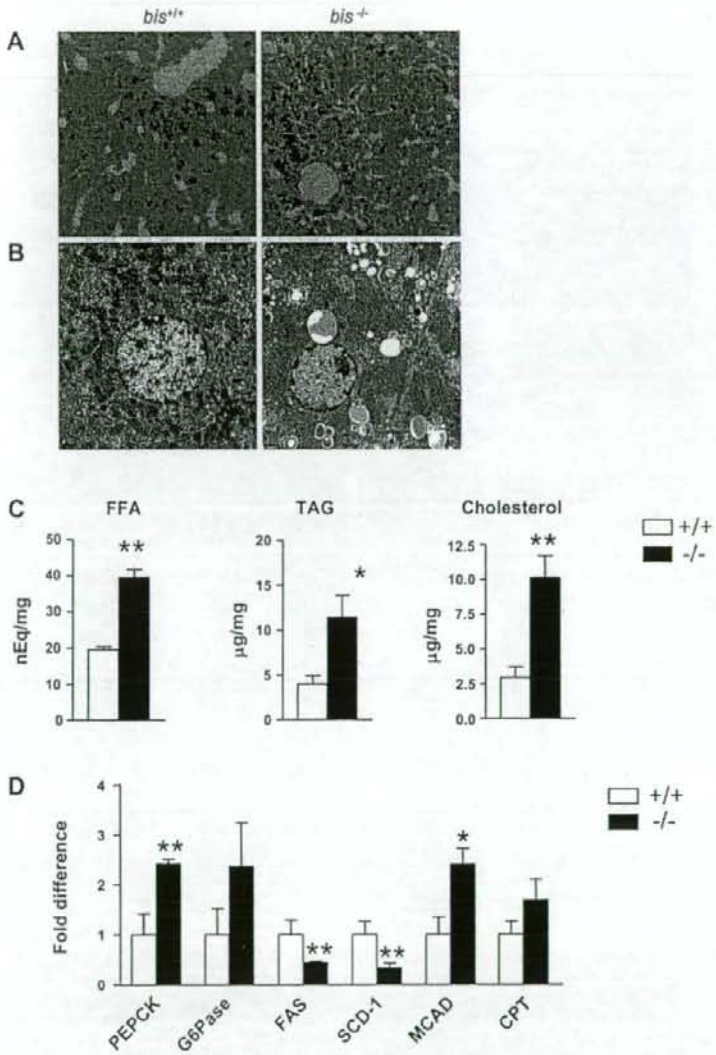

Fig. 3. Lipid accumulation in the liver of *bis*-deficient mice. *A*: Oil Red O staining of histological sections of liver from $bis^{+/+}$ and $bis^{-/-}$ mice. Red staining indicates neutral lipid accumulation. *B*: representative electron micrographs of $bis^{+/+}$ and $bis^{-/-}$ mice livers. Scale bars, 1 μm. *C*: increased levels of free fatty acid (FFA), triglyceride (TAG), and cholesterol in livers of $bis^{-/-}$ mice compared with livers of wild-type littermates. Results are expressed as means ± SE for 5 animals at 16 days of age. $*P < 0.05$, $**P < 0.01$, compared with wild-type littermates. *D*: alteration in mRNA levels of several genes involved in glucose or lipid metabolism in *bis*-deficient mice: quantitative RT-PCR of selected genes from livers of wild-type and *bis*-deficient mice. Data are means ± SE of 3 animals in each group, older than 15 days of age. Data are normalized relative to cyclophilin mRNA in the same samples, and wild-type values were arbitrarily set as 1.0. $*P < 0.05$, $**P < 0.01$, compared with wild-type littermates.

strains result in different degrees of compensatory responses, especially in response to metabolic challenges (2). For the generation of homozygous $bis^{-/-}$ mice we used heterozygous mice that were backcrossed with C57BL6 more than eight generations. Thus the effect of the Sv129 genetic background on the phenotypes of our study appeared insignificant. It is also possible that the metabolic disturbances observed in this study using biochemical and ultrastructure assays were not noticeable in the histological examinations performed by the previous research group.

The cause of death of the $bis^{-/-}$ mice was previously suggested to be respiratory failure, based on the marked degeneration of the diaphragm and intercostal muscle (10). It was also postulated that the decreased cardiac performance and subsequent pulmonary edema may have played a role in the death of the $bis^{-/-}$ mice (10). In the present study, massive apoptosis and degeneration of skeletal muscles were not observed in $bis^{-/-}$ mice (Fig. 4), suggesting that the loss of antiapoptotic activity in muscles is not the primary cause of death in these mice. Instead, the serious metabolic deterioration, such as sustained hypoglycemia and lipid accumulation in the liver, observed in our $bis^{-/-}$ mice model, may be ultimately responsible for the death of the animals.

What causes the perturbations in glucose and lipid metabolism in $bis^{-/-}$ mice? Analysis of the hepatic expression of key enzymes in the pathways of glucose and lipid metabolism

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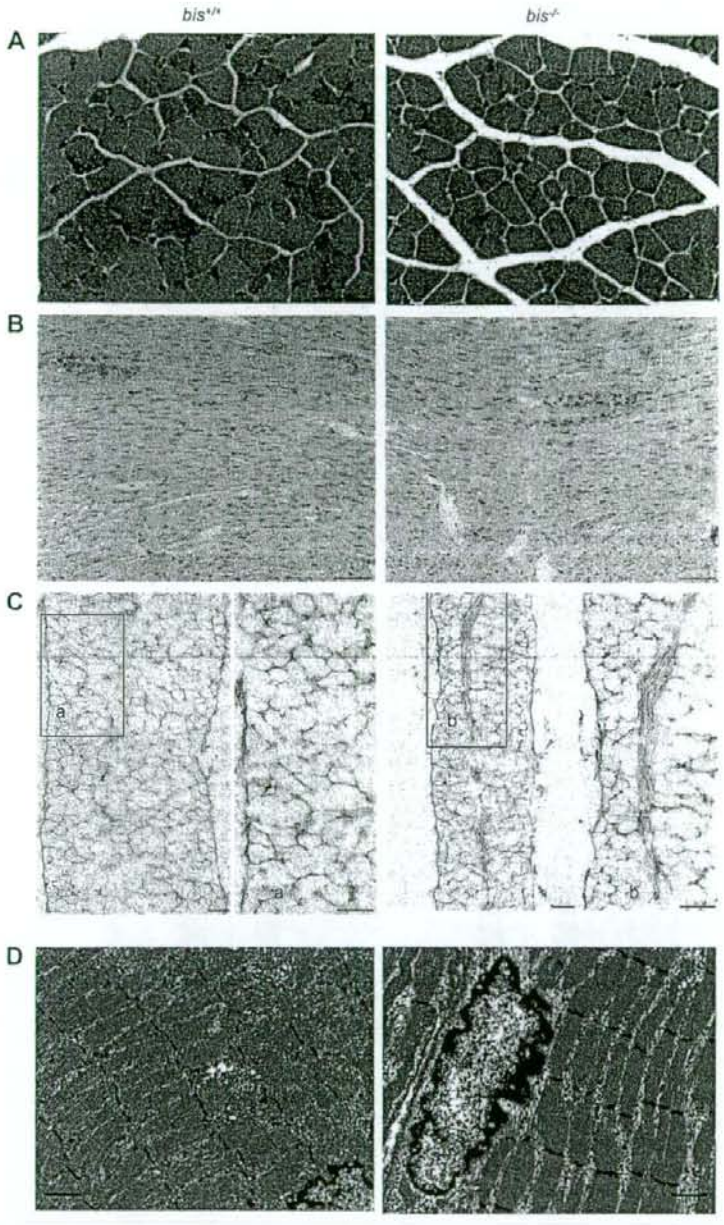

