 0.93 in those from wild SH cells (Fig. 2c). The sensitivity to rasagiline, an irreversible inhibitor of MAO-B, increased by MAO-B transfection as shown by the inhibitor concentration-activity studies (Fig. 2d), indicating that increased MAO activity was as a result of transfected MAO-B. The values of the apparent Michaelis constant, K_m, and the maximal velocity, V_{max}, of MAO-A and MAO-B, are summarized in Table 1.

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.

Table 1 Kinetic parameters of MAO-A in wild SH cells and MAO-B in MAO-B-SH cells

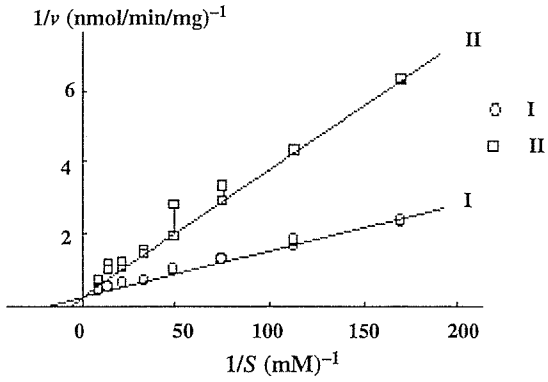
MAO-isomer	K_m value (μM)	V_{max} (nmol/min/mg protein)
Type A	50.9 ± 8.9	3.8 ± 0.9
Type B	109.0 ± 15.6	25.8 ± 5.2

Mitochondria were prepared from wild SH and MAO-B-SH cells for an MAO-A and MAO-B sample, respectively. To measure MAO-B activity, MAO-B sample was pretreated at 37°C for 20 min with 1 μM clorgyline to deplete MAO-A activity. MAO activities were measured at eight different concentrations of kynuramine in triplicate measurements. The values represent the mean and SD of four independent experiments.

Binding site of *N*M(*R*)Sal in MAO-A

The binding site of *N*M(*R*)Sal in MAO-A was examined by the kinetic analysis of MAO activity (Fig. 3). *N*M(*R*)Sal inhibited MAO-A activity in competition to the substrate, and the apparent inhibitor constant, K_i, value was

MAO-A



MAO-B

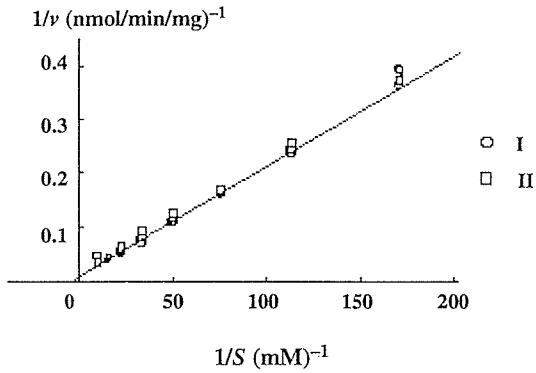


Fig. 3 Kinetic analysis of effects of *N*M(*R*)Sal on activity of MAO-A and MAO-B. To measure MAO-A and MAO-B activity, mitochondria were isolated, respectively, from wild SH or MAO-B-SH cells, the mitochondria from which were pretreated with 1 μM clorgyline at 37°C for 20 min. The amounts of MAO samples used for the kinetical studies were 38 and 52 μg for MAO-A and MAO-B, respectively. The effects of *N*MSal were studied by use of eight graded concentrations of kynuramine in the absence (I) or presence of 100 μM of *N*M(*R*)Sal (II). The reciprocal of the reaction velocity was plotted against that of the substrate concentration according to Lineweaver–Burk. Each spot represents the value measured in duplicate.

59.9 ± 5.4 μM. In contrast, *N*M(*R*)Sal did not inhibit MAO-B activity up to 250 μM.

