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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

Hong Yi,* Yukihiro Akao,* Wakako Maruyama,† Kavin Chen,‡ Jean Shih‡ and Makoto Naoi*

**Department of Neurosciences, Gifu International Institute of Biotechnology, Kakamigahara, Gifu, Japan*

†Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan

‡Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

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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Address correspondence and reprint requests to 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

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′-CG-ATGTGCTGCCTGCCTTTGATGC-3′ (sense) and 5′-GTCTCCAC-CACCCAAGTTTCCTGT-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 ΔΨm

The effects of *N*M(*R*)Sal 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 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

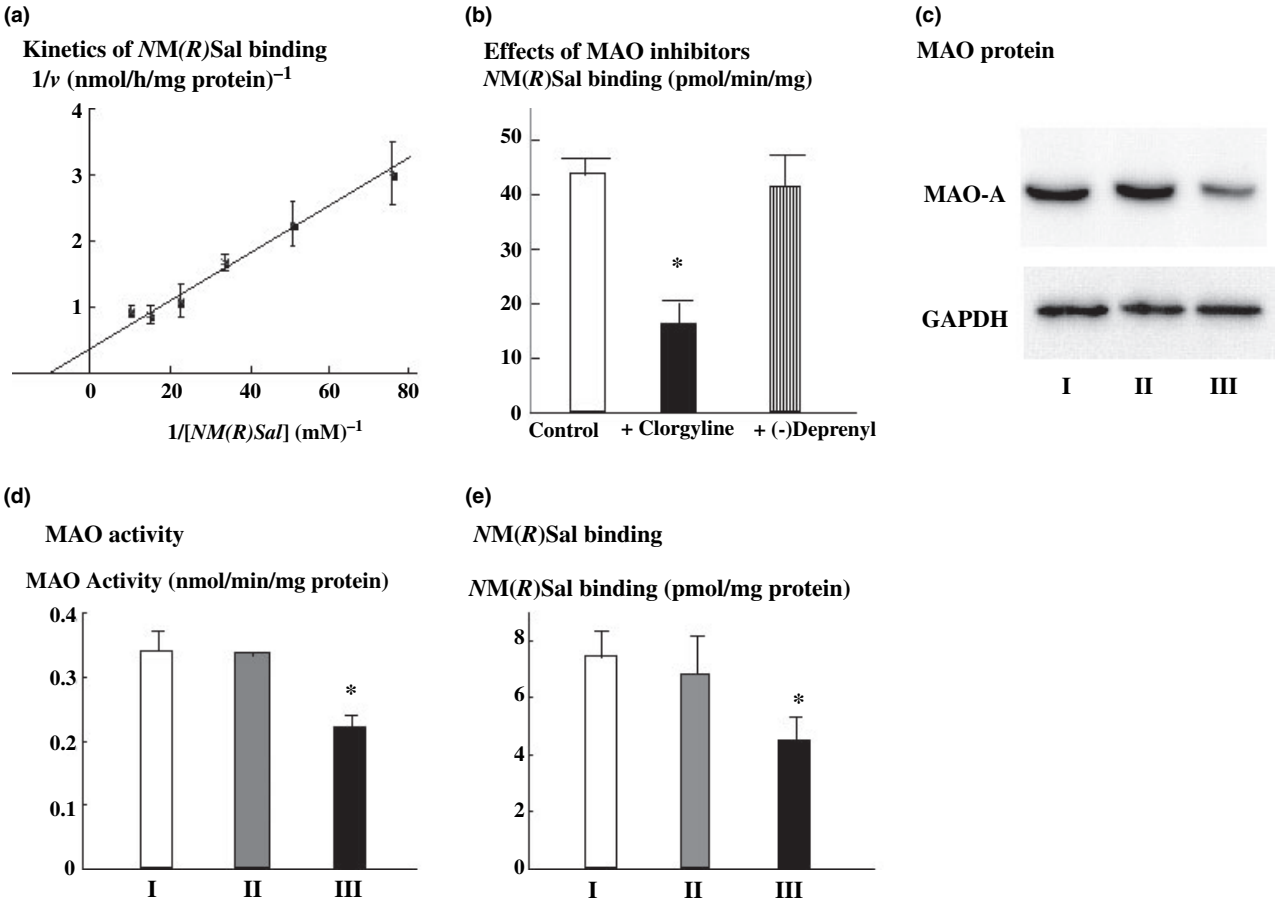

Fig. 1 Binding of *N*M(*R*)Sal to mitochondria and the effects of RNAi targeting MAO in SH-SY5Y cells. (a) *N*M(*R*)Sal binding to mitochondria prepared from wild SH cells. *N*M(*R*)Sal was quantified by HPLC-ECD, as described in Materials and methods. The reciprocal of binding velocity was plotted against that of *N*M(*R*)Sal concentration. The spot and bar represent the mean and SD of triplicate measurements. (b) 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 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) *N*M(*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 *N*M(*R*)Sal binding significantly, but (–)deprenyl did not affect the binding.

In order to confirm whether *N*M(*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 *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.