Fig. 4. No massive apoptotic features in myocytes of *bis*-deficient mice. *A*: hematoxylin and eosin staining of quadriceps femoris muscles of wild-type and *bis*-deficient mice. Scale bars, 30 μm. *B* and *C*: TUNEL staining of ventricle (*B*) and thin sections of diaphragm (*C*) of wild-type and $bis^{-/-}$ mice. *C*, *a* and *b*: Higher magnifications of boxed areas. Scale bars, 50 μm. *D*: transelectron microscopy of quadriceps femoris muscles of wild-type and *bis*-deficient mice. Scale bars, 1 μm.

revealed an increase in gluconeogenesis and lipolysis and a decrease in lipogenesis in $bis^{-/-}$ mice (Fig. 3*D*). These changes, which were also accompanied by a decrease in peripheral fat and serum triglyceride levels (Table 2), are typical of the adaptive response to a scarcity of glucose in serum that supplies the energy for gluconeogenesis in the liver, which is observed after fasting (35). Since we frequently observed that, even throughout their weight loss, $bis^{-/-}$ mice were trying to suckle, it is unlikely that isolation from the feeding mother or loss of appetite was the cause of their hypoglycemia. An impediment in the uptake or absorption of milk possibly caused delayed growth, due to an insufficiency

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of nutrients for normal growth, and substantially metabolic deterioration, the same results of fasting. Since the amounts of milk in the stomachs of $bis^{-/-}$ mice were low at ≥16 days of age and no obvious histological abnormalities were found in the intestines of $bis^{-/-}$ mice (Supplemental Fig. S2), the ingestion of milk, rather than the process of absorption, appears to be impaired in $bis^{-/-}$ mice. The hypothesis that hypophagia or dysphagia is linked to nutritional problems and growth retardation in $bis^{-/-}$ mice is supported by a previous mutation study of *starvin* (*stv*), a *Drosophila* gene encoding a Bag-domain protein (6). The Bag domain is located in the COOH terminal of Bis, shared with several proteins comprising the Bag family (33). Coulson et al. (6) showed that mutation of *stv* results in a failure of larvae to grow after hatching and a severely impaired ability to take up food. The expression of STV was shown to be highly specific in embryonic somatic muscle and tendon cells, suggesting a role in muscle development or function. However, the gross morphology and function of somatic muscles including mouth-hook movement is predominantly normal in *stv* mutants, indicating that the feeding disability of *stv* mutants is not linked to dysfunction of skeletal muscles. Thus, in light of the study of *stv* mutants of *Drosophila*, the malnutrition status observed in *bis* deficiency is associated with impairment in uptake of milk, which is probably not caused by dysfunction of skeletal muscles. However, although obvious apoptotic changes were not found in the skeletal muscles in $bis^{-/-}$ mice, it is possible that Bis deletion caused functional weakness of muscles involved in suckling or swallowing or abnormal esophageal motor function shown in achalasia, a esophageal motility disorder in humans (16). Therefore, the role of Bis in the physiological regulation of swallowing remains to be elucidated.

We also observed a dramatic involution of the thymus and spleen in mice with a homozygous *bis* gene deletion (Fig. 2, *C* and *D*). At present, the direct link between the two representative phenotypes of $bis^{-/-}$ mice, metabolic deterioration and involution of the thymus and spleen, remains unclear. The thymus has been shown to be significantly affected in malnutrition, undergoing a severe atrophy due to apoptosis-induced thymocyte depletion (22, 24, 30). We showed that the reduction in the relative weight of the thymus and spleen was not obvious until 12 days after birth (Fig. 2*E*), at a time when body weight was still increasing and the serum glucose level was within the normal range (Fig. 2*A* and data not shown). Thus the involution of the thymus and spleen appears to be directly or indirectly linked to the nutritional status of $bis^{-/-}$ mice. Shrinkage of the thymus and spleen has also been described in *bcl-2*-deficient mice (13, 36). Since Bis binds Bcl-2 (18), interaction between Bis and Bcl-2 may be required for normal physiology of these lymphoid organs. However, $bcl\text{-}2^{-/-}$ mice have selective lymphopenia, but $bis^{-/-}$ mice have an overall decrease in white blood cells (Table 1). Furthermore, thymic and hepatic levels of Bcl-2 and HSP70, another Bis binding partner (33), were not decreased in protein extracts from *bis*-deficient mice in a Western blot analysis (data not shown). Thus the phenotypes observed in $bis^{-/-}$ mice are not mainly due to the disruption of the interaction between Bis and Bcl-2, or HSP70, but due to the specific effect of ablation of *bis* gene.

Bis has been shown to be highly expressed in lymphocytic leukemia cells, and downmodulation of Bis increases susceptibility to apoptosis in normal and neoplastic leukocytes (26–28). Therefore, our results showing significant decrease in leukocytes in peripheral blood cells from $bis^{-/-}$ mice support the previous reports for survival-sustaining roles of Bis in leukocytes. However, it is not certain whether the absence of Bis affects the viability of peripheral leukocytes or the function of progenitor cells in bone marrow. Thus, with the shrinkage of lymphoid organs, the decreases in the leukocyte numbers in $bis^{-/-}$ mice suggest the expanded roles of Bis in the physiology of hematopoietic cells and in the development of lymphoid organs, not confined to prosurvival activity of lymphocytes.

In this study, we generated *bis*-deficient mice and demonstrated that *bis* ablation resulted in growth retardation and early lethality with serious metabolic deterioration and involution of the thymus and spleen. Our results suggest that Bis is critical for postnatal growth and survival. However, the critical role for Bis in the regulation of feeding and the physiology of the thymus and spleen, which may or may not be linked, remains to be fully defined.

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Neuroprotection by propargylamines in Parkinson's disease: intracellular mechanism underlying the anti-apoptotic function and search for clinical markers

M. Naoi[1], W. Maruyama[2], H. Yi[1], Y. Akao[1], Y. Yamaoka[1], M. Shamoto-Nagai[2]

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

Summary In Parkinson's and other neurodegenerative diseases, a therapeutic strategy has been proposed to halt progressive cell death. Propargylamine derivatives, rasagiline and (−)deprenyl (selegiline), have been confirmed to protect neurons against cell death induced by various insults in cellular and animal models of neurodegenerative disorders. In this paper, the mechanism and the markers of the neuroprotection are reviewed. Propargylamines prevent the mitochondrial permeabilization, membrane potential decline, cytochrome c release, caspase activation and nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase. At the same time, rasagiline induces anti-apoptotic pro-survival proteins, Bcl-2 and glial cell-line derived neurotrophic factor, which is mediated by activated ERK-NF-κB signal pathway. DNA array studies indicate that rasagiline increases the expression of the genes coding mitochondrial energy synthesis, inhibitors of apoptosis, transcription factors, kinases and ubiquitin-proteasome system, sequentially in a time-dependent way. Products of cell survival-related gene induced by propargylamines may be applied as markers of neuroprotection in clinical samples.

Keywords: Apoptosis, propargylamine, rasagiline, mitochondria, permeability transition pore, GDNF, Bcl-2, nuclear transcription factor

Abbreviations

ANT	adenine nucleotide translocator
BDNF	brain-derived neurotrophic factor
BPAP	1-(benzofuran-2-yl)-2-propylaminopentane
CyP-D	cyclophilin-D
CsA	cyclosporin A
$\Delta\Psi m$	mitochondrial membrane potential
FACS	fluorescence-augmented flow cytometry
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDNF	glial cell-line derived neurotrophic factor
R-2HMP	*N(R)*-(2-heptyl)-*N*-methyl-propargylamine
IL	interleukin
MAO-A and *MAO-B*	type A and B monoamine oxidase
MAP	mitogen-activated protein
MEM	Hanks' minimum essential medium
mPT	mitochondrial permeability transition
NM(R)Sal	*N*-methyl(*R*)salsolinol
PD	Parkinson's disease
PI	propidium iodide
TNF	tumor necrosis factor
VDAC	voltage-dependent anion channel

