I. NEUROPROTECTION BY PROPARGYLAMINES: INTRACELLULAR MECHANISM

The development of "neuroprotective drugs" is now gathering attention in order to slow down the disease progress and improve quality of life of the patients with neurodegenerative disorders, such as Parkison's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis. On the other hand, activation of mitochondria-dependent apoptotic signal is considered to account for cell death in neurodegenerative disorders (1,2) and well-conserved and -regulated apoptotic cascade has been proposed to be a target of neuroprotection (3,4). Using the cellular and animal models of neurodegenerative disorders, several candidates of neuroprotective agents have been proposed: antioxidants, inhibitors of monoamine oxidase [MAO, monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4], anti-inflammatory drugs, drugs interfering glutamate excitotoxicity, and growth factors (5–8). These candidates are expected to intervene the death signal transduction and protect neurons from degeneration.

N-Propargyl-1(*R*) -aminoindan (rasagiline) is an inhibitor of type B MAO (MAO-(MAO-B) (9,10), and has been developed as an anti-Parkinson drug (11–13). The phase III clinical trial of rasagiline was now finished for the treatment of parkinsonian patients. The neuroprotective potency of rasagiline has been proved in vivo using animal models induced by neurotoxins, excitotoxicity toxins, ischemic, and closed brain injury (14–16). However, in clinical studies, it requires further results to prove the neuroprotective potency, in addition to the previously confirmed symptomatic effects (17).

Also in vitro rasagiline has been shown to reduce glutamate toxicity in cultured hippocampal neurons (13) and to prolong survival of cultured, serum-derived rat fetal mesencephalic cells (18). The structure–activity relationship suggested that the neuroprotective effect of rasagiline and related compounds did not depend on the MAO inhibitory property, as shown by neuroprotection by the enantiomer of rasagiline, *N*-propargyl-1(*S*-)aminoindan (TVP-1022), which

was 100-fold less active as MAO inhibitor (14). We studied the mechanism behind neuroprotection of rasagiline against cell death induced in human neuroblastoma SH-SY5Y cells by peroxynitrite and neurotoxins, *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] and 6-hydroxydopamine, as a cellular PD model (19,20).

Apoptosis is a death process observed in neurons after exposure to neurotoxins, increased oxidative stress, excitotoxins, and withdrawal of neurotrophic factors. The intracellular process of apoptosis induced by *N*M(*R*)Sal in SH-SY5Y cells was elucidated as follows. Binding of *N*M(*R*)Sal to mitochondrial outer membrane initiates mitochondrial permeability transition (mPT), opening a megachannel called mPT pore, which induces rapid reduction of mitochondrial membrane potential, $\Delta\Psi_m$, and swelling of mitochondria. Then the following apoptotic cascade is activated: release of cytochrome *C* and other apoptosis-inducing factors from mitochondria to cytoplasm, activation of caspase 3, an executer of apoptosis, and translocation of glyceraldehydes-3-phosphate dehydrogenase [GAPDH, D-glyceraldehydes-3-phosphate:NAD; oxidoreductase (phosphorylating), EC 1.2.1.12] from cytoplasm to nuclei. In the final, fragmentation and condensation of nuclear DNA are induced, as shown by nuclei with condensed chromatin and fragmented DNA, and ladder formation of fragmented oligonucleosomal DNA by agarose gel electrophoresis (21,22). Figure 1 summarizes the activation of apoptotic cascade induced by *N*M(*R*)Sal and other stimuli. F1

A series of propargylamines, including rasagiline, (−) deprenyl, and aliphatic (*R*)*N*-(2-heptyl)-*N*-methylpropargyl-amine (*R*-2HMP) inhibits the activation of apoptotic cascade and protects SH-SY5Y cells against apoptosis. The chemical structures of propargylamines with antiapoptotic potency are shown in Fig. 2. As summarized in Fig. 3, these propargylamines prevent collapse in $\Delta\Psi_m$, in isolated mitochondria (23), and SH-SY5Y cells (19), and following activation of apoptotic cascade. These results are quite similar to those observed in SH-SY5Y cells with overexpression of antiapoptotic Bcl-2 protein family, suggesting the involvement of Bcl-2 and related prosurvival protein. Based on these results, we examined whether rasagiline could induce genes coding antiapoptotic protein in neurons. F2 F3

