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Role of the Redox Protein Thioredoxin in Cytoprotective Mechanism Evoked by (−)-Deprenyl

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ABSTRACT

Through the inhibition of monoamine oxidase type B (MAO-B), (−)-deprenyl (selegiline) prevents the conversion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the toxic metabolite 1-methyl-4-phenylpyridinium ion (MPP^+) and also prevents the neurotoxicity in the dopaminergic neurons in animal models. Cumulative observations suggest that selegiline may also protect against MPP^+-induced neurotoxicity, possibly through the induction of pro-survival genes. We have observed that thioredoxin (Trx) mediates the induction of mitochondrial manganese superoxide dismutase (MnSOD) and Bcl-2 during preconditioning-induced hormesis. We therefore investigated whether the redox protein Trx plays any role in the neuroprotective mechanism of selegiline against MPP^+-induced cytotoxicity in human SH-SY5Y neuroblastoma cells and also in primary neuronal cultures of mouse midbrain dopaminergic neurons. After confirming that selegiline protects against MPP^+-induced cytotoxicity, we observed further that selegiline, at 1 μM or less, induced Trx for protection against oxidative injury caused by MPP^+. The induction of Trx was blocked by protein kinase A (PKA) inhibitor and mediated by a PKA-sensitive phospho-activation of mitogen-activated protein (MAP) kinase Erk1/2 and the transcription factor c-Myc. Selegiline-induced Trx and associated neuroprotection were concomitantly blocked by the antisense against Trx mRNA, but not the sense or antisense mutant phosphothionate oligonucleotides, not only in human SH-SY5Y cells but also in mouse primary neuronal culture of midbrain dopaminergic neurons. Furthermore, the redox cycling of Trx may mediate the protective action of selegiline because the inhibition of Trx reductase by 1-chloro-2,4-dinitrobenzene ameliorated the effect of selegiline. Trx (1 μM) consistently increased the expression of mitochondrial proteins MnSOD and Bcl-2, supporting cell survival (Andoh et al., 2002). In conclusion, without modifying MAO-B activity, selegiline augments the gene induction of Trx, leading to elevated expression of antioxidative MnSOD and antiapoptotic Bcl-2 proteins for protecting against MPP^+-induced neurotoxicity.

(−)-Deprenyl (selegiline) was originally developed in Hungary (for review, see Knoll, 2000) and pharmacologically classified as a selective monamine oxidase type B (MAO-B) inhibitor with a broad spectrum in clinical uses including antiparkinsonian and antidepressant activities (Birkmayer et al., 1983; Murphy et al., 1983). The initial use of selegiline in patients with Parkinson's disease is aimed at increasing brain dopamine levels through the inhibition of MAO-mediated oxidative deamination of dopamine during the treatment of patients with levodopa. Clinical observations imply that selegiline might have additional neuroprotective properties reflected by slowing the progression in clinical deterioration (Heinonen and Lammintausta, 1991; LeWitt, 1991). Subsequent clinical trials of selegiline in the DATATOP Parkinson study and in Alzheimer's dementia indicate that this elusive neuroprotective effect of selegiline is observed only in early clinical phases (Tariot et al., 1987; Shoulson et al., 2002). Selegiline is known to delay the time until enough disability develops to warrant the initiation of levodopa therapy in patients with early Parkinson's disease. It has been suggested that this beneficial effect of selegiline is largely sustained during the overall 8.2 years of the DATATOP clin-

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ABBREVIATIONS: MAO-B, monoamine oxidase type B; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Trx, thioredoxin; SOD, superoxide dismutase; DNCB, 1-chloro-2,4-dinitrobenzene; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; MEK, mitogen-activated protein kinase kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PKA, protein kinase A; PKC, protein kinase C; BIM, bisindolylmaleimide; AP-1, activator protein 1.

ical trial (Shoulson et al., 2002). Moreover, a short-term cognitive effect of selegiline in helping Alzheimer's dementia was also reported (Tariot et al., 1987). Furthermore, selegiline has an additional clinical indication for ameliorating depression (Murphy et al., 1983). This possible antidepressive action of selegiline may be useful in the treatment of depressed patients suffering chronic neurodegenerative disorders including Alzheimer's dementia and Parkinson's disease.