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

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

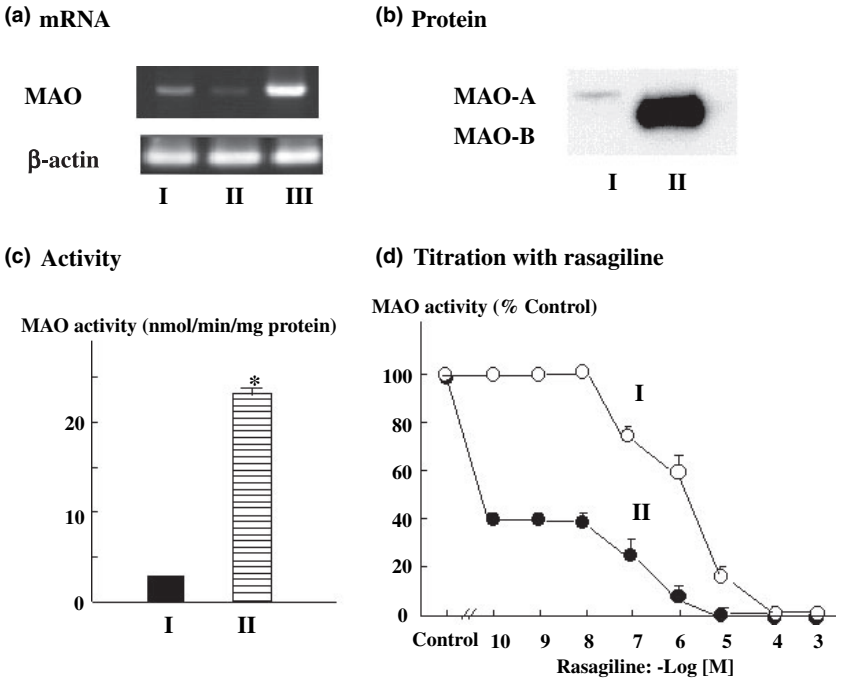

Fig. 2 Establishment of SH-SY5Y cells transfected with human MAO-B. SH-SY5Y cells were transfected with MAO-B as described in Materials and methods. (a) mRNA isolated from wild SH cells (I), cells transfected with IRES vector alone (II), and with full-length MAO-B cDNA (III). β-Actin was used as control. (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

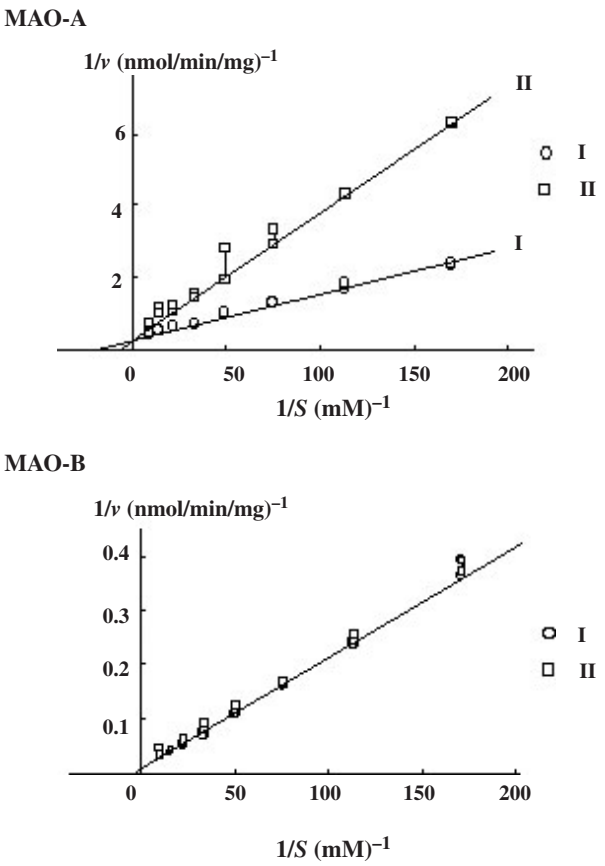

Fig. 3 Kinetic analysis of effects of *N*M(*R*)Sal on activity of MAO-A and MAO-B. To measure MAO-A and MAO-B activity, mitochondria were isolated, respectively, from wild SH or MAO-B-SH cells, the mitochondria from which were pretreated with 1 μM clorgyline at 37°C for 20 min. The amounts of MAO samples 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

