

Conclusion

The results of this article suggest that mitochondrial dysfunction might induce the degeneration of dopamine neurons through the modification and inactivation of the proteasome system and the subsequent aggregation of oxidized proteins. At present, the precise mechanism behind the induction of cell death requires further investigation, but it might be quite relevant to consider that the inactivation of proteasome might play a critical role in activation of apoptotic signal. In neurodegenerative disorders, the environmental and genetic factors might induce a malignant cycle among the mitochondrial dysfunction, increased oxidative stress, and the reduced activity of proteasome in neurons, resulting in the typical pathological features, cell death, and formation of inclusion body. Our recent studies on the ubiquitin-proteasome system in the substantia nigra of human brains further support our hypothesis. In the Parkinsonian brain, the 20S β -subunit precipitated with the specified antibody was modified with acrolein, whereas that from the control was not modified.

Future studies on the mitochondrial signaling, protein oxidation, and proteasome system will give rise a new insight in the involvement of oxidative stress in neuronal cell death. The results will provide us with further development of neuroprotective agents for 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 MAO-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, *NM*(*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). *NM*(*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 *NM*(*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; *NM*(*R*)Sal, and *NM*(*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.

Naai *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 (NMSal), 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 NM(*R*)Sal in the mitochondrial membrane.

Previously we reported that NMSal inhibits monoamine oxidase [MAO, monoamine: oxygen oxidoreductase (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*-methyl-*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*-propargyl-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 NM(*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

NM(*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 (Sémmellweis 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% CO₂. 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-CACCAAGTTTCCTGT-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 NM(*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 TNGGQERKRVGGSGQ, 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 pIRES1neo-MAO-B, the full-length human MAO-B gene included in pECE vector (Lan *et al.* 1989) was digested with *Hind*III 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 μ g 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'-GGACCAACCCAGAATCGTAT-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 μ M 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 NMSal 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 NM(R)Sal to mitochondria

Mitochondria were prepared and suspended in 100 μ L of 10 mM Tris-HCl buffer, pH 6.0, and incubated with 10–100 μ M NM(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 μ L of 10 mM perchloric acid solution containing 0.1 mM EDTA, mixed, centrifuged, filtered through a Millipore HV filter (pore size 0.45 μ m), 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 NM(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 μ M NM(R)Sal for 3 h at 37°C. The effects of 5-HT and β -PEA were also examined by addition of 100–500 μ M 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 NM(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 μ M NM(R)Sal at 37°C for 24 h, and treated with trypsin, gathered, and washed with PBS. The cells were stained with 75 μ M 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 NM(R)Sal or clorgyline for 24 h in the presence of 100 μ M-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 NM(R)Sal to mitochondria and the effects of MAO-A gene silencing by siRNA in SH-SY5Y cells