During the past 2 decades, basic studies revealed that selegiline prevents the parkinsonism phenomena caused by MPTP and nigral injury evoked by MPP^+ in cells and animal models (Burns et al., 1983; Cohen et al., 1984; Mytilineou and Cohen, 1985; Wu et al., 1995). Similar neuroprotective effects of selegiline have been observed in animals and cells treated with other neurotoxins, such as methyl-β-acetoxyethyl-2-chloroethylamine, 6-hydroxydopamine, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine, 5,6-dihydroxyserotonin, and 3,4-methylenedioxymethamphetamine, by a mechanism that cannot be explained solely by its inhibitory effect on MAO-B (Heinonen and Lammintausta, 1991; Sprague and Nichols, 1995).

We, among other research groups, investigated further this elusive neuroprotective mechanism of selegiline. For instance, selegiline scavenges reactive oxygen species and suppresses the generation of cytotoxic hydroxyl radicals and the peroxidation of brain lipids produced by MPP^+ in the rat brain in vivo (Wu et al., 1995). It also prevents the iron-catalyzed auto-oxidation of dopamine to melanin. Furthermore, selegiline protection of brain neurons could be augmented by its unique capacity to induce Cu/ZnSOD, MnSOD, catalase, Bcl-2, GDNF, and NGF (Carrillo et al., 1991; Vizuete et al., 1993; Kitani et al., 1994; Semkova et al., 1996; Tatton et al., 1996; Revuelta et al., 1997; Kunikowska et al., 2002). Selegiline may also bind to glyceraldehyde-3-phosphate dehydrogenase, which is known to be associated with apoptotic cell death (Tatton et al., 2003). However, molecular mechanisms underlying the gene induction caused by selegiline are not fully understood.

We reported recently that the redox protein thioredoxin (Trx) mediates the induction of MnSOD and Bcl-2 during preconditioning-induced hormesis (Andoh et al., 2003) thus antagonizing MPP^+-induced cyto- and neurotoxicity (Andoh et al., 2002b; Bai et al., 2002). Trx contains two redox-active cysteine residues that can be oxidized to form intramolecular disulfide bonds. Reduction of the oxidized Trx is catalyzed by Trx reductase with NADPH as electron donor. The thiol oxidoreductase activity of Trx, when coupled with either methionine sulfoxide reductase or Trx peroxidases, functions as a potent antioxidant via removing H_2O_2 (Kang et al., 1998; Moskovitz et al., 1999). In addition, the reduced form of Trx is known to inhibit the apoptosis signaling-regulating kinase 1 (Saitoh et al., 1998) and to enhance DNA binding to transcription factors such as nuclear factor-κB and AP-1; the latter is achieved in conjunction with the redox protein Ref-1 by reducing a specific cysteine residue in the DNA binding domain of Jun and Fos dimers (Abate et al., 1990). Thus, Trx may act as an effective neuroprotector by suppressing hydroxyl radical generation, lipid peroxidation, caspase activation, cytochrome *c* release, and thus apoptosis (Andoh et al., 2002a; 2003). In this study, human neuroblastoma SH-SY5Ycells were employed to investigate the mechanism by which selegiline exerts its neuroprotective effect, other than its capacity to inhibit MAO-B.