Figure 1 Mitochondria-dependent apoptosis cascade activated by an endogenous neurotoxin, *N*M(*R*)Sal in Sh-SY5Y cells. AQ2

II. INDUCTION OF NEUROPROTECTIVE PROTEIN

As mentioned above, some kinds of proteins have been proposed to alleviate neuronal loss through suppression of oxidative stress, prevention of apoptotic signal transduction, and promotion of cell survival. Rasagiline, (−) deprenyl, and aliphatic propargylamines were found to increase the activity of antioxidative enzymes, superoxide dismutase (SOD), and catalase, in the rat brain after the continuous injection

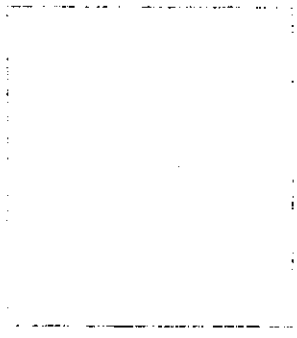

Figure 2 Chemical structures of propargylamines with neuroprotective potency. Rasagiline contains a cyclic benzylamine structure, (−)deprenyl β-phenyethylamine structure, and 2-HMP aliphatic hydrophobic structure.

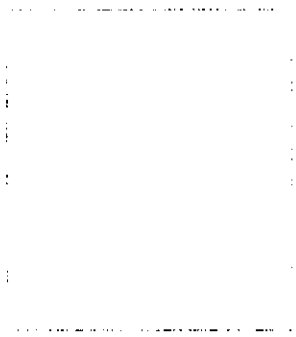

Figure 3 The summary of the mechanism underlying neuroprotection by propargylamines.

(24,25). (−) Deprenyl and desmethyldeprenyl were reported to increase mRNA level of SOD 1 and 2, Bcl-2 and Bcl-xL, nitric oxide synthase, c-JUN, and nicotinamide adenine dinucleotide dehydrogenase in PC12 cells (26).

Bcl-2 and related proteins are known to prevent mPT induction and activation of apoptotic cascade in a variety of physiological and pathological contexts (27,28). The family of Bcl-2-related proteins constitutes one of most relevant regulatory gene products against apoptosis. We found that rasagiline increased mRNA and protein levels of *bcl-2* and *bcl-xL* in SH-SY5Y cells.

Neurotrophic factors, such as nerve growth factor, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CDTF), have been proposed as agents preventing neuronal loss (29,29a). Recently, we found that rasagiline induced mRNA and protein of GDNF, which protects or promotes survival of dopamine neurons selectively.

These results suggest that rasagiline may activate an intracellular signal transduction common for induction of genes coding these antiapoptotic proteins, antioxidative enzymes, antiapoptotic Bcl-2 family protein, and GDNF.

II.A. Induction of Bcl-2 by Rasagiline

Bcl-2 family proteins play key roles in regulating apoptosis (28) and they may be either death antagonists (Bcl-2, Bcl-xL, Bfl-1,

A1, and Mcl-1) or agonists (Bax, Bak, Bad, Bid, Bik, and Hrk). The proteins form homo- or heterodimers between anti- and proapoptotic members and determine cellular sensitivity to apoptotic stimuli by titrating one another's function. Bcl-2 is mainly localized in the mitochondrial inner membrane and it can promote survival in neurons and other cells undergoing apoptosis (27). Overexpression of Bcl-2 protects various neuron paradigms in vivo and in vitro from death induced by neurotoxins and other insults. Bcl-2 regulates apoptosis induced by *N*M(*R*)Sal, as proved by preventing apoptosis in Bcl-2-overexpressed SH-SY5Y cells (19) and also $\Delta\Psi_m$ decline in isolated mitochondria prepared from liver of Bcl-2 overexpressed mice (23). These results suggest that Bcl-2 protein in mitochondria may mediate the neuroprotection by rasagiline. The induction of mRNA and protein of antiapoptotic Bcl-2 family proteins was examined in SH-SY5Y cells either by reverse transcription (RT)-PCR or Western blot analysis.