The binding of NM(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

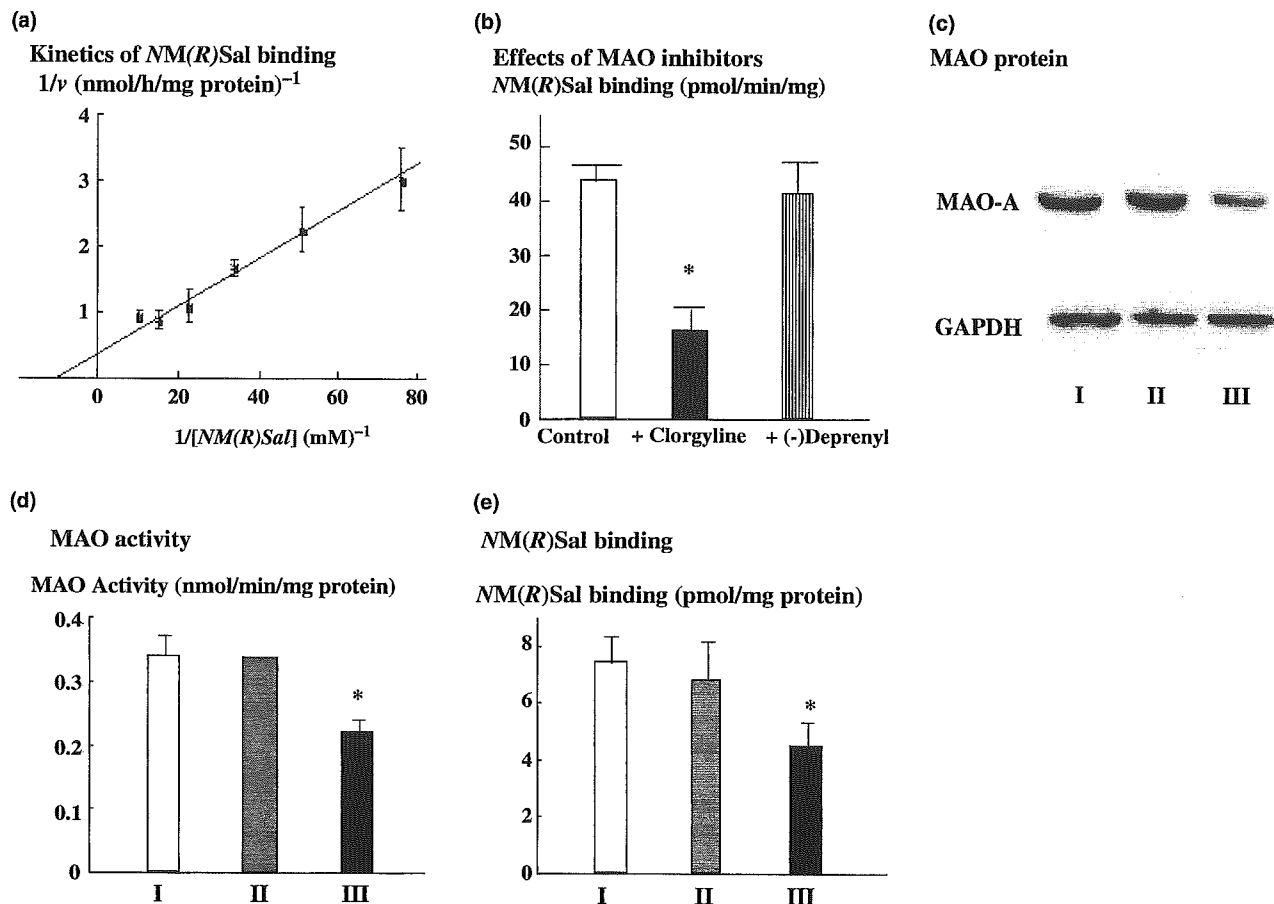


Fig. 1 Binding of *NM(R)Sal* to mitochondria and the effects of RNAi targeting MAO in SH-SY5Y cells. (a) *NM(R)Sal* binding to mitochondria prepared from wild SH cells. *NM(R)Sal* was quantified by HPLC-ECD, as described in Materials and methods. The reciprocal of binding velocity was plotted against 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 μ M MAO inhibitors for 20 min at 37°C, then incubated with 10 μ M *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 μ M 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 $80 \pm 15 \mu$ M 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

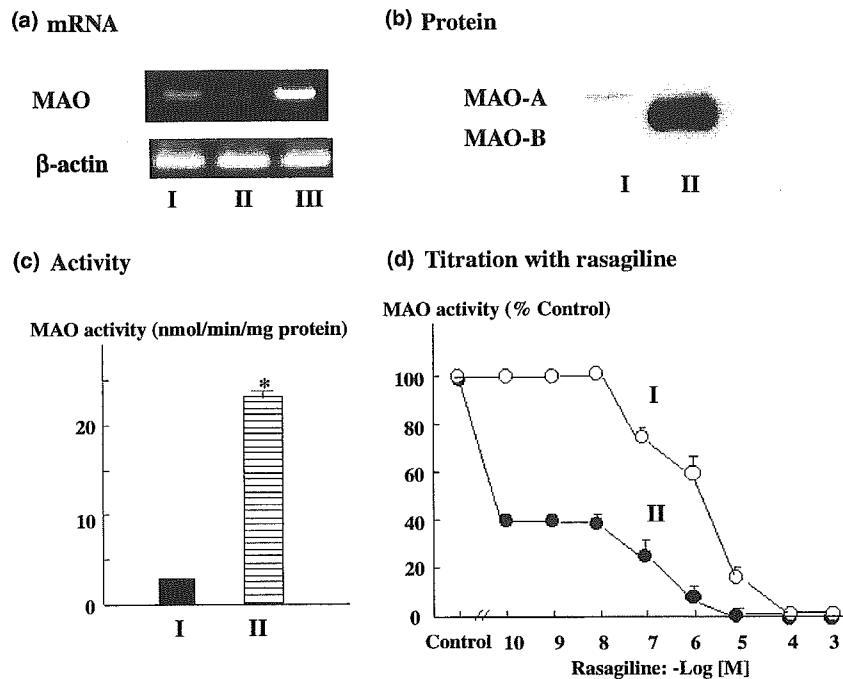


Fig. 2 Establishment of SH-SY5Y cells transfected with human MAO-B. SH-SY5Y cells were transfected with MAO-B as described in Materials and methods. (a) mRNA isolated from wild SH cells (I), cells transfected with IRES vector alone (II), and with full-length MAO-B cDNA (III). β -Actin was used as control. (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 μ M 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 μ M 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 *NM(R)Sal* to mitochondria prepared from wild SH and MAO-B-SH cells was examined. The binding velocity of *NM(R)Sal* to mitochondria isolated from wild SH and MAO-B-SH cells were 163.6 ± 52.6 and $150.1 \pm$

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

MAO-isomer	K_m value (μ M)	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

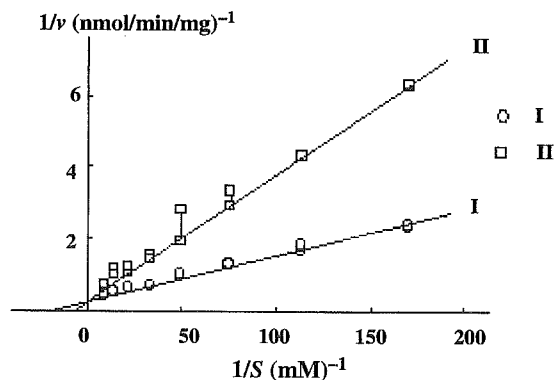
Mitochondria were prepared from wild SH and MAO-B-SH cells for an MAO-A and MAO-B sample, respectively. To measure MAO-B activity, MAO-B sample was pretreated at 37°C for 20 min with 1 μ M clorgyline to deplete MAO-A activity. MAO activities were measured at eight different concentrations of kynuramine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

20.9 pmol/min/mg protein, respectively. The transfection of MAO-B did not increase *NM(R)Sal* binding.

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

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

MAO-A



MAO-B

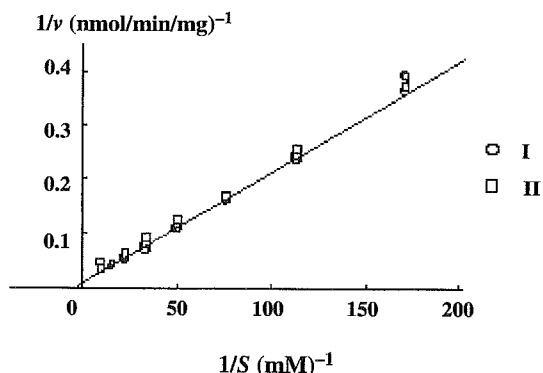


Fig. 3 Kinetic analysis of effects of *NM(R)Sal* on activity of MAO-A and MAO-B. To measure MAO-A and MAO-B activity, mitochondria were isolated, respectively, from wild SH or MAO-B-SH cells, the mitochondria from which were pretreated with 1 μM clorgyline at 37°C for 20 min. The amounts of MAO samples used for the kinetical studies were 38 and 52 μg for MAO-A and MAO-B, respectively. The effects of *NMSal* were studied by use of eight graded concentrations of kynuramine in the absence (I) or presence of 100 μM of *NM(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.

