

Overexpression of amyloid precursor protein induces susceptibility to oxidative stress in human neuroblastoma SH-SY5Y cells

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Received October 12, 2004; accepted April 9, 2005
Published online June 15, 2005; © Springer-Verlag 2005

Summary. In Alzheimer's disease amyloid β peptide ($A\beta$) produced from amyloid precursor protein (APP) is considered to induce cell death. To clarify the molecular mechanism underlying $A\beta$ neurotoxicity, we established the cell line overexpressing wild or mutant (His684Arg) APP in human SH-SY5Y cells. This paper presents that overexpression of wild-APP in the cells (SH/w-APP) increased the levels of APP and $A\beta_{1-40}$ but not $A\beta_{1-42}$, and reduced Bcl-2 level and proteasome activity with increased susceptibility to oxidative stress. The intracellular levels of reactive oxygen species in SH/w-APP increased significantly by H_2O_2 treatment. The level of Bcl-2 protein, but not mRNA, was markedly decreased in SH/w-APP cells, which was inversely correlated with APP expression among subcloned SH/w-APP cells. These results indicate that increased expression of wild type APP renders neuronal cells more vulnerable to oxidative stress leading to cell death.

Keywords: Alzheimer's disease, amyloid precursor protein, apoptosis, oxidative stress, proteasome inhibitor, Bcl-2.

Abbreviations

AD Alzheimer's disease; *AMC* 7-amino-4-methyl-coumarin; *APP* β -amyloid precursor protein; *$A\beta_{1-40}$* β -amyloid₁₋₄₀; *$A\beta_{1-42}$* β -amyloid₁₋₄₂; *CM-H₂DCFDA* 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate; *BOC* *t*-butyloxycarbonyl; *DCF* 2',7'-dichlorofluorescein; *FACS* fluorescence augmented flow cytometry; *GDNF* glial cell line-derived neurotrophic factor; *GSH* reduced glutathione; *PBS* phosphate-buffered saline; *PD* Parkinson's disease; *PT* permeability transition; *RNS* reactive nitrogen species; *ROS* reactive oxygen species; *RT* reverse transcription; *Z* benzyloxycarbonyl.

Introduction

In Alzheimer's disease (AD), abnormal protein-protein interactions may induce precipitation of protein, forming intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and extracellular aggregates of senile plaques containing β -amyloid ($A\beta$) (Selkoe and Schenk, 2003). The major constituent of the core of senile plaques is

A β , a 39–43 amino acid peptide, produced from β -amyloid precursor protein (APP) by a series of proteases, β - and γ -secretases. Under normal conditions, APP is processed by a non-amyloidogenic pathway, where α -secretase produces soluble form of APP, whereas by an amyloidogenic pathway β - and γ -secretases generate A β with 40 or 42 amino acids in the length, β -amyloid_{1–40} or _{1–42} (A β _{1–40}, A β _{1–42}). The involvement of A β _{1–40} and A β _{1–42} in AD pathogenesis has been shown further by studies on mutations in *APP* gene causing early-onset autosomal-dominant AD. *APP* mutations increase the ratio A β _{1–42}/A β _{1–40} (Miravelle et al., 2000), and A β _{1–42} is prone to precipitation and aggregation constituting amyloid fibrils. This suggests that abnormal processing of APP and increase in A β may play a major role in the pathogenesis of the hereditary forms and also that of sporadic AD, since the pathological features of both forms of AD are almost the same. In addition, genetic variability in A β catabolism and cleavage was reported to contribute to the risk of late-onset AD (Wavrant-DeVrieze et al., 1999). The “amyloid hypothesis” (Hardy and Selkoe, 2002) elucidates most of the sequential pathological processes in AD; A β _{1–42} deposition in plaques, activation of microglia and astrocytes, and dysfunction of synapse and neurons.

Recently in AD, apoptosis has been implicated as the potentially important mechanism underlying neuronal cell loss (Cotman and Anderson, 1995; Stadelmann et al., 1999). A β and presenilins 1 and 2 were found to drive cells into apoptosis (Loo et al., 1993). A number of hypotheses have been proposed to elucidate the mechanisms behind the induction of apoptosis by A β in AD; direct toxicity, such as increased oxidative stress, mitochondrial dysfunction, perturbed ionic homeostasis, and altered activities of kinases and phosphatases (Mattson et al., 1992; Behl et al., 1994; Ghosh et al., 1999; Avila, 2000). At the same time, A β increases vulnerability of neuronal cells to various insults including

oxidative stress (Goodman et al., 1994). More recently, new mechanistic aspects for the cell death in AD have been gathering attention. A β activates the mitochondrial apoptotic cascade, through opening permeability transition pore (PTP), which Bcl-2 protein family regulates, either in an anti-apoptotic way (Bcl-2, Bcl-xL) or in a pro-apoptotic function (Bax, Bad) (Zhang et al., 2002). In addition, A β induces dysfunction in the proteasome system, which is responsible for the cellular proteolysis of most ubiquitinated, oxidized protein and regulates oxidative stress (Keller et al., 2000).

In this paper, the effects of increased synthesis of APP, and A β _{1–40} and A β _{1–42} were studied in concern to the morphological characteristics of the cells, the susceptibility to oxidative stress and proteasome inhibition. For the present study we established human SH-SY5Y cells overexpressing the wild and the mutated APP at the 684th amino acid from His to Arg, which is within A β _{1–40} and A β _{1–42} region. The results are discussed in relation to the interaction among cellular factors, anti-oxidant capacities and mitochondrial Bcl-2 levels, the reduction of which may lower threshold to cell death.

Materials and methods

Chemicals

Peroxynitrate-generating 3-(4-morpholinyl)sydonimine (SIN-1) was purchased from Dojindo (Kumamoto, Japan); geneticin from Invitrogen (San Diego, CA, USA); a proteasome inhibitor, benzyloxycarbonyl (Z)-L-isoleucyl- γ -*t*-butyl-L-glutamyl-L-alanyl-L-leucinol (PSI), 7-amino-4-methyl-coumarin (AMC), substrates for proteasome, *t*-butyloxycarbonyl (Boc)-Leu-Arg-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Leu-Leu-Glu-MCA from Peptide Institute (Osaka, Japan). Hoechst 33342 and 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Hydrogen peroxide (H₂O₂) and other chemicals were purchased from Wako (Tokyo, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan) supplemented with fetal calf serum in an atmosphere of 95% air-5% CO₂

at 37°C. Antibodies were purchased as follows: anti- β -tubulin antibody from Sigma (St. Louis, Mo, USA), rabbit polyclonal anti-APP from Cell Signaling (Beverly, MA, USA), and mouse monoclonal anti-Bcl2, anti-Bcl-xL, and rabbit polyclonal anti-Bax from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmid construction and DNA transfection of APP gene

Transfectants overexpressing wild APP were prepared with a pIRES1neo eukaryotic expression vector (Invitrogen, San Diego, CA, USA). For construction of pIRES1neo-APP vector, the coding region of human APP gene was obtained by reverse transcription (RT)-PCR from cDNA of SH-SY5Y cells and then inserted into the EcoRI-cleaved pIRES1neo vector. pIRES1neo-APP was confirmed to include wild-type APP gene by DNA sequencing. SH-SY5Y cells were transfected with pIRES1neo or pIRES1neo-APP by using liposomes (LipofectAMINE, GIBCO BRL, Rockville, MD, USA). The cells were selected using the culture medium containing 0.7 mg/ml geneticin (GIBCO BRL), and individual clones were isolated by limiting dilution, and characterized by RT-PCR and Western blot analysis. The stable clones overexpressing APP protein and control clone containing pIRES1neo vector were named as SH/w-APP and SH/IRES, respectively. The construction for an APP mutant, His684Arg, was performed by site-directed mutagenesis (BD Clontech, Franklin Lakes, NJ, USA) and the mutation was confirmed by DNA sequencing. The stable clone expressing APP mutant, His684Arg, was named as SH/m-684. In SH/w-APP cells, we further subcloned them by limiting dilution.