Rasagiline was prepared as reported previously (9) and kindly donated by Teva Pharmaceutical (Netanya, Israel). SH-SY5Y cells were cultured in the presence of various concentrations (10 μM–1 pM) of rasagiline for 24 hr 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. cDNA was generated by reverse transcription of the total RNA, and the cDNA fragments were amplified using the PCR primers. PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin cDNA was used as an internal standard. The mRNA levels of *bcl-2* and *bcl-xL* were quantified by computer-assisted image analysis using NIH imaging software.

Rasagiline was confirmed to enhance expression of *bcl-2* and related genes. Reverse transcription-PCR analyses revealed increased levels of *bcl-2* mRNA after treatment with 100 nM rasagiline in a time-dependent way (Fig. 4A). The *bcl-2* mRNA levels began to increase after 3 hr of the treatment with rasagiline and the increase continued further to about threefold at 24 hr. Western blot analyses showed that Bcl-2 protein level increased from 6 to 24 hr of the treatment. Figure 4B shows that rasagiline increases *bcl-2* mRNA level, F4

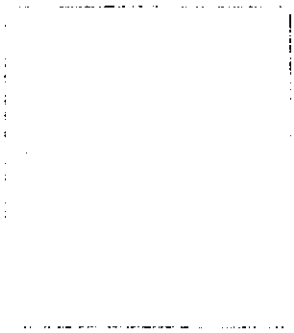

Figure 4 Effect of rasagiline on levels of mRNA and protein of *bcl-2* family. (A) SH-SY5Ycells treated with 100 nM rasagiline for 3, 6, and 24 hr and the mRNA levels were assayed by RT-PCR and the protein levels by immunoblot analysis using antibody against Bcl-2 protein. (B) The cells were treated with 100, 10, and 1 nM rasagiline for 24 hr and the *bcl-2* and *bax* mRNA were assayed by RT-PCR.

but not *bax* mRNA at 100–1 nM. Figure 5A shows the quantitative analyses of *bcl-2* mRNA levels increased by rasagiline treatment, and the relative value of *bcl-2* mRNA to *β-action* mRNA increased to about 150% of control after incubation with rasagiline. Among *bcl-x* isoforms, a 337 base pair fragment corresponding to the *bcl-xL* also increased by 100 nM rasagiline treatment, whereas the mRNA levels of *mcl-2* and *bax* were not affected (Fig. 5B). Other MAO-A and -B inhibitors, clorgyline, and pargyline, did not affect the mRNA level at the concentrations examined (10 μM–1 pM). These results clearly indicate that rasagiline inducts prosurvival Bcl-2 protein family, but not apoptosis-promoting Bax family. F5

II.B. Induction of Glial Cell-Line-Derived Neurotrophic Factor

Glial cell-line-derived neurotrophic factor is a member of the transforming growth factor-β superfamily and effectively protects dopaminergic neurons against cell death in various animal models of PD prepared with 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (30,31).

Figure 5 The effects of rasagiline and sulfasalazine on *bcl-2* and related genes. SH-SY5Y cells were incubated with rasagiline for 24 hr and mRNA was extracted and applied for RT-PCR. (A) The level of *bcl-2* mRNA in the cells treated with various concentration of rasagiline was quantified by NIH imaging software and compared to control cells without the treatment of rasagiline. (B) The effect of rasagiline and the pretreatment with sulfasalazine. Rasagiline at 100 nM increased mRNA of *bcl-2* and *bcl-xL* but did not affect *mcl-1* or *bax*. Pretreatment of 100 μM sulfasalazine inhibited the increase of *bcl-2* and *bcl-xL* by rasagiline. β-Actin was used as control.