$59.9 \pm 5.4 \mu\text{M}$. In contrast, *NM(R)Sal* did not inhibit MAO-B activity up to 250 μM .

NM(R)Sal induced $\Delta\Psi\text{m}$ decline in mitochondria prepared from wild SH cells and apoptosis in SH cells

The role of MAO-A in apoptosis by *NM(R)Sal* was shown by competition with 5-HT, a substrate of MAO-A. 5-HT prevented *NM(R)Sal*-induced $\Delta\Psi\text{m}$ decline in isolated mitochondria and apoptosis in wild SH cells (Figs 4a and b). The number of apoptotic cells after *NM(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 $\Delta\Psi\text{m}$ decline by *NM(R)Sal*
Involvement of MAO-A and MAO-B in *NM(R)Sal*-induced reduction of $\Delta\Psi\text{m}$ was examined using mitochondria prepared from wild SH and MAO-B-SH cells. Figure 5(a and b) show that *NM(R)Sal* reduced $\Delta\Psi\text{m}$ in mitochondria containing MAO-A, but did not affect $\Delta\Psi\text{m}$ in those prepared from MAO-B-SH cells. The fluorescence intensity of MitoTracker Orange representing $\Delta\Psi\text{m}$ reduced to 71.1% of control in mitochondria containing MAO-A, while $\Delta\Psi\text{m}$ in mitochondria from MAO-B-SH cells was not affected; 98.9% of control. β -PEA, an MAO-B substrate, did not prevent *NM(R)Sal*-induced $\Delta\Psi\text{m}$ reduction in mitochondria prepared from wild SH cells (Fig. 5c). In addition, the antibody against MAO reduced $\Delta\Psi\text{m}$ 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, *NM(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 (Maccocci *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'Carroll *et al.* 1988). In addition, inhibitors of MAO-B, rasagiline and (-)-deprenyl, prevent cell death in

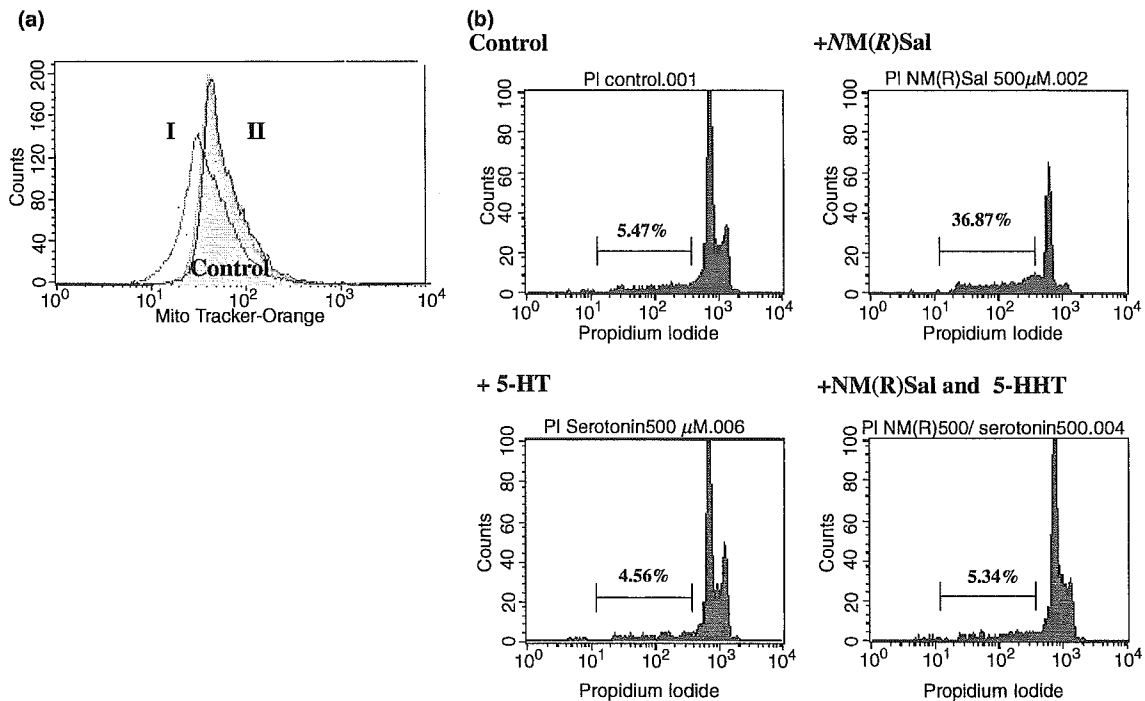


Fig. 4 Effects of 5-HT on $\Delta\Psi_m$ decline in isolated mitochondria and apoptosis in wild SH cells. (a) Mitochondria isolated from wild SH cells were incubated with 100 μM *NM(R)Sal* in the absence (I) and presence of 100 μM 5-HT (II). $\Delta\Psi_m$ was measured by FACS after staining with MitoTracker Orange and Green. (b) Wild SH cells were incubated with 500 μM *NM(R)Sal* overnight and apoptotic cells were