Quantitative real time RT-PCR

Total RNA was isolated using a Total RNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, USA). RNA samples were reverse transcribed by Super Script II RNase H-reverse transcriptase (Invitrogen) using oligo(dT) primer (Invitrogen). Prepared cDNA samples were purified by a PCR Purification kit (QIAGEN, Hilden, Germany) and used for PCR.

Quantitative real time PCR was performed using a LightCycler Fast Start DNA Master SYBR Green I kit (Roche, Mannheim, Germany) in a LightCycler (Roche) according to the manufacturer's instruction. Primers for APP were as follows: APP forward, 5'-AAGAAGGCAGTTATCCAGCATTTTC-3'; for APP reverse, 5'-TTGTAGAGCAGGGAGAGAGACTGA-3'. Primers for Bcl-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were commercially obtained from Roche. The PCR products were evaluated from the melting curve.

Quantitation of A β by the Sandwich ELISA

The culture medium of the cells cultured for 3 days was used for quantitative analyses of A β by the Sandwich ELISA for A β using A β ELISA kit (Biosource International, Camarillo, CA, USA). The values of A β concentrations in the medium were expressed as pg/ml. All ELISAs were carried out in duplicate.

Cell viability and morphological study

The vector-transfected SH-SY5Y cell clones grown in the presence of geneticin (G418, 0.7 mg/ml) were subjected to cell growth and morphological observation. Viable cell number was assessed by the trypan-blue dye exclusion test.

Immunocytochemistry

The cells were immunostained for β -tubulin and F-actin as described in detail previously (Matsumoto et al., 2002). The cells were incubated with anti- β -tubulin antibody overnight at 4°C. After washing with PBS, the cells were incubated with the Alexa488-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. F-actin staining was performed with Alexa546-Phalloidin (Molecular Probes) for 30 min at room temperature. As negative control, we used the cells reacted with only the secondary antibody. The stained cells were observed with a fluorescence microscope, Olympus BX-51 (Olympus, Tokyo, Japan).

Detection of APP, Bcl-2, Bcl-xL and Bax by Western blotting analyses

Five micrograms of protein of each sample was analyzed for APP and Bcl-2 protein family, as previously described by Matsumoto et al. (2003). β -Tubulin was used as an internal control.

Measurements of production of reactive oxygen–nitrogen species by FACS

The production of reactive oxygen and nitrogen species (ROS-RNS) was quantitatively measured by the use of CM-H₂DCFDA, which detects H₂O₂, nitric oxide, and peroxynitrite. After treated with or without H₂O₂, the cells were washed twice with PBS, and CM-H₂DCFDA (10 μ M in the final concentration) was added to the cell suspension in 25 mM HEPES, pH 7.5. After 30 min incubation at 37°C, the amount of 2',7'-dichlorofluorescein (DCF) produced by CM-H₂DCFDA oxidation in the cell was determined by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Beckton Dickinson, San Jose, CA,

USA). The fluorometric measurement was performed with excitation at 488 nm and emission at 515–545 m.

Measurement of proteasome activity

The cells were suspended in the lysis buffer (50 mM Tris-HCl buffer, pH 7.75, 1 mM DTT, 0.5 mM EDTA

and 5 mM MgCl₂) and incubated on ice for 30 min. The supernatants of centrifugation at 6000 g for 20 min were used as enzyme samples. Protein amount was measured with a DC Protein assay kit (Bio-Rad, Heocolos, CA, USA). Sample (10 μg protein) was incubated for 60 min at 37°C with MCA-conjugated substrates for three types of proteasome, Boc-Leu-