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

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease and affects 1–2% of the aged population. PD is pathologically characterized by selective cell death of dopamine neurons in the substantia nigra pars compacta, and biochemically by depletion of dopamine neurotransmitter in the striatum. The etiology for the sporadic form of PD remains enigmatic, whereas a growing understanding of responsible genes for familiar forms of PD suggests that the processes leading to neuronal loss may be common with those in the sporadic form of PD (Eriksen et al., 2005; Vila and Przedborski, 2004). The loss of nigral dopamine neurons in PD is hypothesized as the mutations in genes detected in the familiar form sensitizes the neurons to intrinsic and extrinsic insults. Increased oxidative stress, mitochondrial dysfunction, impaired ubiquitine-proteasome system, abnormal inflammatory cytokines, and excitotoxicity are considered to cause cell death in dopaminergic neurons, in which dopamine itself should be involved by not fully clarified mechanisms. At present, available therapies for patients with PD are limited to ameliorate the symptoms. Dopamine replacement relieves the major symptoms at least for the beginning several years. However,

progressive loss of dopamine neurons results in motor fluctuation and cognitive dysfunction, hallucinations, depression and dementia. A therapy intervening the disease progress itself is now seriously required, and "neuroprotective" therapy to rescue neurons from cell death and "neurorestrorative" therapy to restore deteriorated neurons to a normal state have been proposed (Dawson and Dawson, 2002). The therapy should target intracellular death cascade, which is activated rather slowly for decades to the end point showing the clinical signs and regulated by well-conserved and -regulated cell death system (Riederer, 2004). Using cellular and animal PD models, the molecular mechanisms behind neuronal loss have been intensively studied, and several agents have been confirmed to prevent the cell death processing. In order to ameliorate the pathogenic factors, neuroprotective agents have been proposed, including antioxidants, neurotrophic factors, anti-inflammatory drugs, mitochondria supplement, inhibitors of monoamine oxidase (MAO), and drugs interfering glutamate excitotoxicity. Since signal proteins for apoptosis increase in the nigral neurons of Parkinsonian brains, anti-apoptotic agents altering apoptotic signal pathway have been gathering attention (Maruyama et al., 2002a; Mandel et al., 2003; Simpkins and Jankovic, 2003; Youdim et al., 2006). The anti-apoptotic function is confirmed in inhibitors of type B MAO (MAO-B) and caspase inhibitors, immuno-modulators, Co-Q10, NMDA receptor antagonists and neurotrophic factors in cellular and animal model systems. Recently, several clinical trials were reported to examine effects of propargylamine MAO-B inhibitors, rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Youdim et al., 2001) and (−)deprenyl [selegiline, *N*, α-dimethyl-*N*-2-propynylbenzene-ethanolamine], in Parkinsonian patients, and beneficial effects were confirmed to slow the progression of the symptoms (Parkinson Study Group, 2004, 2006; Pälhagen et al., 2006). However, the final conclusion about the neuroprotective efficiency remains to be clarified (Riederer et al., 2004; Schapira and Olanow, 2004; Suchowersky et al., 2006).

Rasagiline and (−)deprenyl were applied in PD to increase dopamine availability through inhibiting the oxidative deamination by MAO (Birkmayer et al., 1977). In addition, MAO-B inhibitors inhibit the oxidation of protoxicants to toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-phemylpyridinium ion (MPP^+), scavenge reactive oxygen species, and prevent the lipid peroxidation and the formation of toxic dopamine quinone. Later clinical observations suggest that they may protect neurons against cell loss in PD, AD and other neurodegenerative disorders. We studied the mechanism behind protection of rasagiline against apoptotic or necrotic cell death induced in human neuroblastoma SH-SY5Y cells by oxidative stress (Maruyama et al., 2002c) and neurotoxins, such as *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] (Naoi et al., 2002a) and 6-hydroxydopamine (6-OHDA) (Maruyama et al., 2001b, 2002b). *N*M(*R*)Sal binds to type A MAO (MAO-A) in mitochondrial outer membrane, opens a mega-channel called mitochondrial permeability transition (mPT) pore, initiates rapid reduction of mitochondrial membrane potential, $\Delta\Psi m$, and swelling of mitochondria (Akao et al., 2002a; Maruyama et al., 2002a; Naoi et al., 2006; Yi et al., 2006a). Induction of mPT results in the cytochrome c release signaling subsequent apoptosis, or the loss of ATP production leading to necrosis. Bcl-2 protein family in mitochondria directly regulates the apoptotic pathway, and intracellular signaling strictly regulates the synthesis and posttranslational modification. Neuroprotective agents intervene these apoptotic processes, either by suppressing apoptogenic factors or increasing pro-survival, anti-apoptotic factors in cells.

In this paper, our recent understanding on the mechanism underlying anti-apoptotic function of propargylamines is reviewed. The effects of propargylamine derivatives were examined in relation to the regulation of mPT and the induction of pro-survival proteins, Bcl-2 and neurotrophic factors. To confirm the involvement of cell signaling, gene expression by the propargylamines was studied by cDNA array analyses. Hitherto clinical studies indicate that the more quantitative, biochemical and molecular evaluation is required to confirm the neuroprotection in Parkinsonian patients. Our recent results by use of primate suggest that gene products increased by rasagiline in the CSF and serum may be used as clinical markers to quantify the potency of putative neuroprotective drugs in clinical samples. The expected future development of neuroprotective therapy is discussed.

Materials and methods

Materials

Rasagiline and related compounds were kindly donated by Teva Pharmaceutical (Netanya, Israel). *N*-Propargylamiine and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA); JC-1, Hoechst33342, MitoTracker Orange and Green, and Rhodamine 123 from Molecular Probes (Eugene, OR, USA). Anti-Bcl-2 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); anti-β-actin antibody from Oncogene (Boston, MA, USA); mouse monoclonal anti-GAPDH antibody from Chemicon International (Temecyla, CA, USA). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO_2. Bcl-2 was overexpressed in SH-SY5Y cells as reported previously (Akao et al., 2002a). Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999).

Determination of apoptosis

Apoptotic and necrotic cell death were assessed quantitatively using fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA) (Yi et al., 2006a). To determine apoptotic cells, the cells were stained with PI solution in phosphate-buffered saline (PBS) containing 1% Triton X-100 and subjected to FACS analysis. Cells with a lower DNA content showing less PI staining than G1 were defined to be apoptotic (subG1 peak) according to Eckert et al. (2001).

Measurement of mitochondrial membrane potential, $\Delta\Psi m$

The $\Delta\Psi m$ in isolated mitochondria was quantified by FACS using MitoTracker Orange and Green. The mitochondria were treated with agents at 37°C for 3 h, and stained with 100 nM MitoTracker Orange and Green, then 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. In other experiments, mitochondria were prepared from male Donryu rat liver or transgenic mice expressing human Bcl-2 in the liver, as previously described (Shimizu et al., 1998). $\Delta\Psi m$ was assessed also by measurement of reduction in Rhodamine 123 fluorescence, which was ascribed to $\Delta\Psi m$-dependent uptake of Rhodamine 123 into the mitochondria (Narita et al., 1998).

Measurement of mRNA and protein of Bcl-2 family proteins

SH-SY5Y cells were cultured in the presence of various concentrations (10 μM–1 pM) of rasagiline for 24 h or for a various incubation time with 100 nM rasagiline. The whole cells were gathered and the total RNA was extracted by the phenol/guanidinium thiocyanate method. The cDNA was generated by reverse transcription of the total RNA, and the cDNA fragments were amplified using the PCR primers (Akao et al., 2002b). PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin cDNA was used as an internal standard.

Quantitative measurement of mRNA and protein of GDNF

SH-SY5Y cells were treated with rasagiline in 96 well plates with Hanks' minimum essential medium (MEM). The effect of sulfasalazine (100 μM), an inhibitor of IκB, was examined by adding the inhibitor 30 min before the treatment with rasagiline. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). Samples or standard were added to GDNF antibody-coated wells, and incubated for 12–18 h at 4°C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boehringer Mannheim) for 1 h. The enzyme activity in each well was measured by incubation with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. The fluorescence intensity of produced 4-methylumbelliferone was measured at 360 nm with excitation at 448 nm. The mRNA of GDNF was measured by reverse transcription-polymerase chain reaction (RT-PCR), as reported (Maruyama et al., 2004a).