quantified by FACS after staining with PI. Control cells were incubated with *NM(R)Sal* (+ *NM(R)Sal*) or 500 μM 5-HT (+ 5-HT) or *NM(R)Sal* and 5-HT (+ *NM(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 *NM(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 *NM(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 *NM(R)Sal* to MAO initiates the activation of apoptotic signalling, as shown in this paper and also proposed in our previous study (Naoui *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 $\Delta\Psi_m$ and following apoptosis by *NM(R)Sal*. In addition, the results of clorgyline and (-)-deprenyl on *NM(R)Sal* binding support further the role of MAO-A in apoptosis induced by this neurotoxin. As reported previously, *NM(R)Sal* is not oxidized by MAO, but by another amine oxidase (Naoui *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 $\Delta\Psi_m$

decline and apoptosis by *NM(R)Sal*. The binding of *NM(R)Sal* to the active site of MAO and the opening of mPT pore. The decline in $\Delta\Psi_m$ 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, *NM(R)Sal*, and similar, but less marked, effects on $\Delta\Psi_m$ were observed also with MPP⁺, an oxidation product of MPTP. These results suggest that selective MAO-A inhibitors, *NM(R)Sal*, its oxidation product, 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion and MPP⁺, might activate mitochondrial apoptotic signalling through binding to MAO-A (Naoui *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 neurodegenerative and psychiatric disorders, such as bipolar emotional disorders (Lim *et al.* 1994) and X-linked mental retardation (Brunner *et al.* 1993).

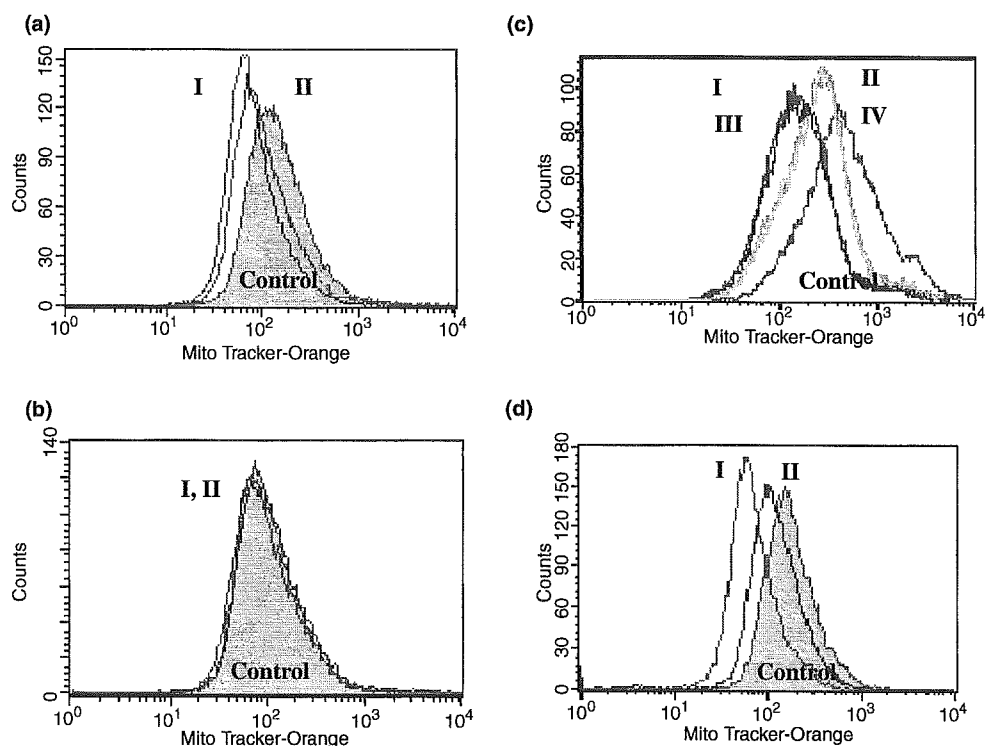


Fig. 5 *MM(F)Sal* reduced $\Delta\Psi_m$ in isolated mitochondria. (a, b) Mitochondria were prepared from wild SH (a) and MAO-B-SH cells (b), and incubated with 500 μM (I) and 250 μM *MM(F)Sal* (II) for 3 h. (c) Mitochondria from wild SH cells were treated with 500 μM (I) or 250 μM *MM(F)Sal* (II) without 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. $\Delta\Psi_m$ was visualized with MitoTracker Orange and measured by FACS in mitochondrial fraction gated with MitoTracker Green.

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The effect of neuromelanin on the proteasome activity in human dopaminergic SH-SY5Y cells

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Summary. In Parkinson's disease (PD), the selective depletion of dopamine neurons in the substantia nigra, particular those containing neuromelanin (NM), is the characteristic pathological feature. The role of NM in the cell death of dopamine neurons has been considered either to be neurotoxic or neuroprotective, but the precise mechanism has never been elucidated. In human brain, NM is synthesized by polymerization of dopamine and relating quinones, to which bind heavy metals including iron. The effects of NM prepared from human brain were examined using human dopaminergic SH-SY5Y cells. It was found that NM inhibits 26S proteasome activity through generation of reactive oxygen and nitrogen species from mitochondria. The mitochondrial dysfunction was also induced by oxidative stress mediated by iron released from NM. NM accumulated in dopamine neurons in ageing may determine the selective vulnerability of dopamine neurons in PD.