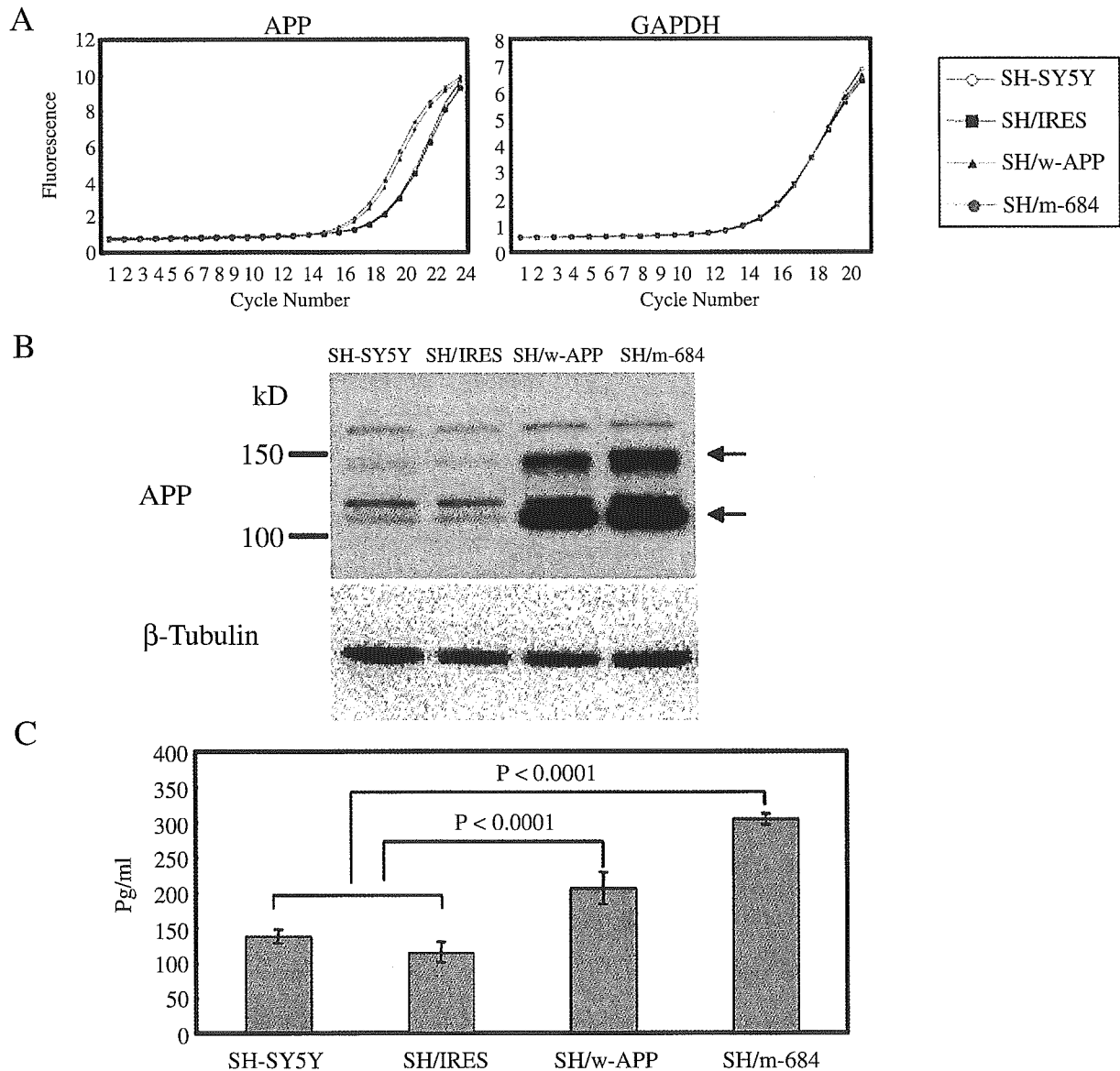


Fig. 1. Establishment of APP-overexpressing SH-SY5Y cells and assessment of the produced Aβ. **A** Real-time RT-PCR of *APP* and *GAPDH*. Cycle number and the fluorescent intensity corresponding to PCR product were shown. **B** Western blot analysis of APP. β-Tubulin was used as an internal standard. The arrows indicate the bands of APP. A band with the higher molecular weight corresponds to a modified APP probably by glycosylation and the lower one APP itself. **C** The amounts of Aβ₁₋₄₀ in the medium after 3 days' culture were assessed by ELISA. Difference from SH-SY5Y and SH/IRES is statistically significant

Arg-Arg-MCA for trypsin like, Suc-Leu-Leu-Val-Tyr-MCA for chymotrypsin like, and Z-Leu-Leu-Glu-MCA for postglutamyl peptidase-like activity. The released AMC fluorescence was measured in a fluoromicroplate reader, MTP-600F (CORONA, Hitachinaka, Japan), with excitation at 380 nm and emission at 450 nm. The enzymatic activity was expressed as pmole/h/mg protein.

Measurement of intracellular GSH level

Intracellular reduced glutathione (GSH) content was quantitatively measured by using a GSH Assay Kit (Calbiochem, San Diego, CA). After cells were sonicated in 5% metaphosphoric acid and centrifuged at 3000 g for 10 min, the supernatant was used to quantify GSH according to the manufacturer's instruction. The pellet dissolved in 1 M NaOH was used for the measurement of protein content. The GSH content was expressed as nmole/mg protein. Values were represented as the mean of three independent experiments.

Statistics

Differences were statistically evaluated by one way ANOVA followed by Fisher's PLSD. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Establishment of APP-overexpressing SH-SY5Y cells

Real time RT-PCR study using APP specific P1 and P2 primers showed that the amounts of the products increased markedly in SH/w-APP transfected with wild APP, and SH/m-684 cells with mutated APP, compared to SH/IRES cells (Fig. 1A). The amount of APP protein of approximately 110 kDa and also modified form of APP with 140 kDa increased markedly in SH/w-APP and SH/m-684 cells, as determined by Western blot analysis (Fig. 1B). The amounts of $A\beta_{1-40}$ excreted in the culture medium are shown in Fig. 1C. $A\beta_{1-40}$ amounts significantly increased in SH/w-APP and SH/m-684 cells (Fig. 1C). The amount of $A\beta_{1-42}$ also increased in the culture medium of SH/m-684 cells to be 35 pg/ml, whereas it was under detection limit, 10 pg/ml, in that of other cell lines.

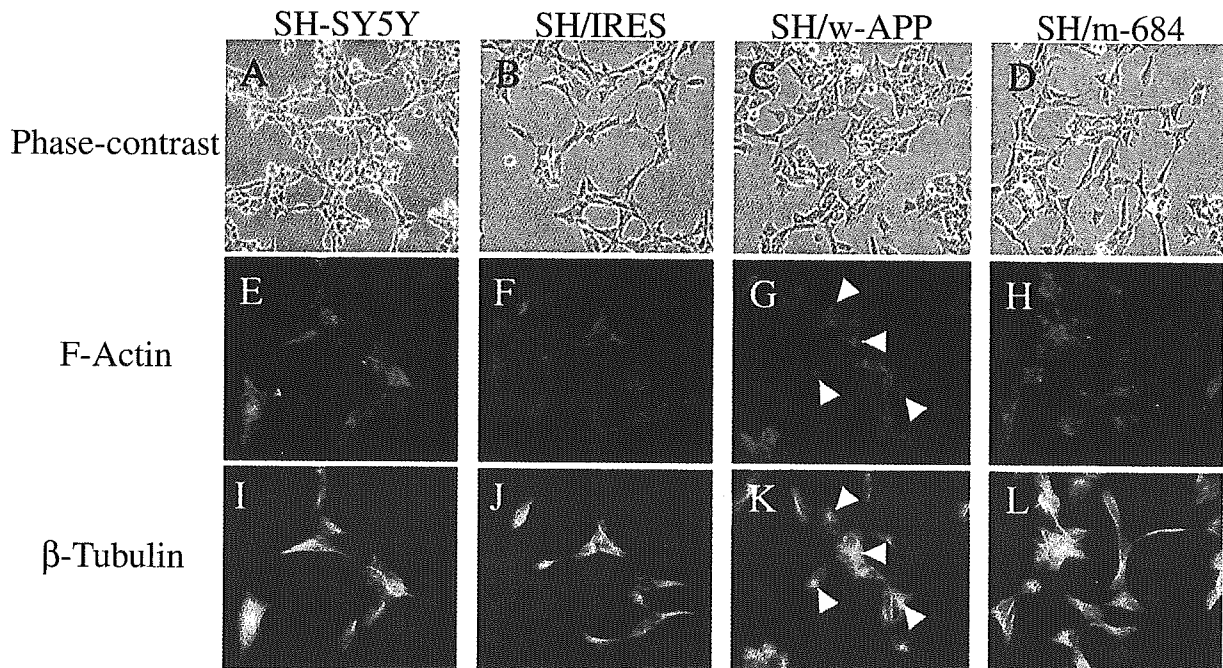


Fig. 2. Morphological study of the transfectants. *Upper panel:* phase-contrast aspects. *Middle and lower panel:* immunocytochemistry for F-Actin and β -Tubulin, respectively. The triangles indicate marked condensed regions of cytoskeleton in SH/w-APP cells

*Alteration of neurite net work formation
in cells overexpressing wild-type APP*

SH-SY5Y cells are usually sending out 1–2 long neurites, which contact with those of adjacent multiple cells forming a network with several cells. However, the morphological properties of SH/w-APP were markedly different from those of SH/IRES or SH/m-684 cells (Fig. 2A–D). SH/w-APP cells shaped polyhedral and had shorter and less branched neurites than other cell lines, which showed spindle form with neurites multi-directionally connected with adjacent cell bodies, as indicated in the staining of anti-F-actin and β -tubulin antibody (Fig. 2C).