Since GDNF and other neurotrophic factors cannot penetrate into the brain through the blood–brain barrier, several trials have been reported to deliver GDNF in the substantia nigra by direct administration (32,33), gene therapy (34,35), and cell implant (36,37). There were several controversial results about the effectiveness of GDNF supplement therapy in parkinsonian patients, mostly because of the technical difficulties to deliver GDNF to nigral dopamine neurons. However, recently GDNF injected directly to the putamen improved the symptoms in a part of parkinsonian patients (38). We confirmed that rasagiline induced GDNF in SH-SY5Y cells and the mechanism was also clarified.

The effect of rasagiline on levels of GDNF mRNA was studied by RT-PCR, and on those of GDNF protein was quantified using the enzyme immunoassay (EIA), as reported previously (39,40). Glial cell-line-derived neurotrophic factor mRNA was virtually not detectable in SH-SY5Y cells, but

after the treatment with 0.1 μM of rasagiline for 3 hr considerable amount of GDNF mRNA was detected. As summarized in Table 1, the amount of GDNF protein in the cells increased most markedly after being treated with 0.1 μM of rasagiline. The GDNF protein was less than 1 pg/mL before rasagiline treatment, but it increased more than 100 pg/mL after the treatment for 3 hr (Table 1). AQ3 T1

II.C. Activation of NF-κB Transcription Factor by Rasagiline

NF-κB is the common transcription factor to induce antiapoptotic *bcl-2*, neurotrophic GDNF, and antioxidative SOD, all of which were increased by rasagiline (24,40,41). As shown in Fig. 6, NF-κB consists of two subunits of 65 kDa (p65: RelA) F6

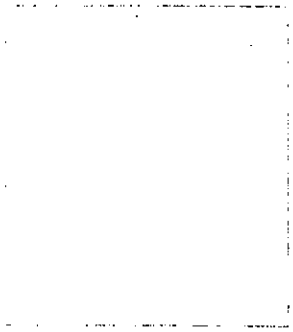

Figure 6 The mechanism behind the activation of NF-κB. By the various exogenous and endogenous stimuli, IκB kinase is activated and it phosphorylates IκB in the inactive NF-κB complex. Phosphorylated IκB is degraded by ubiquitin–proteasome system and active NF-κB dimmer consists of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52) translocates into the nuclei to bind κB sites.

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.

The translocation of activated p65 subunit was studied by Western blot analysis of the subcelluar fractions of SH-SY5Y cells after treatment with 1 and 0.1 μM of rasagiline for 30 and 60 min. Rasagiline treatment increased p65 subunit in the nuclear fraction in a time- and dose-dependent way, whereas that in the cytoplasmic fraction decreased. The translocation of activated NF-κB was also examined by immunohistochemistry using the p65 antibody (Fig. 7B), and F7 nuclear staining with Hoechst 33342 (Fig. 7A). After 3 hr treatment with rasagiline, nuclear translocation of p65 was confirmed by merging the two figures (Fig. 7C).

The activation of NF-κB and the increased binding activity were examined also by ELISA, using NF-κB p65 transcription assay kit according to Kretz-Remy et al. (42). The

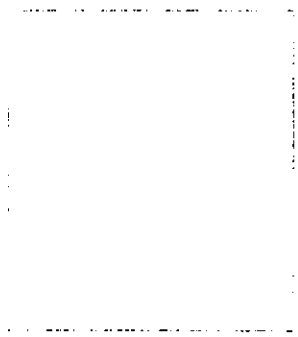

Figure 7 Nuclear translocation of p65 NF-κB subunit by the treatment with rasagiline. SH-SY5Y cells were treated with 0.1 μM of rasagiline for 3 hr and fixed in paraformaldehyde. The sample was stained with Hoechst 33342 nuclear staining (A) and immunostaining using anti-p65 antibody (B). (C) shows the merge of (A) and (B).

principle of this assay is to measure the binding of activated NF-κB p65 to an oligonucleotide containing the NF-κB consensus-binding site. Rasagiline increased the binding activity of NF-κB p65 to the oligonucleotides and it was competitively inhibited by pretreatment with wild oligonucleotide containing the NF-κB binding site, but not the mutated one, indicating the selective binding to the NF-κB binding site.