Abbreviations

DMSO dimethyl sulfoxide, *DTT* dithiothreitol, *ECF* enhanced chemofluorescence, *MEM* minimum essential medium, *NM* neuromelanin, *PBS* phosphate-buffered saline, *SOD*

superoxide dismutase, *PD* Parkinson's disease, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *UPS* ubiquitin-proteasome system, *ZsGFP* a green fluorescence protein homologue from *Zoanthus* sp.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorders in the aged and recently several genes responsible for the familial PD have been reported. However, the etiology of sporadic PD is still an enigma, and oxidative stress, impairment of mitochondrial function and the ubiquitin-proteasome system (UPS) are suggested to initiate a "malignant cycle" resulting in the cell death (Dexter et al., 1994). Especially, recent evidences suggest that failure of the UPS leads to aggregation and accumulation of abnormal proteins to form the inclusion bodies and induce neuronal cell death in the familial case of PD (Polymeropoulos et al., 1997; Shimura et al., 2000; Leroy et al., 1998). Dysfunction of the UPS is found to occur also in the sporadic form of PD also (McNaught and Olanow, 2003). However, the factors, which determine the selective vulnerability of dopamine neurons are still

unknown. One possibility is the involvement of dopamine and the metabolites themselves. Dopamine is a highly reactive catecholamine and is oxidized enzymatically and non-enzymatically. Autoxidation of dopamine produces superoxide and quinones, and polymerized quinones are interacted with proteinacious components and lipids (Gerlach et al., 1995; Dzierzega-Leczna et al., 2004; Fedorow et al., 2005), to produce highly aggregated conjugates, neuromelanin (NM).

NM is a dark pigmented granule, which accumulate in catecholaminergic neurons of the substantia nigra and locus ceruleus. NM has been supposed to be a mere "waste box" in the neurons, but recently it was found to function as a reservoir of trace metals, such as iron (Zecca et al., 2001a). The involvement of iron in dopaminergic degeneration during ageing has been suggested repeatedly and recent studies show the content of iron in the substantia nigra was higher than that in the locus ceruleus (Zecca et al., 2004). Iron possibly induces cell death in nigral dopamine neurons through oxidative stress, as confirmed by studies on postmortem parkinsonian brains and on the cellular and animal PD models (Jenner, 2003). In the substantia nigra of PD brains, increased oxidative stress is suggested by increase in oxidative modification of lipids, proteins and DNA (Yoritaka et al., 1996). These results suggest that the effect of NM and iron should be examined on mitochondrial dysfunction, oxidative stress and the UPS.

In this paper we report the effects of NM prepared from control human brain on the production of reactive oxygen and nitrogen species (ROS, RNS) in mitochondria, the UPS activity using neuroblastoma SH-SY5Y cells. The *in situ* ubiquitin-26S proteasome activity was examined using the cells transfected with a proteasome sensor vector. The results are discussed in relation to the role of NM in selective vulnerability of dopaminergic neurons in PD and ageing.

Materials and methods

Materials

NM was isolated and purified from the substantia nigra of control human brains, as described previously (Gerlach et al., 1995) and was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration (Shamoto-Nagai et al., 2004). L-Cysteine was purchased from Sigma (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Molecular Probes (Eugene, OR, USA), deferoxamine mesylate (DFX) and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma (St. Louis, MO, USA), and anti-polyubiquitin monoclonal antibody from NBT (Tokyo, Japan). A proteasome sensor vector, pZsProSensor-1, was purchased from BD Biosciences (Palo Alto, CA, USA). 2',7'-Dichlorofluorescein (DCF), N-acetyl cysteine (NAC), L-cysteine and catalase from bovine liver, minimum essential medium (MEM) and other reagents were from Wako (Kyoto, Japan).

Measurement of in situ 26S proteasome activity in SH-SY5Y cells expressing a proteasome sensor vector

SH-SY5Y cells were cultured as reported (Shamoto-Nagai et al., 2004). Transfectant with a proteasome sensor vector was established using a pZsProSensor-1 eukaryotic expression vector, designed to express ZsGFP fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome, by lipofection technique as reported previously (Shamoto-Nagai et al., 2004). The culture medium was changed with the medium containing L-Cyst-DMSO solution without (control) or with NM and the cells were cultured for 3 days. In addition, the cells transfected with proteasome sensor vector was incubated with various concentrations of iron in the presence or absence of 25 μ M DFX or antioxidants for 20 h. The fluorescence of ZsGFP in the living cells was measured as described before (Shamoto-Nagai et al., 2004), and the fluorescence intensity of the cells was expressed as arbitrary fluorescence unit/mg protein. The protein amount was measured according to Bradford (1976).

Detection of polyubiquitinated proteins in the cells treated with NM

After treatment with NM for 1 or 3 days, the cells were gathered, washed with PBS, and lysed in the RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Fifty μ g of protein was subjected to SDS-polyacrylamide electrophoresis

using 12.5% polyacrylamide gel (Bio Craft, Tokyo, Japan), and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Polyubiquitinated proteins were visualized using anti-polyubiquitin antibody and enhanced chemofluorescence (ECF) Western blotting kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously (Shamoto-Nagai et al., 2003).

Isolation of mitochondria from SH-SY5Y cells

Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999). The cells were gathered, washed with PBS and suspended in the isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.5) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The mitochondrial fraction was prepared by homogenization and two steps of centrifugation.

Measurement of ROS-RNS with H₂DCFDA

The mitochondria were suspended in PBS and the production of ROS-RNS was quantified fluorometrically by measuring DCF produced from H₂DCFDA (Crow, 1997). H₂DCFDA was added to be 50 μM to the mitochondria suspension in the presence or absence of NM suspension (1–5 μg/ml) in dark at 37°C. The increase in DCF fluorescence at 504 nm with excitation at 520 nm was measured at every 30 min for 3 h in a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan). The generation of ROS-RNS was expressed as mol DCF per min per mg protein. The effects of DFX and anti-oxidants were also examined in the same way, after 15 min pre-incubation with DFX or the antioxidants.