Quantitation of activated NF-κB

Activation of NF-κB was determined by NF-κB binding to κB sites using NF-κB p65 transcription assay kit (Active Motif, Carlsbad, CA, USA) (Maruyama et al., 2004a). Five μg of the extract of Hela cells stimulated with TNF-α for 30 min was used as a positive control. The activation of NF-κB was expressed as % of the positive control.

cDNA array for gene expression in apoptosis

The cells were incubated with 100 nM rasagiline for 6, 12, and 24 h, and the total RNA was extracted. Using AMV reverse transcriptase, total RNA isolated from the sample and control was labeled with Cy3- or Cy5-dUTP. The levels of gene expression were quantitatively analyzed by cDNA expression array using TaKaRa IntelliGene Human Apoptosis CHIP (Takara Biomedicals, Ohtsu, Japan).

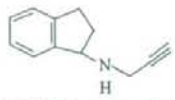
Statistics

Experiments were repeated at least 4 times and the results were expressed as mean and SD. Difference was 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

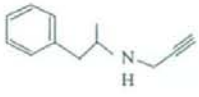
Stabilization of mitochondrial contact sites by propargylamines

A series of propargylamines, rasagiline, (−)deprenyl, aliphatic (*R*)*N*-(2-heptyl)-*N*-methylpropargylamine (*R*-2HMP) and free *N*-propargylamine, prevent the activation of apoptotic cascade and protect SH-SY5Y cells against apoptosis induced by neurotoxins, *N*M(*R*)Sal and 6-OHDA, and oxidative stress caused by dopamine oxidation and a peroxynitrite-generating agent, SIN-1 (Akao et al., 2002a; Maruyama et al., 2002a, b, c; Yi et al., 2006b). Figure 1 shows the chemical structure of examined propargylamines. An endogenous neurotoxin *N*M(*R*)Sal induces the mPT and apoptosis (Naoi et al., 2002b, 2006). As summarized in Fig. 2, these propargylamines completely suppress opening of mPT pore caused by neurotoxins and oxidative stress. Rasagiline inhibits mitochondrial swelling and $\Delta\Psi m$ reduction (Akao et al., 2002a), and prevents release of cytochrome c, caspase 3 processing and nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Maruyama et al., 2002a). Rasagiline protected MAO-A-expressing SH-SY5Y cells from apoptosis and transfection-enforced expression of MAO-B did not increase the sensitivity to rasagiline, indicating that neuroprotective function does not depend on the MAO-B inhibition (Yi et al., 2006a). On the other hand, clorgyline [*N*-methyl-*N*-propargyl-3(2,4-diclorophenpxy)-propylamine] did not prevent, but induced mPT. Table 1 shows the results on the structure-activity relationship for direct stabilization of mPT among propargylamine derivatives with different hydrophobic structure, indanyl (rasagiline), phenyl (deprenyl), aliphatic (2-HMP) and benzofuranyl groups [1-(benzofuran-2-yl)-2-propylaminopentane, BPAP]. The aminoindan derivatives are the most active followed by the phenyl derivatives, and the derivatives with aliphatic and benzofuranyl structures require rather high concentrations for preventing mPT. The modification of aminoindan ring does not affect the potency to stabilize mPT pore, as shown

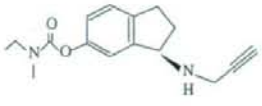


N-Propargl-1(*R*)-aminoindan (Rasagiline)
(*S*)- (TV1022)

N-Propargylamine

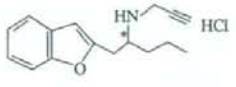


(-)Deprenyl (selegiline)



TV 3326 (*R*-), TV 3279 (*S*-)

N-(2-Heptyl)-*N*-methylpropagylamine
(2-HMP)



FPFS-1180 (*R*-), FPFS-1182 (*S*)

Fig. 1. Chemical structures of propargylamines with neuroprotective potency

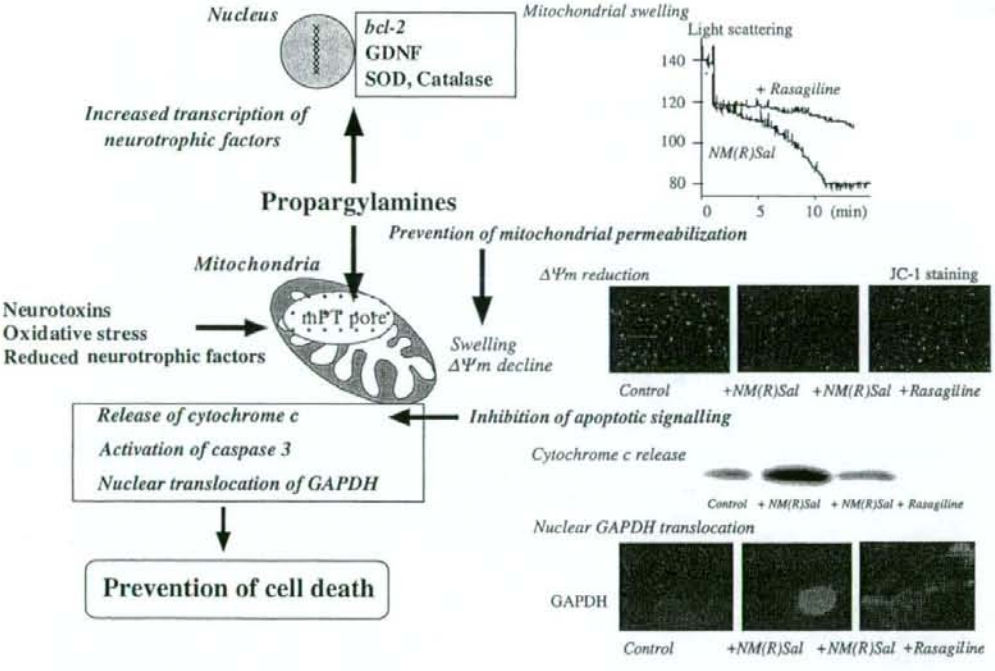


Fig. 2. Target sites of neuroprotective propargylamines in apoptosis cascade. Rasagiline and related compounds suppress mPT, as shown by prevention of mitochondrial swelling and $\Delta\Psi$m reduction. They inhibit also cytochrome c release, caspase 3 activation and nuclear GAPDH translocation. In addition, the propargylamines increase the expression of anti-apoptotic Bcl-2 family protein, neurotrophic factors (GDNF, BDNF), and antioxidant enzymes (SOD, catalase)

with TV 3326 [(*N*-propargyl)-(3*R*)-aminoindan-5-yl]-ethymethyl carbamate and its hydroxyl metabolite, TV 3294 (Maruyama et al., 2003). In general, the *R*-enantiomers are more potent to prevent the mPT than the *S*-enantiomers (Maruyama et al., 2001a, b). The *S*-enantiomer of rasagiline, TV1022, lacks the MAO inhibiting function, but it still

Table 1. *Structure and neuroprotective characteristics of propargylamines*

Name [Structure]	Prevention of mPT	Induction of Bcl-2	Induction of GDNF
Rasagiline [*R*(+)-*N*-propyl-1aminoindan]	10 μM–1 nM	10 μM–1 nM, 10–1 pM	1 μM–100 pM
TV1022 [*S*(−)-*N*-propyl-1-aminoindan]	1 μM–100 nM	–*	–
Aminoindan	–	–	–
N-Propargylamine	1 μM–10 nM	100–1 nM	N.D.**
N-Methylpropargylamine	–	–	N.D.
Propiolaldehyde	–	–	N.D.
(−)Deprenyl	1 μM–100 nM		1 μM–10 nM
(+)Deprenyl	10 μM	–	–
Desmethyldeprenyl	10–1 nM		1 μM–10 nM
TV3326 [5-ethyl ethyl carbamate-rasagiline]	100–10 nM	–	–
TV3294 [5-hydroxyl-rasagiline]	100–10 nM	–	–
R-*N*-(2-Heptyl)-*N*-methylproparylamine	1 μM–100 nM	N.D.	N.D.
S-*N*-(2-Heptyl)-*N*-methylproparylamine	10 μM	N.D.	N.D.
R-*N*-(2-Heptyl)-propargylamine	1 μM–100 nM	N.D.	N.D.
R-3-(2-Heptylamine)-*N*-methylpropionic acid	–	N.D.	N.D.
R-(−)-BPAP	–	100–1 nM	1 nM***
S-(+)-BPAP	1 μM–10 nM	–	N.D.
R-(+)-*N*-(2-propynyl)-BPAP	1 μM–10 nM	100–1 nM	N.D.
S-(−)-*N*-(2-propynl)-BPAP	–	–	N.D.