*Overexpression of wild APP sensitized
the cells to oxidative stress,
reduced proteasome activity,
and increased GSH level*

The effects of overexpression of wild and mutated APP on cell vulnerability were examined by exposing the cells to oxidative stress using H_2O_2 , SIN-1 (a peroxynitrite donor), and PSI (an inhibitor of proteasome activity). The cells were treated with the agents for 12 h. The number of viable cells was shown in Fig. 3 and SH/w-APP was more sensitive to H_2O_2 than SH/IRES and SH/m-684. In addition, SIN-1 and PSI also induced cell death more markedly in SH/w-APP than in other two cell lines.

The effects of APP overexpression on the ROS-RNS levels and the anti-oxidative capacity were examined by adding H_2O_2 to the culture medium. The intracellular ROS-RNS levels before and after H_2O_2 treatment were quantitatively determined as DCF produced from CM- H_2 DCFDA (Fig. 4). The intracellular ROS-RNS levels of SH/w-APP cells significantly increased in a dose-dependent way (the middle panel), whereas in SH/IRES (the upper panel) and SH/m-684 cells (the lower panel) DCF levels were not affected by H_2O_2 (50 to 100 μ M).

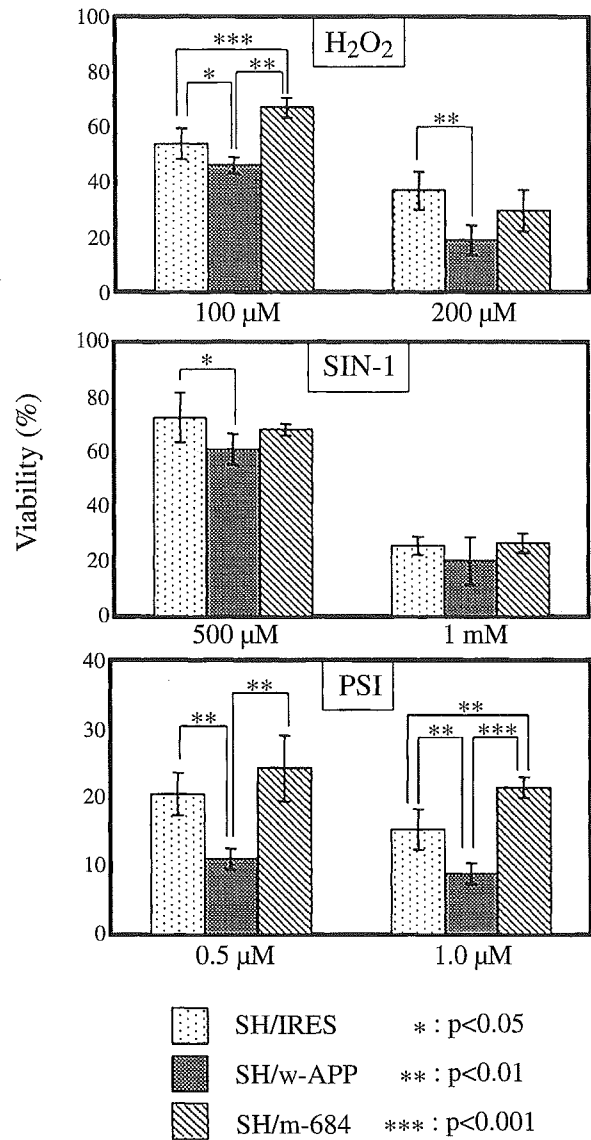


Fig. 3. The cell death induced by H_2O_2 , SIN-1 and PSI in the transfectants. Viable cell number was measured by trypan-blue dye exclusion test at 24 h after the treatment. *Upper panel:* Cells were treated with 100 and 200 μ M H_2O_2 . *Middle panel:* Treated with 500 μ M and 1 mM SIN-1. *Lower panel:* Treated with 0.5 and 1 μ M PSI. Data are expressed as means \pm SD of four different experiments, and P values are evaluated by two-way ANOVA followed by Fisher's PLSD

The effect of APP overexpression on the activity of proteasome was examined in the cell lysate using synthetic fluorescent substrates without addition of ATP (Fig. 5). Trypsin-like activity of proteasome was significantly

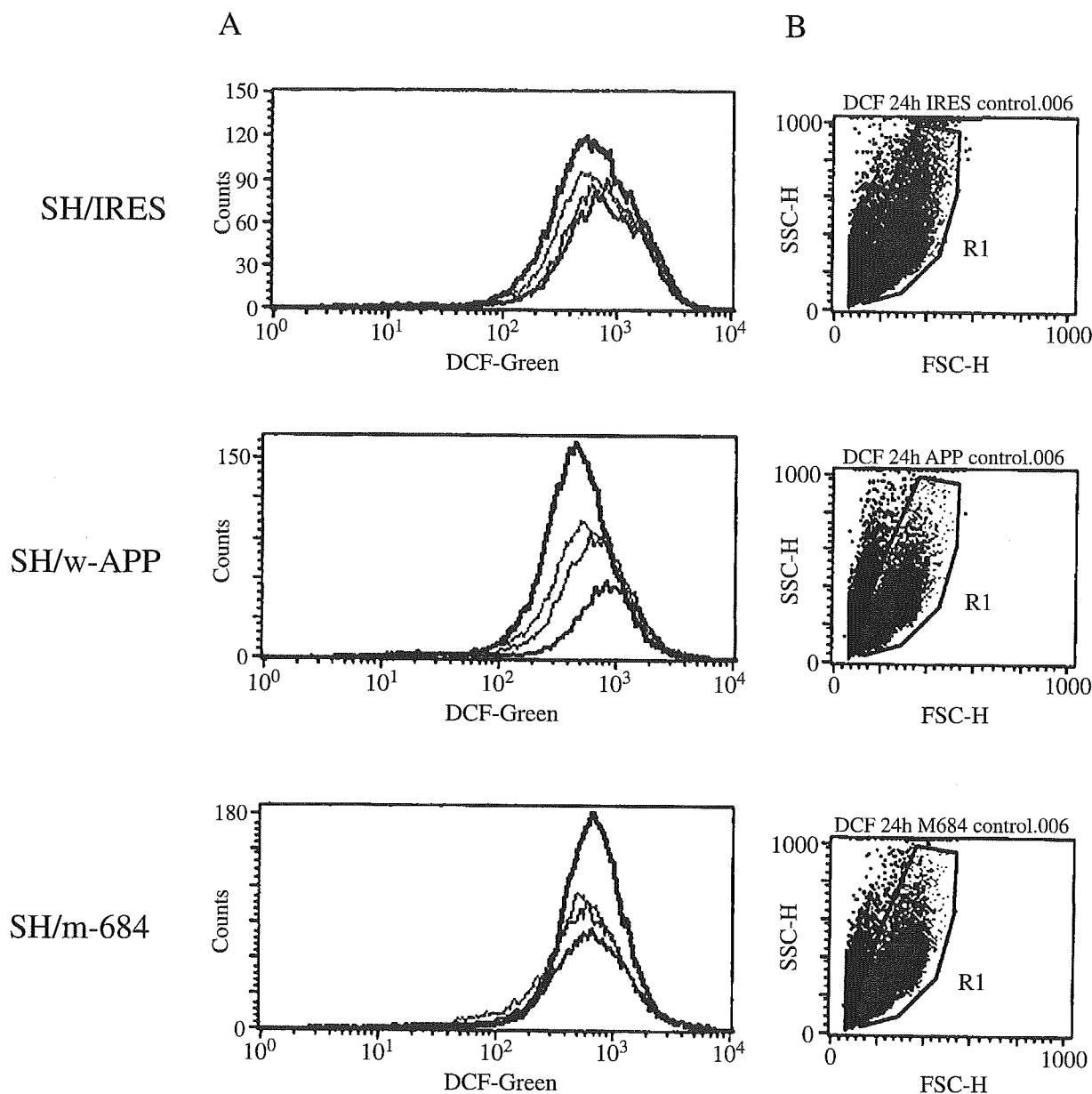


Fig. 4. The intracellular ROS-RNS levels after H_2O_2 treatment. The cells were treated with H_2O_2 for 24 h in serum-free medium. **A** The intracellular ROS-RNS levels measured by FACS using CM- H_2 DCFDA. **B** Living cells were selected by FSC (forward scattered light)-H and SSC (side scattered light)-H (right panels)

reduced in SH/w-APP cells, whereas chymotrypsin-like and postglutamyl peptidase-like activities were only slightly reduced in SH/m-684 cells.