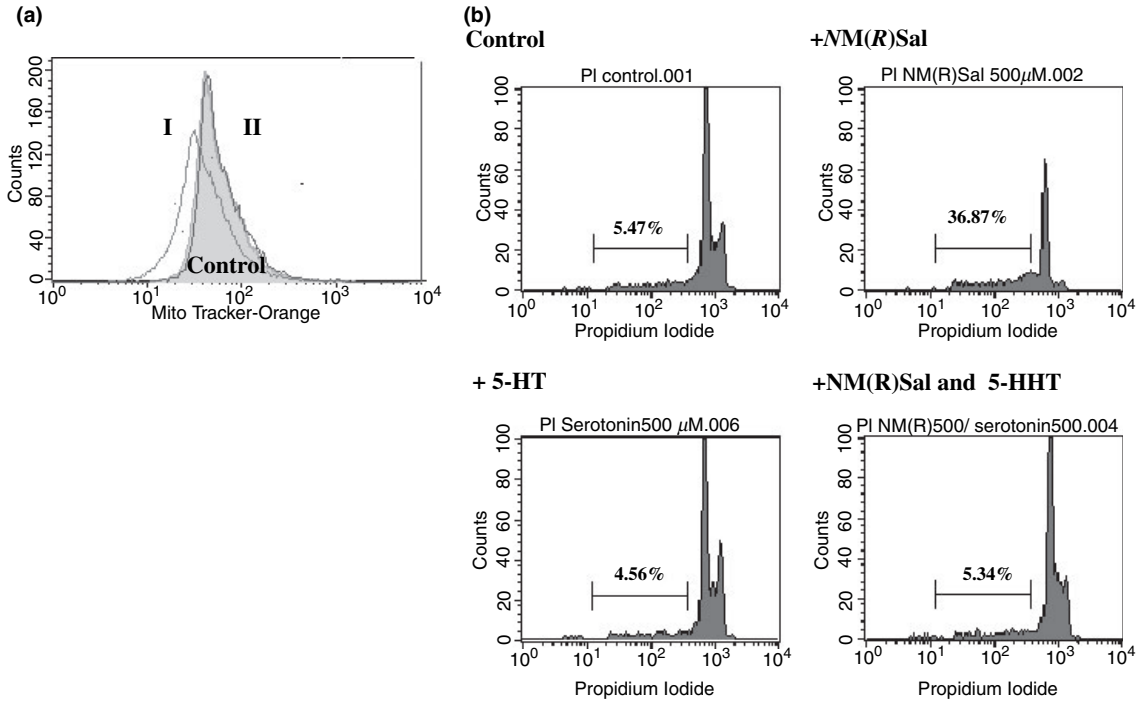

Fig. 4 Effects of 5-HT on ΔΨm decline in isolated mitochondria and apoptosis in wild SH cells. (a) Mitochondria isolated from wild SH cells were incubated with 100 μM *N*M(*R*)Sal in the absence (I) and presence of 100 μM 5-HT (II). ΔΨm was measured by FACS after staining with MitoTracker Orange and 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).

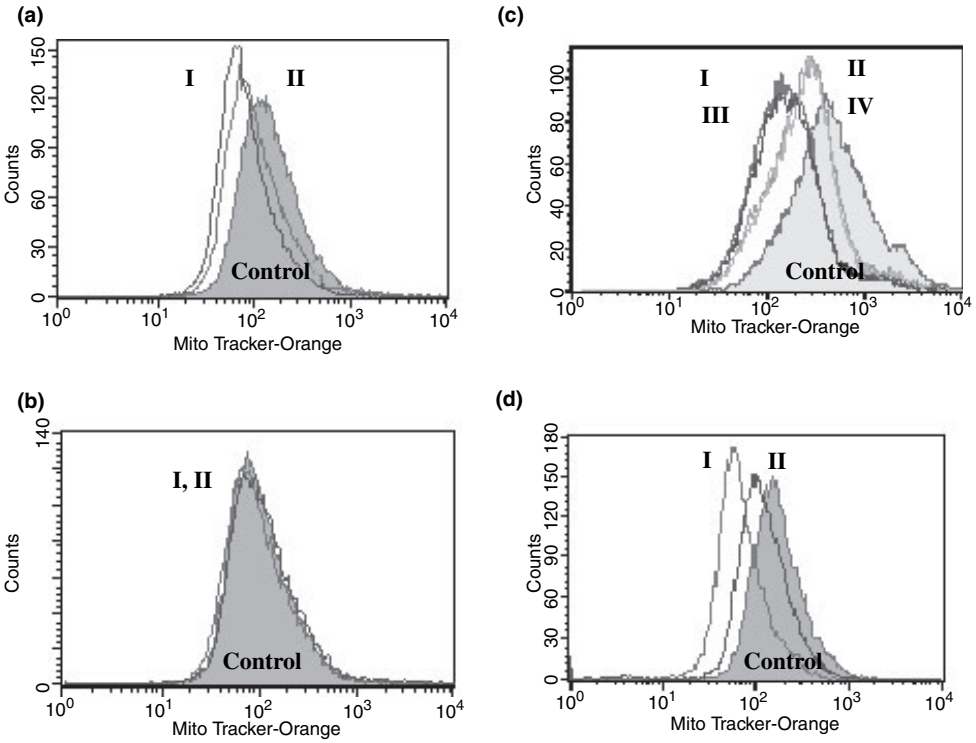

Fig. 5 *N*M(*R*)Sal reduced ΔΨm in isolated mitochondria. (a, b) Mitochondria were prepared from wild SH (a) and MAO-B-SH cells (b), and incubated with 500 μM (I) and 250 μM *N*M(*R*)Sal (II) for 3 h. (c) Mitochondria from wild SH cells were treated with 500 μM (I) or 250 μM *N*M(*R*)Sal (II) without 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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