* Not affective, ** not determined, *** Hirai et al. (2005).

prevents mPT, suggesting again that the anti-apoptotic function is not related to the MAO inhibition. In the case of the benzylfuranyl derivatives, the stabilization of mPT pore depends on the absolute structure of propargylamines. The compounds with dextro-rotation prevented $\Delta\Psi m$ decline by neurotoxins, whereas the corresponding enantiomer with levo-rotation did not (Maruyama et al., 2004b). The propargylamine group is essentially required for the activity as in the case with free *N*-propargylamine itself, whereas the analogues without a propargyl residue, aminoindan and *R*-3-(2-heptylamino)-propionic acid, did not prevent mPT. The methylation of the amino residue in *N*-propargylamine abolished the activity to prevent $\Delta\Psi m$ reduction (Yi et al., 2006b).

The precise mechanism leading to the permeabilization of mitochondria is still unclear, even though several models have been proposed. The mPT pore is primarily composed of adenine nucleotide translocator (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane, which binds to ANT at the contact sites between the inner and outer membrane. In addition, peripheral benzodiazepine receptor (PBR) and MAO in outer membrane and hexokinase at the contact site are associated with the mPT pore. Cyclophilin-D (CyP-D) binds to the matrix site of ANT and induces conformation change to form a non-specific pore leading to release of any molecules of less than 1.5 kDa, and metabolic gradients across the inner membrane are dissipated, with accumulation of Ca^{2+}. Opening of the mPT pores results in swelling of the matrix and rupture of the outer membrane, which leads to the release of apoptogenic factors (cytochrome c, apoptosis-inducing factor, Smac/DIABLO, Omi/HtrA2) resulting in activation of caspase system. Oxidative stress and other insults facilitate the mPT pore opening though cross-linking of thiol groups of cysteine residues in ANT and increases the binding of CyP-D to the ADP binding site (McStay et al., 2002). Neurotoxins, PBR ligands (PK11195, protophorphirin IX), bax and other pro-apoptotic Bcl-2 protein family, heavy metals, inorganic phosphate, fatty aids, quinones and uncouplers of mitochondrial oxidative phosphorylation system induce mPT. On the other hand, viral proteins, such as HIV viral protein R (Jacotot et al., 2001) and myxoma poxvirus protein, M11L (Everett et al., 2002), bind to the CyP-D binding site and prevent the pore formation. Another model of mPT is that Bcl-2 interacts directly with VDAC and regulates ANT activity, which was proved in a model system composed of VDAC in liposomes (Shimizu et al., 1999; Tsujimoto and Shimizu, 2000). According to this model, VDAC interacts with apoptogenic Bax and Bak, functions as "VDAC modulators", changes its conformation leading to formation of a megachannel to allow cytochrome c to pass through, whereas anti-apoptotic Bcl-xL closes the channel. In this case, the outer membrane might be intact without rupture. More recently, lipid bilayer was proposed to play an important role in mPT by interacting with ANT or other mitochondrial components (Lucken-Ardjomande and Martinou, 2005).

*N*M(*R*)Sal binds to MAO-A in the outer membrane and opens mPT pore, which CsA and bongkrekic acid antagonize through binding to CyP-D and ANT. *N*M(*R*)Sal, dopamine and its oxidation product quinone, neuromelanin, and peroxynitrite modify sulfhydryl (SH) groups in mitochondria

and induce mPT (Yi et al., in preparation). Rasagiline prevents the reduction of free SH residues in mitochondria and the mPT, regardless of the types of insults leading to mPT (toxins, PBR ligands and oxidative stress). Rasagiline is bound to MAO-B, MAO-A, or other components in mPT pore, stabilizes the contact site and prevents the conversion of ANT into a pro-apoptotic pore. The study is under way whether rasagiline can bind directly to ANT or CyP-D. In addition, propargylamines bind to several other proteins in cells. (−)Deprenyl and its analogue TCH346 [CGP3466, dibenzo(*b,f*)oxepin-10-yl-methyl-methyl-prop-2-ynyl-amine], bind to GAPDH, and prevent the *S*-nitrosylation of GAPDH, the binding to Siah and its nuclear translocation (Hara et al., 2006). Another candidate binding site is poly(ADP-ribose)-polymerase-1 (Brabeck et al., 2003). However, in apoptotic processes these putative binding sites are downstream of mPT and our results demonstrate that the binding of rasagiline to mitochondrial protein and the regulation of mPT are the primary events in preventing apoptosis.

Induction of neuroprotective Bcl-2 family proteins

It is well known that some kinds of protein, Bcl-2 family protein, anti-oxidants and neurotrophic factors, alleviate neuronal loss through suppression of oxidative stress, prevention of apoptotic signal transduction and promotion of cell survival. Rasagiline, and (−)deprenyl increase the activity of anti-oxidative enzymes, superoxide dismutase (SOD) and catalase, in the rat brain after the systemic administration (Carrillo et al., 2000, Kitani et al., 2000). (−)Deprenyl and desmethyldeprenyl increase mRNA level of SOD 1 and 2, Bcl-2 and Bcl-xL, nitric oxide synthase, c-JUN, and NAD dehydrogenase in PC12 cells (Tatton et al., 2002). Our and Youdim's group have clarified the detailed mechanism underlying the induction of anti-apoptotic proteins by rasagiline analogues.

The family of Bcl-2-related proteins constitutes one of biologically most relevant regulatory gene products against apoptosis through controlling mitochondrial permeabilization (Kroemer, 1997). Bcl-2 family proteins are subdivided into three groups on the basis of the pro- and anti-apoptotic function and the Bcl-2-homology (BH) domains (BH1 to BH4). Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) have 4 BH domains, whereas pro-apoptotic multidomain protein (Bax, Bak, Bok/mtd) lacks BH4. BH3 only proteins (Bid, Bim/Bod, Bad, Bmf) are also pro-apoptotic and link specific apoptotic stimuli to mPT. Bcl-2 is mainly localized in the mitochondrial inner membrane, and the family proteins form homo- or hetero-dimers between anti- and pro-apoptotic members and determine cellular sensitivity to apoptotic stimuli by titrating one another's function. Anti-apoptotic Bcl-2 family proteins prevent apoptosis either by inhibiting pro-apoptotic Bcl-2 members directly, controlling endoplasmic reticulum and mitochondrial homeostasis, or defending against oxidative stress. On the other hand, pro-apoptotic Bcl-2 family proteins induce mPT and trigger the release of mitochondrial apoptogenic factors into the cytosol, as discussed above.