Moreover, as summarized in Table 1, the intracellular GSH levels were higher in SH/w-APP and SH/m-684 cells than control.

Effects of APP transfection on levels of Bcl-2 protein family

To clarify more detailed mechanism underlying oxidative stress-induced cell death, we examined the expression of Bcl-2 family protein regulating mitochondrial apoptotic mechanism in these transfectants. Among

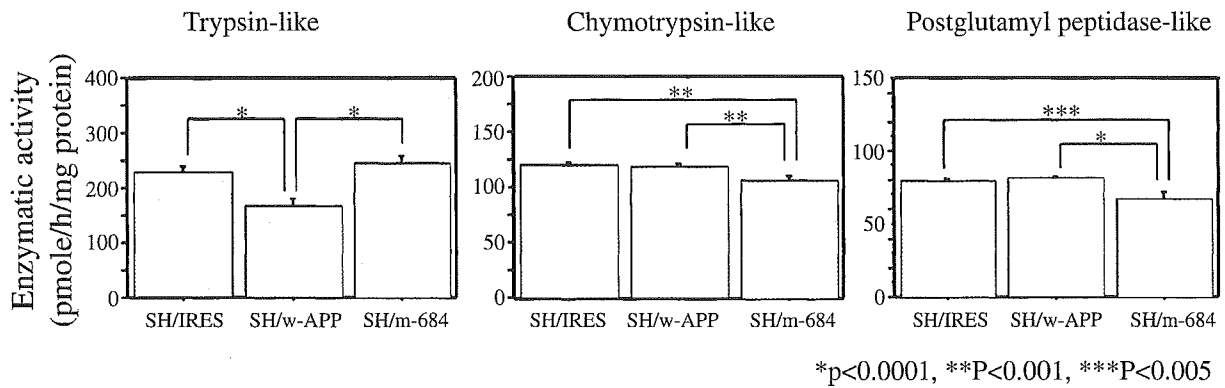


Fig. 5. The proteasome activity of the transfectants. The activities of trypsin-like, chymotrypsin-like and post-glutamyl peptidase-like proteasome were quantified by use of respective fluorescent substrates. The column and bar represent the mean and SD of four independent experiments. Difference is statistically significant

the Bcl-2 family tested, only the level of Bcl-2 protein was decreased exclusively in SH/w-APP cells, whereas it did not change in other transfectants (Fig. 6A). On the other hand, the levels of Bcl-xL and Bax were not affected by the transfection of wild and mutated APP vectors. However, the level of *bcl-2* mRNA measured by the quantitative real time RT-PCR was almost same among the transfectants (Fig. 6B). The effects of APP overexpression on the modification of Bcl-2 protein were examined in SH/w-APP cells, but we could not detect any changes in amounts of phosphorylated form of Bcl-2 protein. In the subcloned cells of SH/w-APP cells, the levels of APP were inversely correlated with those of Bcl-2, as shown in Fig. 6C.

Discussion

In this paper, it was studied whether the excess synthesis of wild-type APP/A β or of A β ₁₋₄₂ or A β ₁₋₄₀ is truly toxic and involved in neuronal cells. In SH/w-APP cells with

increased wild type APP and A β ₁₋₄₀, formation of synaptic connections was impaired. Previously, we exposed SH-SY5Y cells to synthetic A β ₁₋₄₂, but did not detect any morphological change or cell death, suggesting that increased *in situ* synthesis of APP itself may be responsible for such morphological changes. This finding may be relevant with the fact that the similar changes were detected in brains at the early stage of AD (Arendt, 2001).

In the brains from patients with AD, increased oxidative stress has been confirmed by detection of carbonyls (Smith et al., 1996), nitrated protein (Good et al., 1996; Smith et al., 1997), and protein adducts with lipid peroxidation endproducts (Sayre et al., 1997). Oxidative stress has been considered either as causes or consequences of A β production (Butterfield and Lauderback, 2002), and recently A β was reported to induce mitochondrial dysfunction through the conformational change in binding site with nicotinamide adenine dinucleotide, which may increase

Table 1. Intracellular levels of reduced glutathione in the transfectants

Transfectants	SH/IRES	SH/w-APP	SH/m-684
GSH content (nmole/mg protein)	20.05 \pm 1.62	28.30 \pm 1.73*	27.30 \pm 1.56*