The involvement of phosphorylation of IκB, an inhibitory subunit on the activation of NF-κB, was studied by use of sulfasalazine, an inhibitor of IκB kinase as summarized in Table 1. Also NF-κB binding assay showed that sulfasalazine suppressed the rasagiline-induced increase in the binding capacity, again suggesting the involvement of IκB kinase–NF-κB axis. In addition, Western blot analysis of the subcellular fractions of rasagiline-treated cells demonstrated that sulfasalazine reduced nuclear translocation of activated p65 subunit.

Sulfasalazine abolished 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 genes in common (Fig. 5B).

II.D. Gene Expression by Rasagiline Detected by DNA Array Analyses

SH-SY5Y cells were treated with 0.1 μM of rasagiline for 6, 12, and 24 hr and mRNA was extracted and reverse-transcripted with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The probes were hybridized to a cDNA expression array membrane containing more than 2000 genes related to apoptosis, cell survival, and transcription. The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, provided on the membrane and comparing to the control values obtained by the cells without treatment of rasagiline.

The gene induction was widely surveyed by gene array analysis system to compare the level of mRNA relating apoptosis-survival signal in the cells with or without the treatment of rasagiline (Table2). Rasagiline increased mRNA of the T2

genes relating mitochondria and ATP synthesis, ubiquitin–proteasome system and Bcl-2 in first 6 hr and then, genes relating signal transduction and transcription, including a series of kinases and NF-κB, were increased after 24 hr.

III. DISCUSSION

This paper reports that rasagiline induces neuroprotective genes in SH-SY5Y cells through the activation of transcription factor NF-κB. Rasagiline is a selective inhibitor of MAO-B, but its neuroprotective effect cannot be ascribed to MAO inhibition, because SH-SY5Y cells do not contain MAO-B. Recent study revealed that rasagiline and other structurally related propargylamines rescue neurons from apoptosis by inhibiting the induction of mPT and the reduction $\Delta\Psi_m$ the critical step to initiate apoptosis signal. Rasagiline was found to inhibit PT induced by an endogenous neurotoxoin *N*M(*R*)Sal in isolated mitochondria suggesting its direct interaction to the mitochondrial protein (23). Tatton et al. (43) reported that (−) deprenyl rescued neuronal differentiated PC12 cells from apoptosis induced by serum deprivation. They augmented that nuclear translocation of GAPDH inhibited the transcription of *bcl-2* and *bcl-xL* and resulted in mPT, and that (−) deprenyl interfered GAPDH polymerization into the tetramers, which was essential for the nuclear translocation. However, we found that nuclear translocation of GAPDH was a downstream signal of the induction of mPT (44). In addition, we showed that rasagiline did not suppress the decrease, but even increased the transcription of *bcl-2* and *bcl-xL*. NF-κB is one of the most important transcriptional factor, which regulates the cell death-survival signal and is suggested to be involved in the activation of prosurvival genes in neuronal cells in the preconditioning model of ischemia and amyloid β protein (45,46). Rasagiline activates NF-κB, which was antagonized by sulfasalazine, an inhibitor of IκB kinase. Considering that sulfasalazine abolishes the increase of GDNF, *bcl-2*, and *bcl-xL*, these proteins are induced by IκB kinase–NF-κB pathway. Gene array study of rasagiline-treated cells reveals that rasagiline

increases the genes relating mitochondrial energy synthesis, apoptosis, transcription, and proteasome system by a time course way. At present, the mechanism how rasagiline activates NF-κB transcription factor is not fully clarified, but our recent results suggest that there may be a signal transduction from mitochondria to a kinase, which activates NF-κB pathway. The study to find out the target molecule of rasagiline may give us a clue to develop new neuroprotective drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

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MONOAMINE OXIDASE AND THE INHIBITORS: INVOLVEMENT IN CELL DEATH AND SURVIVAL OF NEURONS

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