Overexpression of Bcl-2 protects various neuron paradigms *in vivo* and *in vitro* from death induced by neurotoxins and other insults. Bcl-2-overexpression in SH-SY5Y cells prevented apoptosis induced by *N*M(*R*)Sal, which is relevant with the results that $\Delta\Psi$m decline induced by *N*M(*R*)Sal was suppressed in mitochondria prepared from Bcl-2 overexpressed mouse liver (Akao et al., 2002a; Maruyama et al., 2002a). These results suggest that rasagiline may induce Bcl-2 protein, in addition to the direct stabilization of the mPT pore. We found that rasagiline increases the mRNA and protein levels of *bcl-2* and *bcl-xL* in SH-SY5Y cells, as shown in Fig. 3 (Akao et al., 2002b). Rasagiline showed a reverse-bell shape curve of the concentration-activity relationship and the increase of Bcl-2 was detected at 10 μM–10 nM, and also at 10–1 pM. Bcl-2 protein level increased from 6 to 24 h of the treatment. Rasagiline induced mRNA levels of anti-apoptotic *bcl-2* and *bcl-xL*, but not those of pro-apoptotic *bax* and *mcl-l*. Other MAO-A and -B inhibitors, clorgyline and pargyline, did not affect the mRNA level at the concentrations examined.

The results of structure-activity relationship of propargylamine derivatives to Bcl-2 induction are summarized in Table 1. Rasagiline and *N*-propargylamine increased Bcl-2 mRNA and protein, whereas aminoindan and *N*-methylpropargylamine did not (Maruyama et al., 2002b; Yi et al., 2006b). The structure required for Bcl-2 induction is the propragylamine group, as in the case for preventing mPT. Also among BPAP derivatives, *R*(−)-*N*-propynyl compound, FDFS-1180, induced Bcl-2, more than FDFS-11169 without proynyl group (Maruyama et al., 2004b). For Bcl-2 induction, *R*-propargylamines are more potent than the *S*-enantiomers.

Induction of neurotrophic factors by propargylamines

Neurotrophic factors, including nerve growth factor, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor, prevent cell death in specified type neurons. GDNF is a member of the transforming growth factor-β superfamily and effectively protects dopaminergic neurons against

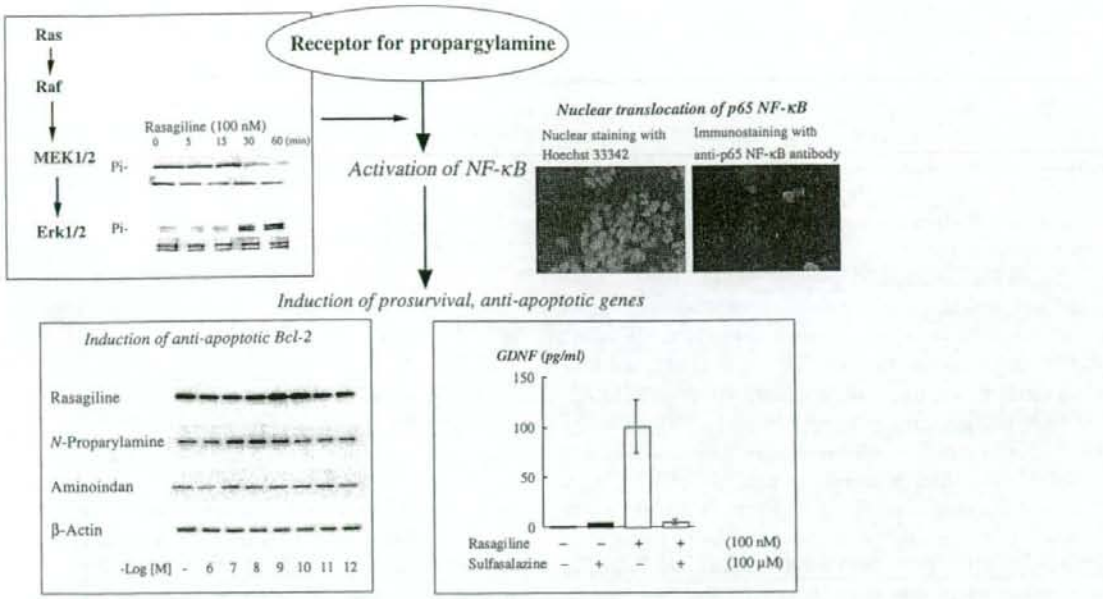


Fig. 3. Rasagiline increases anti-apoptotic Bcl-2 family and GDNF, a dopamine neuron-specific neurotrophic factor, through activation of ERK-NF-κB pathway. Anti-apoptotic propargylamines bind to the putative receptor on the membrane and activate the MEK1/2-ERK1/ERK2 pathway. The activated phosphorylated forms of ERK1/2 were detected after 30 min incubation with 100 μM rasagiline. After 3 h treatment with rasagiline, NF-κB was activated and p65 subunit was translocated into nuclei, as shown by staining using anti-p65 antibody for GAPDH and Hoechst 33342 for nuclei. The involvement of NF-κB in the induction of GDNF and Bcl-2 was also confirmed by use of an inhibitor of IκB kinase, sulfasalazine, which inhibited the increase of GDNF protein in SH-SY5Y cells treated with 100 nM rasagiline. The structure required for the Bcl-2 induction is a propargylamine structure, since aminoindan without a propargyl residue did not increase Bcl-2 levels

cell death in various animal PD models prepared with 6-hydroxydopamine and MPTP. Since GDNF and other neurotrophic factors cannot penetrate into the brain though the blood-brain barrier, several trials have been reported, delivering GDNF in the substantia nigra by direct administration, gene therapy, and cell implant (Bauer et al., 2000; Gill et al., 2003).

As shown in Fig. 3, rasagiline increases GDNF in SH-SY5Y cells. GDNF mRNA was virtually not detectable in SH-SY5Y cells, but after the treatment with 100 nM rasagiline for 3 h considerable amount of GDNF mRNA was detected. GDNF protein level in the control cells was less than 1 pg/ml and increased to be more than 100 pg/ml after rasagiline treatment. Induction of neurotrophic factors, GDNF, BDNF, NGF and neurotrophin-3 (NT-3), by propargylamines was examined in SH-SY5Y cells. Depending on the type of propargylamines, different neurotrophic factors were induced; rasagiline induced GDNF, and (−)deprenyl BDNF (Maruyama et al., in preparation). This result suggests that a specified propargylamine compound can induce a definite neurotrophic factor beneficial for selective type of neurons.

Signal transduction and gene expression by rasagiline for neuroprotection

These results on Bcl-2 and GDNF induction suggest that rasagiline may activate intracellular signals for induction of genes coding these anti-apoptotic proteins. NF-κB is the common transcription factor to induce anti-apoptotic *bcl-2*, neurotrophic GDNF and anti-oxidative SOD, all of which were increased by rasagiline (Carrillo et al., 2000; Akao et al., 2002b; Maruyama et al., 2004a). NF-κB consists of 2 subunits of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52), and is sequestered in the cytoplasm as an inactive complex with NF-κB inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, dissociated from the complex and degraded by the ubiquitin-proteasome system. This reaction allows translocation of free, active NF-κB complex into nuclei, where it binds to specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription, as shown by the p65 binding assay. The translocation of activated p65 subunit into nuclei by rasagiline was confirmed by Western blot analysis of the subcelluar fractions and also by immunohistochemical

observation using the p65 antibody and Hoechst 33342 for nuclear staining (Fig. 3) (Maruyama et al., 2004a). The involvement of phosphorylation of inhibitory IκB subunit on the activation of NF-κB, was demonstrated by use of sulfasalazine, an inhibitor of by IκB kinase (Fig. 3). Sulfasalazine inhibited also the increase of mRNA of *bcl-2* and *bcl-xL* as in the case with GDNF, suggesting the involvement of NF-κB transcription factor in the induction of neuroprotective proteins in common.