Statistics

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

Results

The effect of NM on in situ 26S proteasome activity

After treatment with NM or iron the accumulation of ZsGFP was observed in cyto-

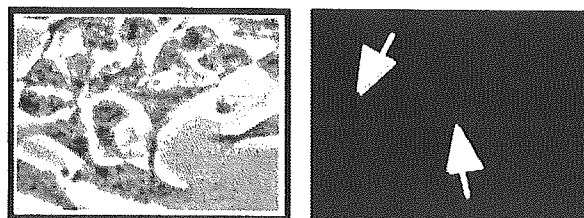


Fig. 1. Effects of NM on the in situ activity of 26S proteasome in SH-SY5Y cells transfected with the proteasome sensor vector. The cells were cultured in the presence of 0.1 μg/ml of NM for 3 days. Upper: morphological observation of the cells treated with NM. Lower: Accumulation of ZsGFP (white arrows) was observed by fluorescence microscopy

plasm by fluorescence microscopy (Fig. 1). After 3 days' incubation, the fluorescence intensity in the cells treated with 0.1 μg/ml

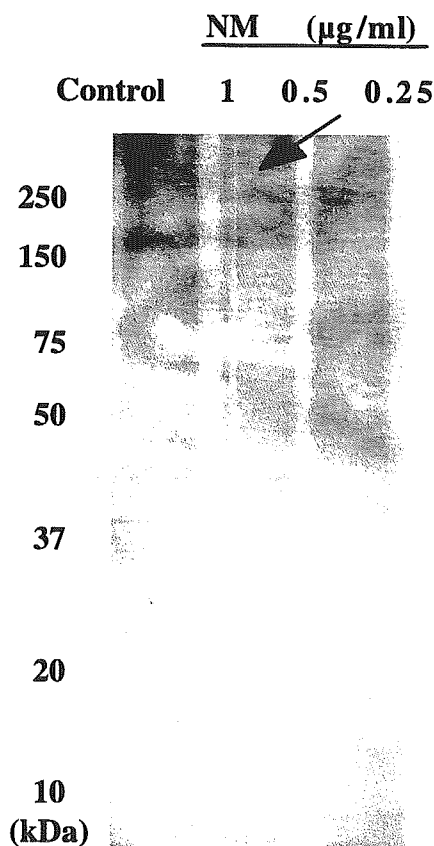


Fig. 2. Accumulation of poly-ubiquitinated proteins in SH-SY5Y cells treated with NM for 3 days (arrow). The cells were cultured with or without NM and lysed, then proteins were separated by SDS-PAGE and immunoblotted using anti-polyubiquitin antibody as described in the Materials and methods

NM increased to be that of 161% of control cells. The accumulation and the increase of fluorescence intensity was confirmed by measuring its fluorescence intensity by Fluorespectrometry also (Fig. 4). The increase of ZsGFP, that reflects the reduced activity of 26S proteasome by iron, was restored by DFX.

Accumulation of polyubiquitinated proteins in SH-SY5Y cells treated with NM

The cells treated with various concentration of NM was gathered and applied to Western blotting using anti-polyubiquitin antibodies. As shown in Fig. 2, in the cells treated with 1 $\mu\text{g}/\text{ml}$ of NM, the accumulation of polyubiquitinated proteins, which is the specific substrate for 26S proteasome, was detected (arrow).

Increase of ROS-RNS in isolated mitochondria by NM

The production of $\cdot\text{OH}$, NO and ONOO^- in mitochondria was quantified fluorometrically using DCF cleaved from H_2DCFDA as an indicator (Fig. 2). In the presence of mitochondria, NM increased DCF fluorescence. In addition, SOD, but not catalase, reduced DCF production in mitochondria themselves. DFX significantly reduced the DCF production from mitochondria enhanced by NM. These results indicate that $\text{O}_2^{\cdot-}$ plays the key role in ROS-RNS production and the involvement of iron in the ROS-RNS production by NM.

Discussion

In this paper, it was clearly shown that NM increased ROS and RNS generation especially superoxide, in mitochondria, which

DCF produced (pmol/min/mg protein)

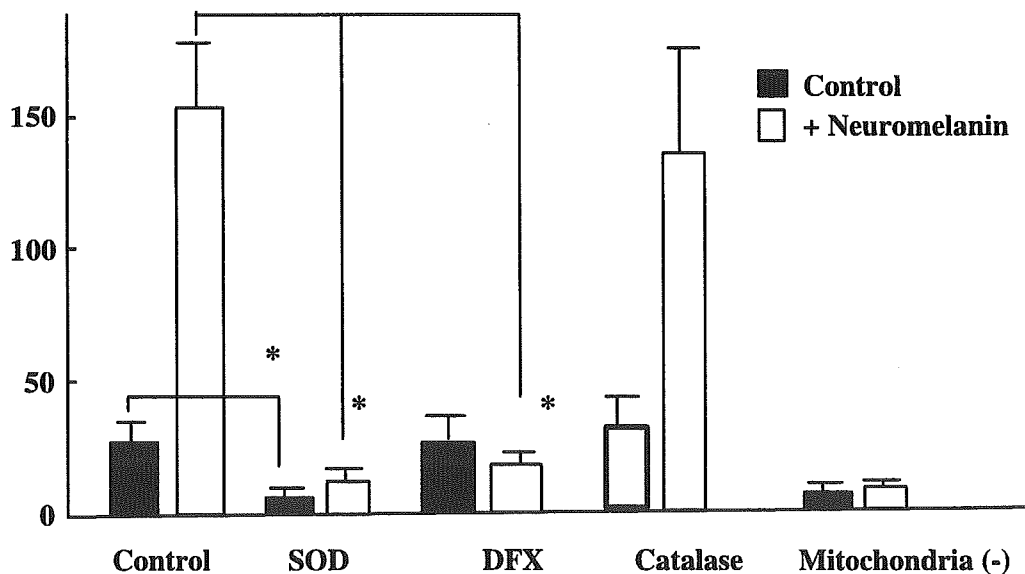


Fig. 3. Effects of SOD, deferoxamine mesylate (DFX), and catalase on DCF production in mitochondria prepared from SH-SY5Y cells. Mitochondria (30 μg protein) were incubated with or without 2.5 $\mu\text{g}/\text{ml}$ NM in PBS, and the effects of SOD (1000 units), DFX (1 μM), and catalase (500 units) were examined. Generated ROS-RNS was quantitatively measured as DCF produced from H_2DCFDA and expressed as pmol/hr. The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. * $P < 0.01$