*N*M(*R*)Sal induced ΔΨm decline in mitochondria prepared from wild SH cells and apoptosis in SH cells

The role of MAO-A in apoptosis by *N*M(*R*)Sal was shown by competition with 5-HT, a substrate of MAO-A. 5-HT prevented *N*M(*R*)Sal-induced ΔΨm decline in isolated mitochondria and apoptosis in wild SH cells (Figs 4a and b). The number of apoptotic cells after *N*M(*R*)Sal treatment was 36.8% of the total and reduced to 5.34% by addition of 5-HT, which was almost the same as in control cells or cells treated with 5-HT alone; 5.43 and 4.56%.

MAO-B was not involved in ΔΨm decline by *N*M(*R*)Sal

Involvement of MAO-A and MAO-B in *N*M(*R*)Sal-induced reduction of ΔΨm was examined using mitochondria prepared from wild SH and MAO-B-SH cells. Figure 5(a 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. The fluorescence intensity of MitoTracker Orange representing ΔΨm reduced to 71.1% of control in mitochondria containing MAO-A, while ΔΨm in mitochondria from MAO-B-SH cells was not affected; 98.9% of control. β-PEA, an MAO-B substrate, did not prevent *N*M(*R*)Sal-induced ΔΨm reduction in mitochondria prepared from wild SH cells (Fig. 5c). In addition, the antibody against MAO reduced ΔΨm in a dose-dependent way to 55.6 and 92.6% of control at the concentration of 100- and 500-fold dilution (Fig. 5d).

Discussion

This paper reports the direct involvement of MAO-A in the mPT and the activation of the mitochondrial apoptosis system by an endogenous neurotoxin, *N*M(*R*)Sal. All the papers hitherto discussed the role of MAO in neuronal degeneration mainly in relation to the enzymatic oxidation of monoamines and the induction of oxidative stress, as this addressed an important pathogenic issue of age-related neurodegenerative disorders. Regarding the role of MAO-A in apoptosis, there have been only a few papers. Higher MAO-A levels were expressed in apoptosis induced by depletion of nerve growth factor in PC12 cells through the p38 mitogen-activated protein kinase signal pathway, and increased ROS generation was considered to potentiate apoptosis (De Zutter and Davis 2001). Our results show that the binding of the neurotoxin to MAO-A activates the mitochondrial apoptotic system. However, 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. In addition, clorgyline and pargyline were reported to prevent the mPT induced by tyramine, a substrate for MAO-A and MAO-B, in mitochondria isolated from rat liver (Marcocci *et al.* 2002). The protective function of MAO-A inhibitors was suggested to be as a result of the maintenance of mitochondrial homeostasis by a direct effect on mPT pore in addition to the inhibition of monoamine oxidation and ROS generation, but the detailed mechanisms were not presented. These results suggest the participation of MAO-A in the regulation of mitochondrial apoptotic signalling, either in a promoting or suppressing way. In contrast, MAO-B is commonly considered to play a major role in the cell death of PD, as in human basal ganglia MAO-B is more abundant than MAO-A and accounts for about 80% of the total MAO activity (O'Caroll *et al.* 1988). In addition, inhibitors of MAO-B, rasagiline and (–)deprenyl, prevent cell death in