Rasagiline and related propargylamines protect cellular and animal models of neurodegenerative disorders, including PD, AD and ischemia (Mandel et al., 2003, 2005). By screening the signal factors activated rasagiline, we found that extracellular-regulated kinase-1/2 (ERK1/ERK2) was activated as an upper signal of NF-*k*B activation (Maruyama et al., 2004a) (Fig. 3). After treatment with 100 nM rasagiline, phosphorylated ERK1/ERK2 was increased in a time-dependent way, which PD98059, an inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase-1 (MEK 1/2), inhibited. CF10923x and Calphosin, inhibitors for protein kinase C (PKC), suppressed the increase of Bcl-2 and activated NF-κB by rasagiline, suggesting the involvement of the pathway through activation of PKC, Ras/Raf and MEK 1/2 in the induction of these proteins. Youdim and his group reported detailed data concerning the activation PKC system by rasagiline, which up-regulates MAP kinase/ERK cascades (Youdim et al., 2003a; Mandel et al., 2005; Weinreb et al., 2004). Recently, in mice treated with MPTP rasagiline was reported to activate signal pathway from neurotrophic factor responsive-tyrosine kinase receptor to phosphatidylinositol 3 kinase protein (Sagi et al., 2007). However, as shown later in DNA array studies, kinases may be activated not only primarily by rasagiline itself, but also secondarily by the following death-regulating processes. At present, it requires further studies to identify the initial signal to induce anti-apoptotic genes.

To screen the gene induction by rasagiline, we examine the time-dependent expression of genes by rasagiline. SH-SY5Y cells were treated with 100 nM rasagiline for 6, 12 and 24 h and mRNA was extracted and reverse-transcribed with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, and comparing to the control values obtained by the cells without treatment of rasagiline (Table 2). Rasagiline increased 108, 57 and 82 genes (>1.5 compared to control) and reduces 37, 54 and 104 genes (<0.5) after 6, 12 and 24 h treatment, respectively. Rasagiline affected genes with different cellular function in a time-dependent way. After 6 h treatment, mRNA of *bcl-2*, and genes related to NF-κB related transcription factors, cytokines and the receptors [interleukin (IL) receptors], mitochondrial ATP synthesis (cytochrome c oxidase, NADH-coenzyme Q reductase, ATP synthase, aconitase) and the ubiquitin-proteasome system were increased. In addition, genes of mPT pore components (ANT, VDAC and MAO-A) were also increased. On the other hand, genes coding growth factor (BDNF, transforming growth factor), cytokines and receptors [tumor necrosis factors (TNF), IL, fibroblast growth factor] were reduced. At 12 h of the treatment, most marked increase was observed in MAP-KK and cytokine receptors. In addition, rasagiline increased mRNA for ANT, VDAC and mitochondrial proteins (complex I–IV, mitochondrial transcription factor A). On the other hand, kinases associated with death signal (MAP kinase activating death domain, MAPKKK 4, TNF receptor associated factor 5, death-associated protein kinase-1), growth factors (NGF), and cytokines decreased. It is interesting that mRNA of *bcl-2*, MAO-B and also transcription factors were reduced significantly at this point. Rasagiline treatment for 24 h enhanced significantly the genes for *bcl-2*, apoptosis inhibitors (apoptosis inhibitors 1, 2 and 4, neuronal apoptosis inhibitory protein) and cell signals, including kinases (MAPK, MAPKK, cyclin-dependent kinase), cytokines and the receptors, and the transcription factors. It may be hypothesized that rasagiline sequentially increases ATP-dependent activation of kinases and transcription factors, the ubiquitine-proteasome system, which degrades the cleaved phosphorylated inhibitors of kinases and transcription factor, increases cytokines and the receptors, and finally induces pro-survival genes.

Table 2. *Gene induction in SH-SY5Y cells by rasagiline*

Rasagiline (100 nM) treatment for		
6 h	12 h	24 h
Increased genes	Increased genes	Increased genes
ATP-synthesis-related mitochondrial mPT pore related	Kinases	Bcl-2
Cytokine receptors	Cytokine and IL receptors	Apoptosis inhibitors
NF-κB related transcription factors	Mitochondrial complex I–IV	TNF and receptors
Ubiquitin-proteasome system	mPT pore related	Growth factors
Reduced genes	Reduced genes	
IL and TNF	Bcl-2	
Cytokin-related transcription factors	Kinases	
Growth factors	IL and TNF	
	Transcription factors	
	Growth factors	

Discussion

The clinical trials to prove the neuroprotective function of rasagiline and (−)deprenyl were reported, but the results are still contradicting, and biomarkers to estimate the progression of neuronal loss should be invented (Michell et al., 2004). The markers for the disease progression and treatment efficiency are based on clinical evaluation of symptoms, PET and SPECT imaging, transcranial ultrasound and some biochemical tests. However, blood tests for PD progression are limited to monitor the pathogenic factors, such as increased oxidative stress (malondialdehyde, superoxide radicals, 8-hydrox-2′-deoxyguanosine), or the reduced complex I (Schapira et al., 1990) and increased MAO-B activity in platelets (Zhou et al., 2001). (−)Deprenyl may reverse the increase in MAO-B and the subsequent reduction of β-phenylethylamine in plasma, but these markers represent MAO inhibitory function of (−)deprenyl, but not the neuroprotective activity. α-Synuclein and its phosphorylated proteins were proposed as the markers, but the recent results did not support this view. In CSF, increased levels of 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-guanosine and malondialdehyde were detected (Abe et al., 2003). However, these markers do not present information for progression of selective neuronal loss in PD.

At present, mechanistic makers for factors intervening the disease progress may be the only available markers to assess the neuroprotective potency. As described above, rasagiline induces GDNF in cultured cells, suggesting that the levels of neurotrophic factors specific for dopamine neurons may be used as markers. Indeed, we examined the change in neurotrophic factors in monkey CSF after systemic treatment of rasagiline (Maruyama et al., in preparation). The results proved the validity of our view, which was supported further by the analyses of the CSF from Parkinsonian patients before and after treatment of (−)deprenyl, even the limited number of the samples (Maruyama et al., in preparation). We are now examining the candidates of the biomarkers for the neuroprotective function in serum and CSF from Parkinsonian patients and primate models.

Recently, an increasing number of evidences indicate that rasagiline and related compounds can ameliorate pathogenic processes in AD and other neurodegenerative disorders. Rasagiline analogues with inhibitor potency to cholinesterase, TV 3326, and its *S* enantiomer TV 3279 were reported to regulate the processing of amyloid precursor protein (APP) and increase the soluble APP secretion through activation of α-secretase activity and the reduction of holo-APP protein (Youdim et al., 2003b; Yogev-Falach et al., 2006). Their results suggest that propargylamines intervene the pathogenic processes in neurodegenerative disorders in general and ameliorate the disease process.

The stereo-chemical and enantiomeric specificity of the propargylamine for their neuroprotective activity suggests the occurrence of the target protein in mitochondria and other cell components. The identification of the binding site of neuroprotective propargylamines may give us a clue to find the most adequate chemical structure for the function, and develop new drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

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Oxidation of polyunsaturated fatty acids induces protein oligomerization and may initiate neuronal death process in Parkinson's disease

Masayo Shamotmo-Nagai[1], Wakako Maruyama[1*], Yoji Kato[2], Toshihiko Osawa[3]

1. Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan
2. Graduate School of Human Science and Enviroment, University of Hyogo, Himeji, Hyogo, Japan
3. Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya, Aichi, Japan

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Correspondence author:: Wakako Maruyama, Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka-cho, Obu, Aichi, 474-8511, Japan
Phone 81-562-46-2311 (ex 5551), Fax 81-562-46-8264, E-mail maruyama@nils.go.jp