The number represents mean \pm SD of 6 experiments. * p < 0.01 from SH/IRES

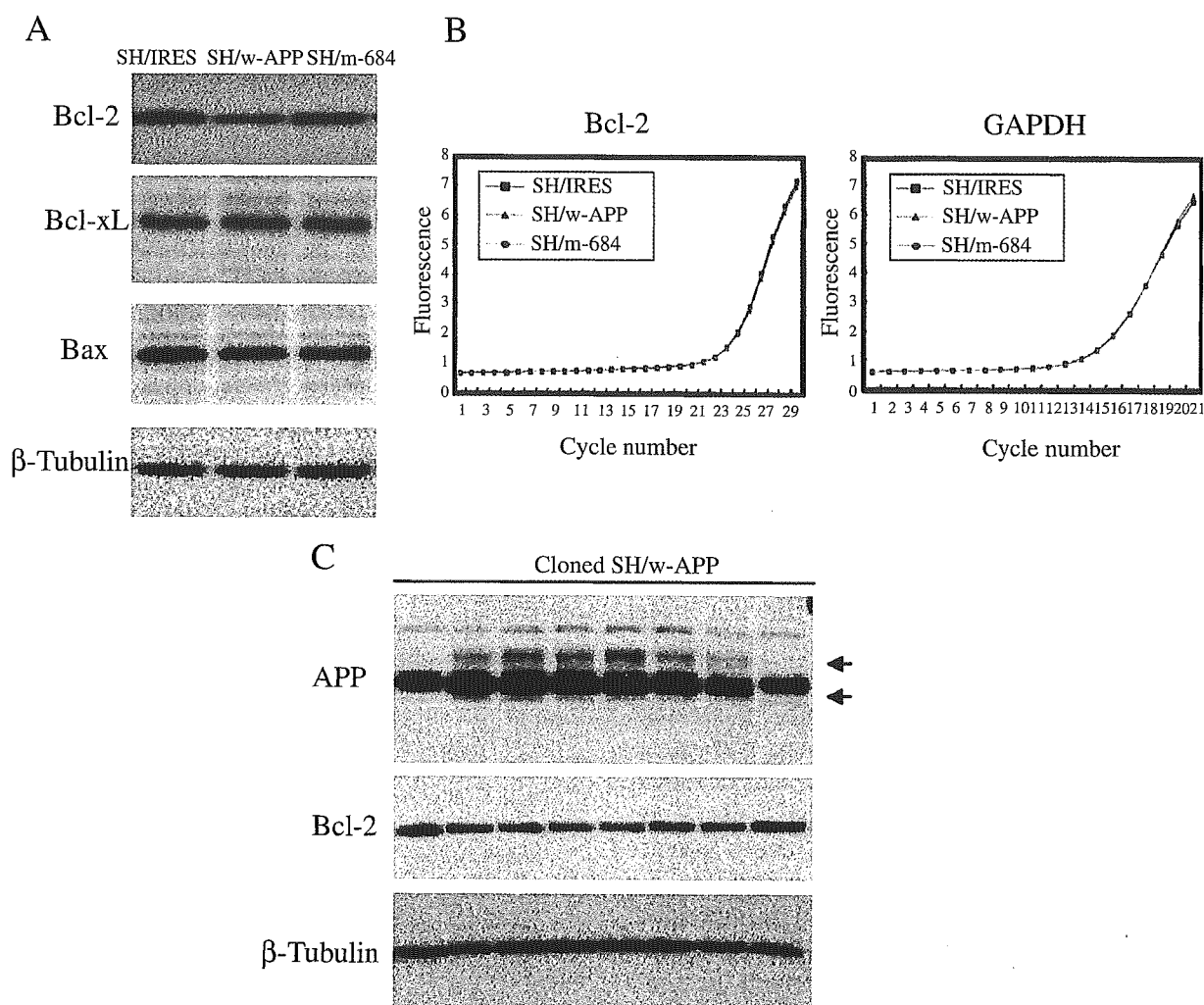


Fig. 6. The expression of apoptosis-related Bcl-2 family in the transfectants and the association of the expression between APP and Bcl-2 expression in SH/w-APP cells. **A** Western blot analysis of Bcl-2, Bcl-xL and Bax and β -tubulin used as an internal standard, in SH/IRES, SH/w-APP and SH/m-684 cells. **B** Real-time RT-PCR of *Bcl-2* and *GAPDH*. Cycle number and the fluorescent intensity corresponding to PCR product were shown.

C Western blot analysis of APP, Bcl-2 and β -tubulin in subcloned cells overexpressing APP

oxidative stress (Lusbader et al., 2004). SH/w-APP cells overexpressing APP could survive and keep proliferation, and the level of ROS-RNS was not increased in steady state, even though anti-oxidative capacity was impaired, since H_2O_2 loading increased ROS-RNS and reduced the activity of proteasome, "the second scavenger" of ROS-RNS. Exposure to H_2O_2 or a proteasome inhibitor turned the compensating state in SH/w-APP cells into un-compensating state, further increased

oxidative stress, and cell death was induced more markedly than in other transfectants.

The levels of anti-apoptotic Bcl-2 protein were reduced only in SH/w-APP, and the reduced levels were in an inverse correlation with the increased APP levels, which may be relevant with a previous report that $A\beta$ peptides down-regulated Bcl-2 and upregulated Bax expression (Paradis et al., 1996). Selective reduction of Bcl-2 protein in SH/w-APP may be relevant with the increased sensitivity

to oxidative stress in this cell line. The ability of Bcl-2 to inhibit apoptosis induced by A β was reported to depend on its expression level (Ivins et al., 1999), supporting further our view. Anti-apoptotic Bcl-2 at sufficient concentrations regulates mitochondrial PT pore, and prevents apoptosis (Maruyama et al., 2002; Akao et al., 2003). It should be emphasized that not the mRNA level but the protein level of Bcl-2 was decreased in SH/w-APP cells. Since the phosphorylated Bcl-2 was not changed in our case, another posttranslational modification(s) may be involved in reduction of Bcl-2 protein. As a conclusion, only overexpression of wild type APP altered functional and morphological properties in the cells, while expression of mutated A β increased A β ₁₋₄₂ excretion, but did not affect cell properties.

Mutations in APP, presenilin 1 and 2, and ApoE4 may increase ROS-RNS in neurons, but for a while the anti-oxidant system including the proteasome system can compensate enhanced oxidative stress. However, further insults, such as aging, other genetic or environmental factors, reduce anti-oxidant capacity further and lower the threshold to apoptosis. If we can control the increased oxidative stress and cell death using anti-oxidants (Gilgun-Sherki et al., 2003), or so-called neuroprotective agents, such as rasagiline, which induce the expression of pro-survival genes coding Bcl-2, Bcl-xL and neurotrophic factors (Akao et al., 2002; Maruyama et al., 2003, 2004; Weinstock et al., 2000), there will be a chance to protect neurons from cell death in AD. More work will be required for dissolving this problem.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (Y. A., No. 09670859), on Scientific Research (C) (W. M.) and Center of Excellence Research Grant (Y. A.) from the Ministry of Education, Culture, Sports and Science and Technology, Japan, Grant-in-Aid for Scientific Research on Priority Areas (C) from Japan Society for the Promotion of Science

(W. M.), Grant for Dementia and Bone Fracture (W. M., and M. N.) from the Ministry of Health, Labor and Welfare, Japan.

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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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Received January 10, 2005; accepted February 26, 2005
Published online April 22, 2005; © Springer-Verlag 2005

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 propioloaldehyde 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*)-sal-solinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *NM(R)Sal*] (Maruyama et al., 1996, 2001b; Naoi et al., 2002b) in human dopaminergic neuroblastoma SH-SY5Y cells. Cell death induced by *NM(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 propionaldehyde.

Materials and methods

Materials

NM(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 propionaldehyde 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% CO₂. 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 μ M *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 μ M–1 nM *N*-propargylamine at 37°C for 30 min, then with 250 μ M *NM(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 μ M 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).

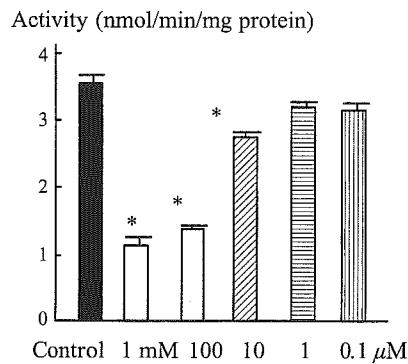
Measurement of $\Delta\Psi_m$ in mitochondria isolated from SH-SY5Y cells

$\Delta\Psi_m$ decline in isolated mitochondria by NM(R)Sal was quantitatively measured by FACS using MitoTracker Orange and Green. The mitochondria were suspended in DMEM and incubated with 10 μ M *N*-propargylamine for 30 min at 37°C, then with 250 μ M NM(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.