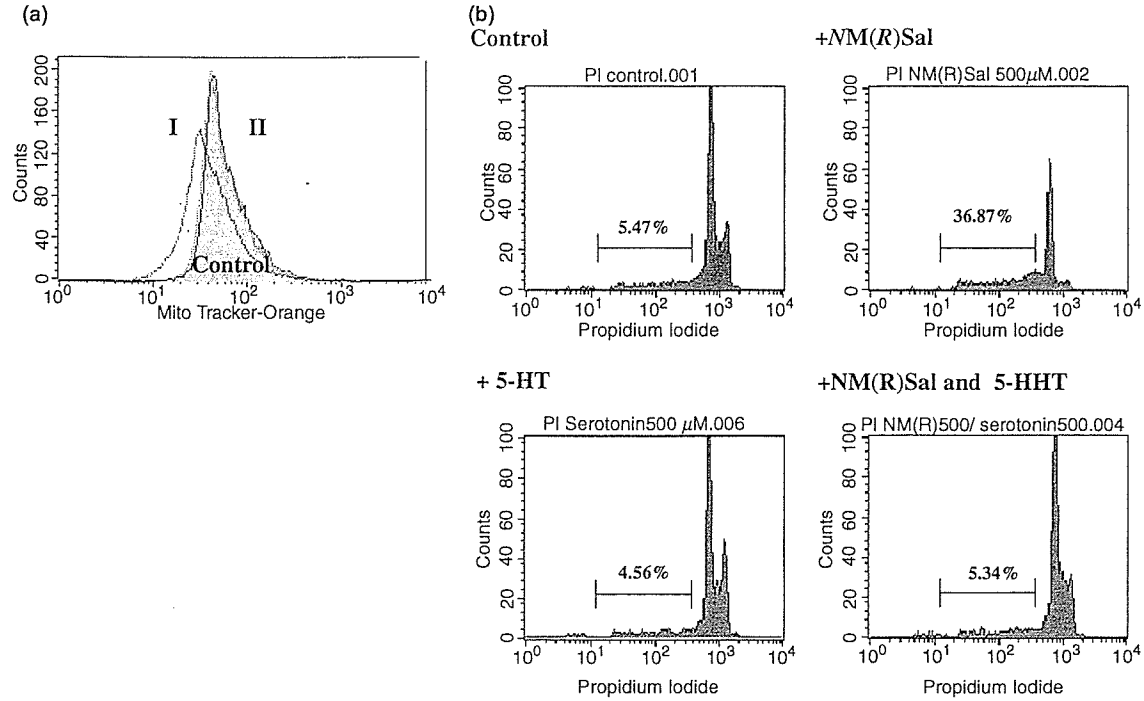


Fig. 4 Effects of 5-HT on ΔΨm decline in isolated mitochondria and apoptosis in wild SH cells. (a) Mitochondria isolated from wild SH cells were incubated with 100 μM *N*M(*R*)Sal in the absence (I) and presence of 100 μM 5-HT (II). ΔΨm was measured by FACS after staining with MitoTracker Orange and Green. (b) Wild SH cells were incubated with 500 μM *N*M(*R*)Sal overnight and apoptotic cells were quantified by FACS after staining with PI. 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). The cells with lower DNA content showing less PI staining than G1 were defined to be apoptotic. The number in Fig. 5(b) represents the number of apoptotic cells in the total (%).

in vivo and *in vitro* models of neuronal cell death. However, it remains to be clarified whether MAO-B itself may mediate the apoptotic or neuroprotective processing.

Our results confirm a novel direct involvement of MAO-A in mitochondrial apoptotic mechanism, in addition to generation of ROS. RNAi targeting MAO-A reduced the *N*M(*R*)Sal binding to mitochondria, to almost the same degree as the reduction of the protein amount and enzymatic activity of MAO. Kinetic studies on the inhibition of MAO-A activity by *N*M(*R*)Sal suggest its binding to the substrate binding site in MAO, as shown by competition with 5-HT, an MAO-A substrate, but not β-PEA, an MAO-B substrate. The binding of *N*M(*R*)Sal to MAO initiates the activation of apoptotic signalling, as shown in this paper and also proposed in our previous study (Naoi *et al.* 2002a). It is supported further by the fact that overexpression of MAO-B in SH-SY5Y cells did not increase, but rather suppressed the decline in ΔΨm and following apoptosis by *N*M(*R*)Sal. In addition, the results of clorgyline and (–)deprenyl on *N*M(*R*)Sal binding support further the role of MAO-A in apoptosis induced by this neurotoxin. As reported previously, *N*M(*R*)Sal is not oxidized by MAO, but by another amine oxidase (Naoi *et al.* 1995), and does not produce, rather scavenges, hydroxyl radical (Maruyama *et al.* 1995), suggesting that ROS-RNS may not be involved in the ΔΨm decline and apoptosis by *N*M(*R*)Sal. 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 ΔΨm by anti-MAO antibody suggests the direct interference of MAO with the mPT pore. However, at present it requires further studies to clarify the mechanism behind the interaction of MAO with other components of the mPT pore.