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INTRODUCTION

Long chain polyunsaturated fatty acids (PUFA), are enriched in the nervous system. Docosahexaenoic acid (22:6n-3, DHA) is one of the most abundant PUFA in the brain and retina, and is component of phosphatidylethanolamine and phosphatidylserine in the celluler membrane. Administration of DHA to the rodents improves their brain functions (1) and it is suggested DHA in the membrane plays a role in the synaptic plasticity and signal transduction systems in the neuronal cells (2-4). In the human, lipids orally administrated are reconstructed through α-oxidation and elongation reaction mainly in the liver after digestion and absorption. However, human can not synthesize enough amount of DHA, so, the intake of DHA from food, mainly fish oil, is essentially required (essential fatty acid). Brain DHA is derived from the circulating plasma pool across the Blood-Brain-Barrier. In the human brain, the amount of n-6 PUFA, such as DHA is estimated to be 17.1% of the total fatty acids, and on the other hand, the amount of n-3 PUFA, such as arachidonic acid (AA) is estimated to be 9.7%. The concentration of n-3 PUFA is higher than that of plasma, so that, the existence of specific transporter for DHA is suggested in the neural cells and astrocytes (5). DHA is known to be a potent antioxidant, but

simultaneously, it is easily oxidized and produces toxic lipid peroxide. These toxic lipid peroxide and their products, such as aldehyde may form adduct with the proteins to induce degenerated proteins with abnormal conformation. The proteins, which associated with cell membrane, such as cell-surface receptor, membrane anchoring protein, or functionally transmembrane protein are the candidates. The oxidized products are degradated by ubiquitin-proteasome system or autophagy, but according to ageing, the accumulation of these abnormal proteins might perturb homeostasis to induce neuronal death.Parkinson disease (PD) is the second common neurodegenerative disorder and affects 1-2% of aged population over 60 years old. The pathogenesis of PD has not been clarified, but aggregation of protein with abnormal conformational is commonly observed features in the neurodegenerative disorders. In PD, degeneration of dopamine neuron in the substantia nigra and the existence of Lewy boies (LB), are diagnostic pathological features. α-Synuclein (α-Syn) is the main component of LB and is known to exist as membrane-bound form by association with PUFA. The mutation of A53T, A30P, and E46K, or triplication of α-Syn gene have been identified in early-onset familial PD (6-8). It is suggested that accumulation of α-Syn is the cause, not the result of PD. In sporadic PD without α-Syn mutation, some post-translational modification of α-Syn may induce pathological process similar to familial PD. α-Syn is a 140-amino acid protein expressed ubiquitously in the neuron and accounts for 0.1-1% of brain cytosolic proteins. α-Syn is suggested to play many roles in nervous system, including regulation of synaptic vesicle mobilization, chaperone activity, modulation of dopamine transporter and dopamine biosynthesis (9-11). In this paper, the oxidative modification of α-Syn by lipid peroxide and aldehyde derived from PUFA was examined. The possibility that lipid peroxidation in the membrane-composing PUFA and adduct formation with α-Syn was investigated. The results are discussed in relation to the role of oxidative stress in ageing in the pathogenesis of PD.

MATERIALS AND METHODS

PUFA is oxidized and produce lipid peroxides, then, form adducts with lysine residue in the proteins. DHA is oxidized and split to produces succinyl-lysine adduct (SUL) with carbonyl terminal and propanoyl-lysine adduct (PRL) with amino terminal, respectively. AA is also oxidized, to produce glutaroyl-lysine (GLL) with carbonyl terminal and hexanoyl-lysine (HEL) adduct with amino terminal. The antibodies of these 4 oxidized PUFA products, SUL, PRL, GLL and HEL are prepared as reported previously (12). Recombinant α-Syn is purachased from BIOMOL International, L.P. (Plymouth Meeting, PA.). DHA, AA and oleic acid donated from CAYMAN CHEMICAL (Ann Arbor, MI) and stearic acid are Nu-chek prep, Inc. (Elysian, MN). Other chemicals are from WAKO finechemical.(Osaka, Japan).

Recombinant α-Syn (2 μM) was co-incubated with long chain fatty acids namaly DHA, AA, oleic acid or stearic acid (1-10 mM) for 3 to 7 days. The reaction products were separated by SDS-PAGE and were analyzed by western blotting using anti-α-Syn antibody. The sample incubated

with DHA or AA, the production of oxidized PUFA was examined also, using antibodies described above. The effects of antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), on oligomerization of α-Syn was examined also.

We examined whether DHA is neuroprotective or neurotoxic using human clonal neuralblastoma SH-SY5Y cells. Free DHA (0-100 μM) or DHA (0-100 μM) pre-incubated with 4 folds concentration of Bovine serum albumin (BSA), which acts as lipid carrier protein was added to the culture medium in the presence or absence of tocopherol (100 μM) for 2 days in 5% CO_2-95% atmosphere at 37 °C. Culture medium was COS-Medium 001 (COSMOBIO, Japan) without serum.. Cell death was estimated using LDH assay according to manufacture's procedure. The cells were mechanically harvested and gathered, then homogenized in RIPA Buffer, then centrifuged at 10,000 xg for 15 min. The supernatant and pellet were used as soluble and insoluble fraction, respectively. The amount of lipid peroxides in the soluble and insoluble fraction of the cells was estimated by fluorospectmeter with excitation at 365 nm and emission at 440 nm.

RESULTS

α-Syn oligomerization was found to be enhanced by the existence of DHA and AA in a dose- and time dependent way, but not that of oleic acid and stearic acid. This result indicate that the existence of .double-bounds in long chain fatty acids is essential for enhancement of α-Syn oligomerization. α-Syn oligomers were found to be positive for SUL and PRL in the sample incubated with DHA, and GLL and HEL in that with AA. BHA and BHT were found to reduce α-Syn ologomerization enhanced by DHA and AA. It was indicated lipid peroxide produced by PUFA formed adduct with α-Syn, then oligomerization and aggregation of the protein.

Free DHA at the concentration higher than 20 μM was found to be cytotoxic to SH-SY5Y cells. Antioxidant tocopherol could not prevent the toxicity of freee DHA. DHA re-incubated with BSA (DHA-BSA) was less toxic than free BSA and tocopherol inhibited the toxicity by DHA-BSA completely.

The amount of PRL, which reflects the level of the proteins conjugated with lipid peroxide derived from DHA, was found to increase in the soluble and insoluble fractions in the cells treated with DHA-BSA. On the othe hand, tocopherol reduced the level of PRL significantly.

These results indicated that the cytotoxic effect of DHA-BSA was induced by the conjugation of oxidation product of DHA with cellular proteins. The cytotoxicity of free DHA should be ascribed to its detergent activity.

DISCUSSION.

In this paper we demonstrated that DHA enhanced oligomerization of α-Syn through adduct formation with lipid peroxide derived from DHA (SUL and PRL) with α-SYN *in vitro*. Using cell

culture system, DHA -BSA, showed weak cytotoxic effect on neural cells and adduct formation of the cellular proteins with PRL was also identified. Antioxidant inhibited cytotoxic effect and adduct formation by DHA-BSA simulataneoously. These results suggest that DHA, which has been believed to be neuroprotective, may become neurotoxic in the condition with increased oxidative stress, such as neurodegenerative disorders.

The increased lipid peroxidation of the membrane is suggested to play a important role in the vicious process in ageing. Lipid peroxides in the membrane initiates sequential reaction of PUFA oxidation to increase the amount of oxidized fatty acids to decrease the membrane fluidity and perturb the function of the proteins associated with cellular membrane. In PD, where dopamine neuron degenerated and membrane-associated protein, α-Syn is aggregated, increased oxidative stress, mitochondrial dysfunction and impaired ubiquitin-proteasome system were observed. Oxidation of PUFA, especially DHA may decrease the binding capacity of α-Syn to the membrane. α-Syn released from the membrane to the cytosol can not stabilize its α-helix structure no more.. In addition, lipid peroxide may directly make adduct with α-Syn and induce protein oligomerization as shown in this paper.

Epidemiological study suggests that the intake of fish oil and vegetable decrease the risk of neurodegenerative disorders, such as Alzheimer disease (AD) and PD (13). However, intervention of neurodegenerative disorders by DHA has not been successful. The results of this paper that in the brain of neurodegenerative disorders, where oxidative stress increased, DHA may neurotoxic. DHA may be effective when it is administrated not after but before the onset of the disease. In addition, intake of the food-derived antioxidant such as polyphenol which can prevent the oxidation of DHA may increase its usefulness (Fig.1). The further investigation to clarify the effect of food-derived DHA and polyphenol using clinically available biomarker is now under the way.

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