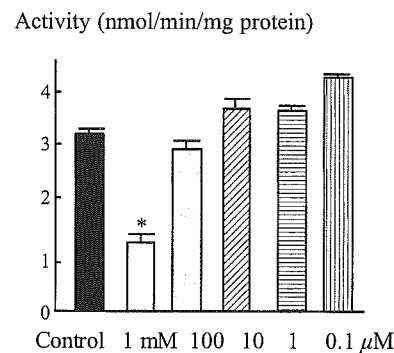
Determination of Bcl-2 protein levels in the cells treated with *N*-propargylamine

SH-SY5Y cells treated with 1 μ M–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 lysed protein (5 μ g) 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

N-Propargylamine



N-Methylpropargylamine



Propiolaldehyde

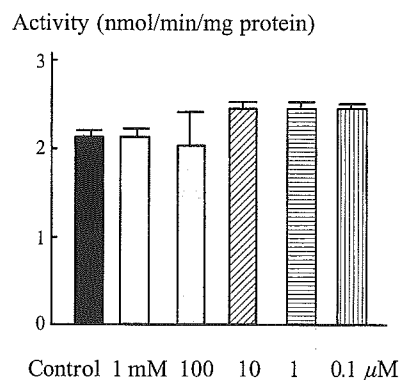


Fig. 1. The effects of *N*-propargylamine analogues on MAO-A activity. Mitochondria prepared from SH-SY5Y cells were used as MAO samples and incubated with 1 mM–0.1 μ M *N*-propargylamine analogues and 100 μ M kynuramine as a substrate at 37°C for 60 min, and the amounts of produced 4-hydroxyquinol were 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 μ M–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 μ g 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.

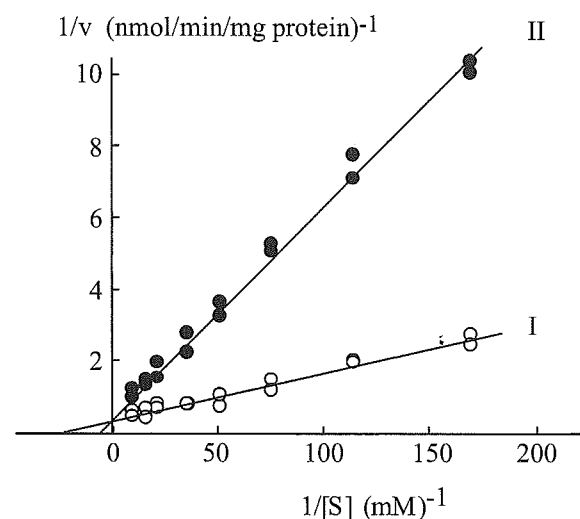


Fig. 2. The effects of *N*-propargylamine on the enzymatic activity of MAO-A. Mitochondria were prepared from SH-SY5Y cells, and MAO activity was measured fluorometrically with 8 different substrate concentrations in the absence (I) or presence of 100 μ M *N*-propargylamine (II). The double reciprocal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

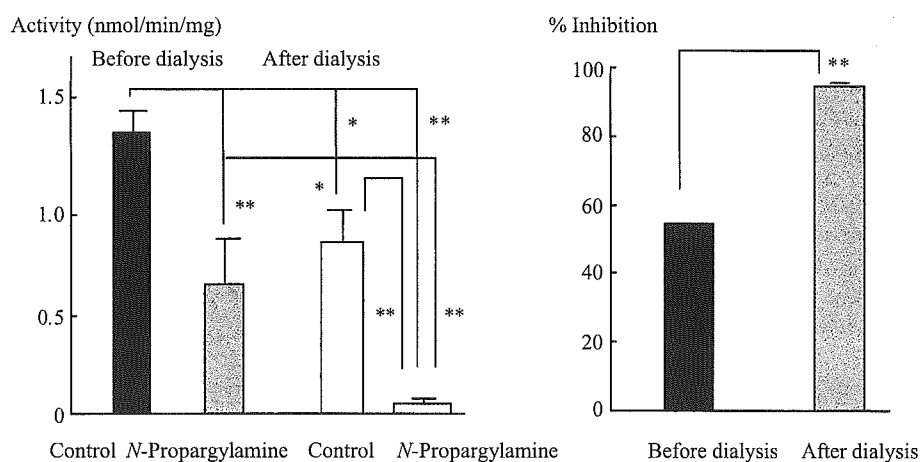
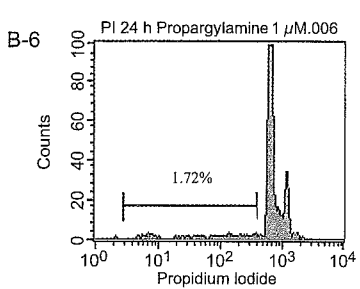
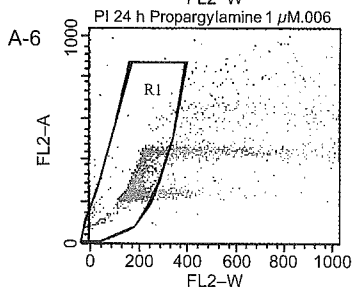
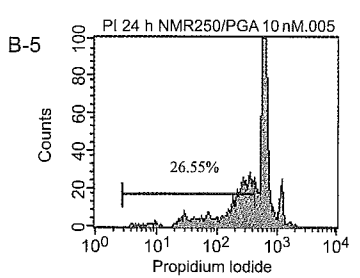
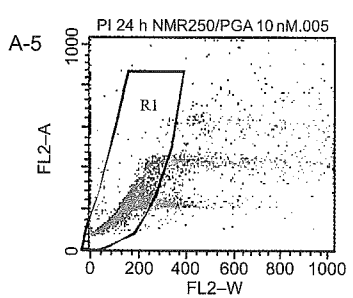
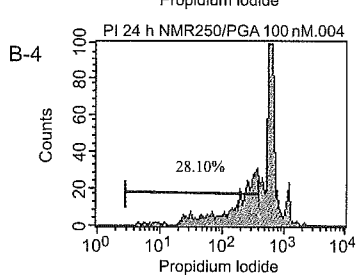
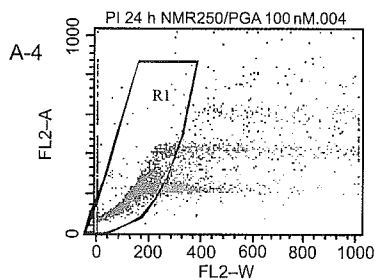
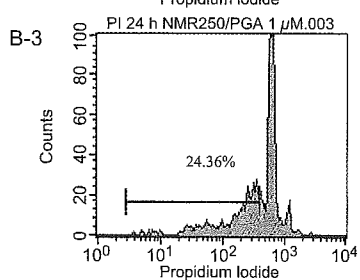
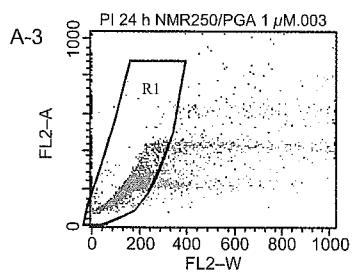
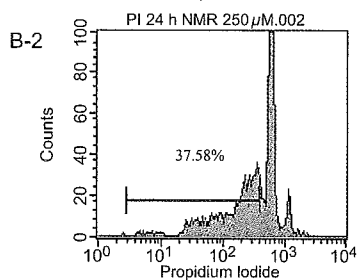
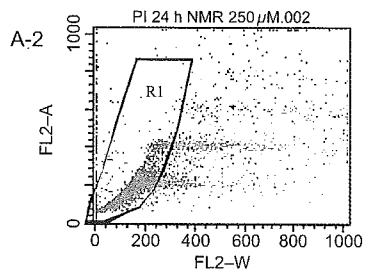
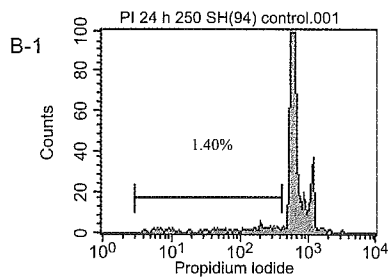
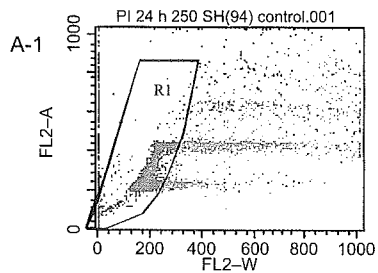