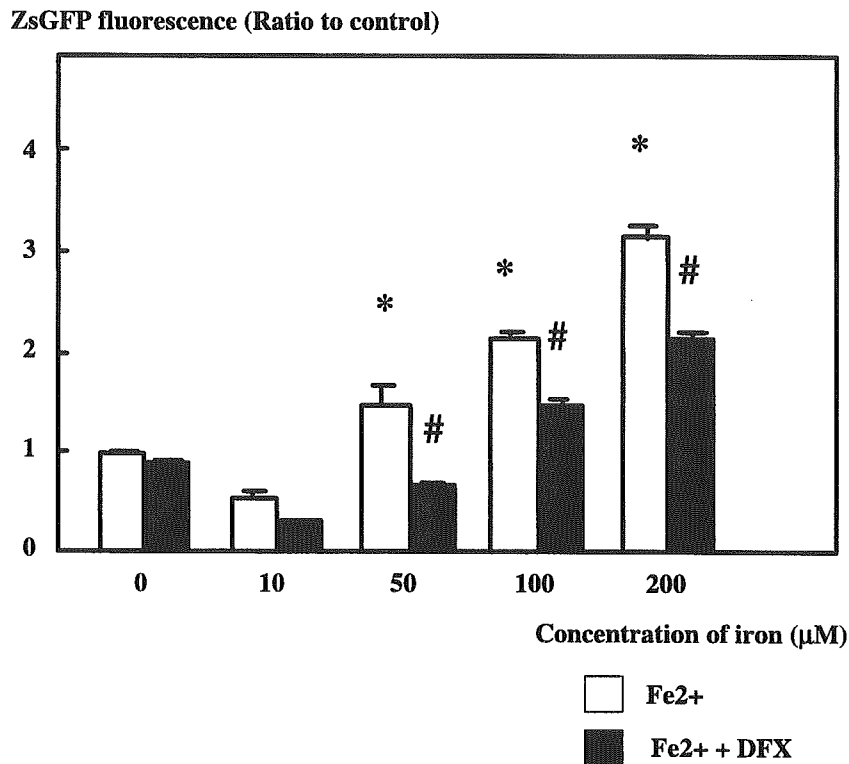


Fig. 4. Iron inhibited in situ activity of 26S proteasome in SH-SY5Y cells transfected with a proteasome sensor vector. After the treatment with iron with or without deferoxamine mesylate (DFX) for 20h, the fluorescence intensity of ZsGFP, which is coded by a proteasome sensor vector, at 505 nm with excitation at 493 nm was quantified and expressed as arbitrary fluorescence unit/mg protein. The column and bar represent mean and SD of 3 experiments. After the treatment with iron, the fluorescence intensity increased in a dose-dependent manner. * $p < 0.05$ compared to the control. This increase was suppressed by 25 μM DFX significantly. # $p < 0.05$ compared to the cells treated with iron without DFX

was mediated by released iron, as shown by the complete suppression of DFX. The onset of oxidative stress may deteriorate the function of mitochondria, in addition to the direct inhibition of mitochondrial respiratory chain enzymes. NM reduced the in situ activities of 26S proteasome, as shown using a green fluorescent protein homologue targeted to 26S proteasome. The mitochondrial toxins, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), were reported to induce neuronal death selective to dopamine neurons with formation of Lewy body-like inclusion body in *in vivo* models of PD (Betarbet et al., 2000; Kowall et al., 2000). The mechanism of cell death has not been elucidated, but the involvement

of reduced UPS activity was suggested. Recently we found that mitochondrial dysfunction caused by rotenone, a complex I inhibitor, increased abnormal oxidative modification of proteins with acrolein, and reduced the activity of proteasome, through binding of aggregated oxidized protein to the catalytic site of 20S proteasome and direct adduction of acrolein to 20S proteasome itself (Shamoto-Nagai et al., 2003). It may be reasonable to assume that mitochondrial dysfunction plays a central role in the pathogenesis of sporadic PD, and impairment of the UPS may be a final executor in the neural degeneration.

Iron is known to induce oxidative stress by enhancing electron transfer in a Fenton