The direct involvement of MAO-A in the apoptotic mechanism was confirmed in cell death induced by a dopaminergic neurotoxin, *N*M(*R*)Sal, and similar, but less marked, effects on ΔΨm were observed also with MPP^+, an oxidation product of MPTP.. These results suggest that selective MAO-A inhibitors, *N*M(*R*)Sal, its oxidation product, 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion and MPP^+, might activate mitochondrial apoptotic signalling through binding to MAO-A (Naoi *et al.* 1994), 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 with the silenced MAO-A gene, and future studies by neurochemical and behavioural analyses may bring new insights to the function of MAO-A in neurodegeneratve and psychiatric disorders, such as bipolar emotional disorders (Lim *et al.* 1994) and X-linked mental retardation (Brunner *et al.* 1993).

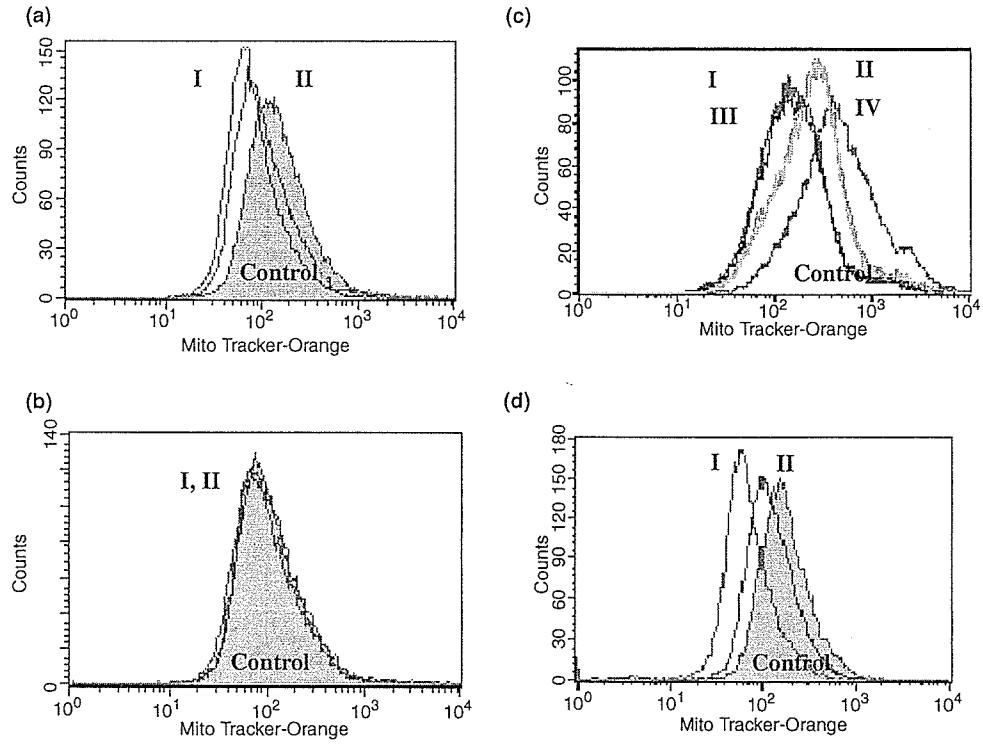


Fig. 5 *N*M(*R*)Sal reduced ΔΨm in isolated mitochondria. (a, b) Mitochondria were prepared from wild SH (a) and MAO-B-SH cells (b), and incubated with 500 μM (I) and 250 μM *N*M(*R*)Sal (II) for 3 h. (c) Mitochondria from wild SH cells were treated with 500 μM (I) or 250 μM *N*M(*R*)Sal (II) without β-PEA, or in the presence of 100 μM β-PEA (III) and (IV). (d) Mitochondria were prepared from wild SH cells and treated with the anti-MAO antibody diluted by 100-fold (I) and 500-fold (II) at 5°C for 30 min. ΔΨm was visualized with MitoTracker Orange and measured by FACS in mitochondrial fraction gated with MitoTracker Green.

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