Fig. 3. Reversibility of MAO inhibition by *N*-propargylamine. MAO samples were treated with 100 μ M *N*-propargylamine for 30 min at 37°C, then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, at 4°C overnight. The MAO activity was measured with 100 μ M kynuramine. The inhibition of MAO activity 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



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 μ M, 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 μ M, whereas the values of the apparent K_m and maximal velocity, V_{max} , were 45.5 μ M 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 μ M *NM(R)Sal* resulted in apoptosis of $37.6 \pm 3.9\%$ 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

NM(R)Sal with or without 1 μ M–1 nM *N*-propargylamine. *N*-Propargylamine reduced the number of apoptotic cells to 63–70% of that of *NM(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.

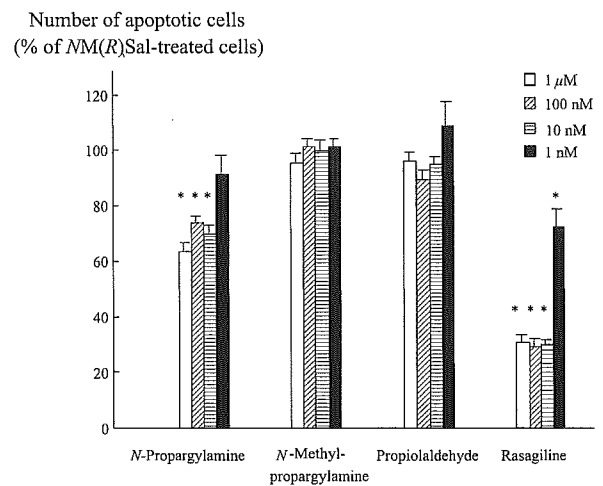


Fig. 5. The anti-apoptotic potency of *N*-propargylamine analogues and rasagiline. SH-SY5Y cells were treated with 1 μ M, 100–1 nM propargylamine derivatives, and then with 250 μ M *NM(R)Sal* overnight. The number of apoptotic cells was determined by FACS as sub G1 peak (Fig. 4) using PI as an indicator. The number of apoptotic cells was expressed as % of apoptotic cells after treated with 250 μ M *NM(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 *NM(R)Sal* alone

Fig. 4. The effects of *N*-propargylamine against apoptosis induced by *NM(R)Sal*. SH-SY5Y cells were treated with 1 μ M–10 nM *N*-propargylamine at 37°C for 30 min, then with 250 μ M *NM(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 μ M *NM(R)Sal*. #3, 4, and 5; cells pretreated with 1 μ M, 100 nM, 10 nM *N*-propargylamine, then 250 μ M *NM(R)Sal*. #6; cells treated with 1 μ M *N*-propargylamine

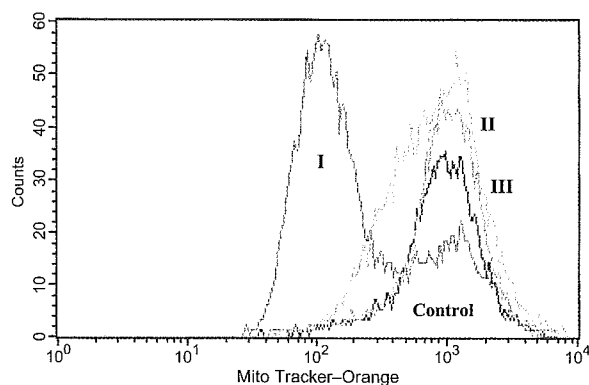


Fig. 6. The effects of *N*-propargylamine on $\Delta\Psi_m$ decline induced by *NM(R)Sal* in mitochondria prepared from SH-SY5Y cells. Mitochondria were treated for 20 min without (I) or with 1 μ M *N*-propargylamine (II), and then with 250 μ M *NM(R)Sal* for 3 h at 37°C. III: Cells treated with 1 μ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

Stabilization of $\Delta\Psi_m$ by *N*-propargylamine

NM(R)Sal at 250 μ M induced $\Delta\Psi_m$ decline in mitochondria isolated from SH-SY5Y cells, as shown by FACS MitoTracker Orange fluorescence representing $\Delta\Psi_m$ (Fig. 6). Pre-

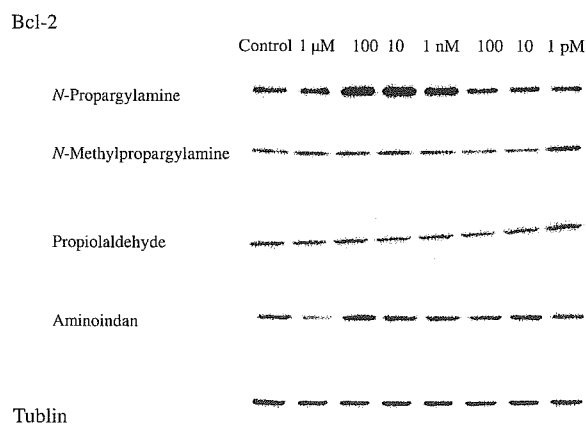


Fig. 7. The effect of *N*-propargylamine analogues and aminoindan on protein levels of Bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μ M–1 pM *N*-propargylamine analogues or aminoindan for 24 h, and Bcl-2 protein was quantified by Western blot analysis. Tublin in the cells was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

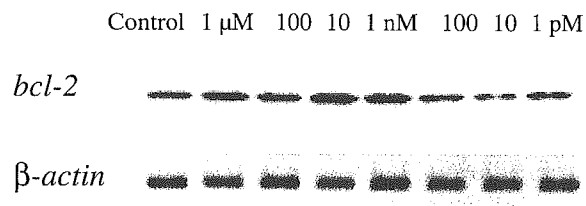


Fig. 8. The effect of *N*-propargylamine on mRNA levels of *bcl-2* in SH-SY5Y cells. The cells were cultured in the presence of 1 μ M–1 pM *N*-propargylamine for 24 h, and mRNA levels were measured by RT-PCR method, as written in Materials and methods. β -Actin mRNA was used as control

treatment of the cells with 1 μ M *N*-propargylamine markedly prevented the decline in $\Delta\Psi_m$, 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 $\Delta\Psi_m$ (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 μ M–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-aminoinidan, 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 rasagiline (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- κ B 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