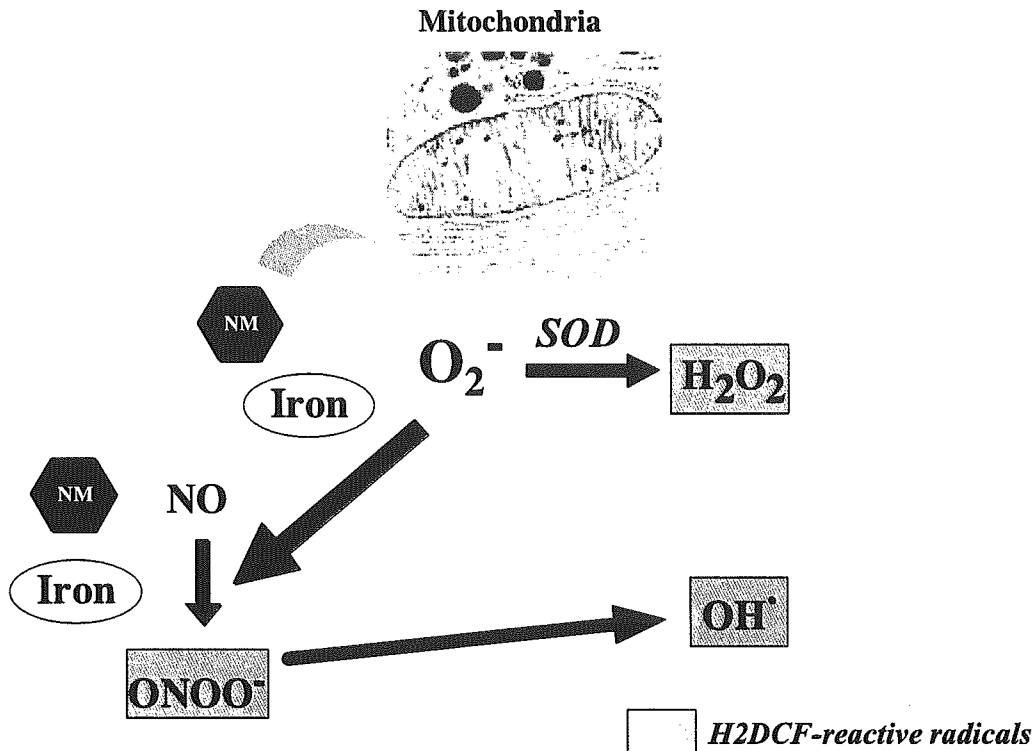


Fig. 5. The scheme how iron increases oxidative stress in mitochondria. Iron released from NM generates superoxide (O_2^-), which is catalyzed by SOD. O_2^- also reacts with nitric oxide (NO) by near-diffusion limit to produce peroxynitrite ($ONOO^-$). $ONOO^-$ is a strong radical itself and is decomposed into hydroxyl radical (OH^\cdot) also

reaction (Fig. 5). In the dopaminergic neurons, almost all the iron exists as bound with ferritin or NM, suggesting that NM may be neuroprotective, by chelating iron and other trace metals (Zecca et al., 2001b). Iron content in the substantia nigra increases by ageing but remained stable after the fourth decade of age, but NM content increases further according to ageing (Zecca et al., 2001a). In the postmortem PD brain, increased iron content was detected (Riederer et al., 1989) and recent results using transcranial ultrasonography revealed that in the substantia nigra of parkinsonian patients, not only the iron content increased, but that of NM decreased (Zecca et al., 2005). These results indicate that in the dopaminergic neurons in PD, the binding capacity of NM with iron was decreased and as a result, cytosolic free iron

increased to inhibit ubiquitin-26S proteasome system, as shown in this paper (Gerlach et al., 2003).

Recent proteomics studies indicate the involvement of endosome-lysosome system as a source of proteinacious components in human NM (Tribl et al., 2005). This means NM production is not only "passive" accumulation of oxidative products, but some enzymatic system is required for the formation of high molecular aggregates. It requires further studies to elucidate whether the characteristics of NM itself or its synthetic pathway are changed in PD. The future studies on the intracellular mechanisms underlying the selective cell death by iron released by NM may bring out new strategies to prevent or rescue the decline in nigral dopamine neurons in PD.

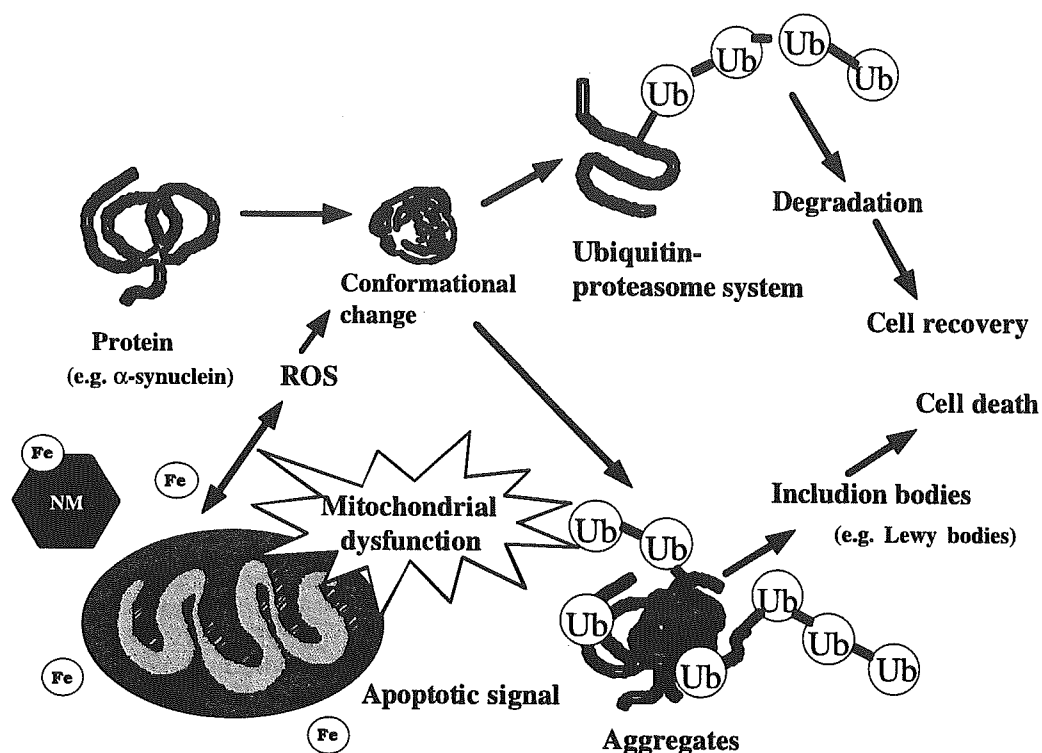


Fig. 6. The possible mechanism of dopaminergic neuronal death in Parkinson's disease. Iron released from NM increases oxidative stress in the mitochondria, and inhibit 26S proteasome system in the cells. Impairment of 26S proteasome increases accumulation of abnormal proteins to induce cell death ■

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