Materials and Methods

Materials. The human neuroblastoma SH-SY5Y cells were kindly provided by Dr. Carol Thiele (National Cancer Institute, National Institutes of Health, Bethesda, MD). Dulbecco's modified Eagle medium, penicillin/streptomycin, and heat-inactivated bovine serum were purchased from Invitrogen (Carlsbad, CA). Hoechst 33258 (bisbenzimide), 1-chloro-2,4-dinitrobenzene (DNCB), H-89, and bisindolylmaleimide were ordered from Sigma Chemical Co. (St. Louis, MO). Oxidized *Escherichia coli* Trx and human catalase antibody were purchased from Calbiochem (San Diego, CA). Sense, antisense, and antisense mutant for human Trx mRNA (nucleotide sequences: antisense, 5′-TCTGCTTCACCATCTTGGCTGCT-3′; sense, 5′-AGCAGCCAAGATGGTGAAGCAGA-3′; mutant antisense, 5′-TCGTTCTCACCATCTTGGTCCGT-3′) and mouse Trx mRNA (nucleotide sequences: antisense, 5′-TCAGCTTCACCATTTTGGCTGTT-3′; sense, 5′-AACAGCCAAAATGGTGAAGCTGA-3′; mutant antisense, 5′-TCCATGTCACCATTTTGGTGTCT-3′) were synthesized as *S*-oligonucleotides by Invitrogen and by Hokkaido Bioscience (Hokkaido, Japan), respectively. Human Trx and mouse Trx antibody were obtained from MBL International (Watertown, MA) and Redox Bioscience (Kyoto, Japan), respectively. Antibodies against phosphorylated and nonphosphorylated c-Jun. MEK1/2, MAPK/Erk1/2, and c-Myc were obtained from Cell Signaling Technology Inc. (Beverly, MA) and BD PharMingen (San Diego, CA). A horseradish peroxidase-linked antibody against IgG was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Cell Culture. Human neuroblastoma SH-SY5Y cells ($\sim 10^6$ cells) were plated and cultured in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. These neurotrophic cells were incubated in 5% CO_2 at 37°C for 2 to 3 days before use. The mouse (C57BL/6J; Japan SLC, Shizuoka, Japan) dopaminergic neuron in primary culture was prepared according to Gille et al. (2002). Ten days after the primary culture, we used these cells for experiments. MPP^+-induced oxidative stress, such as lipid peroxidation, was assayed fluorometrically. Moreover, oxidative stress-mediated apoptotic cell death was assessed using Hoechst 33258 fluorescent dye after exposing these neurotrophic cells to MPP^+ (1 mM) for 24 h ($n = 6$). In some experiments, these human neuroblasts were transfected with *S*-oligonucleotide probes against human Trx mRNA for 48 h before the experiments. After administration, cells with selegiline and MPP^+ Western blotting was used to detect the induction of Trx, phospoactivation of transcription factors, and MAPK caused by selegiline ($n = 3$–6).

Staining of Nuclear DNA in Apoptotic Cells with Hoechst 33258. At the end of 24-h MPP^+ treatment, cells were harvested and fixed with 4% paraformaldehyde in ice-cold phosphate-buffered saline for 30 min. After rinsing with saline, nuclear DNAs were stained with 1 μM Hoechst 33258 fluorescent dye for 5 min at room temperature and observed with a fluorescent microscope (excitation/emission wavelength = 365/420 nm). Apoptotic cells were identified based on the presence of highly condensed and/or fragmented nuclei.

Lipid Peroxidation Assay. Cells were harvested 24 h after MPP^+ application (1 mM) in the presence or absence of Trx (1 μM). These collected cells were washed twice with ice-cold phosphate-buffered saline. After sonicating in 200 μl of saline, the protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). Fluorescent products of lipid peroxidation were extracted from homogenates using solvent extraction procedure. The fluorescent conjugated products of malondialdehyde and amino acids (excitation/emission wave lengths = 356/426 nm) were measured using a Luminescent Spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA) (Andoh et al., 2002b). Results were presented as relative fluorescent intensity. Experimental data were defined as relative fluorescent units per milligram of protein.

Transfection of Cells with Antisense, Antisense Mutant, and Sense Phosphorthionate Oligonucleotides. *S*-oligonucleotide probes of sense, antisense, and antisense mutant designed by Saitoh et al. (1998) for hybridizing human Trx mRNA were used. For transfection, 2 μM concentrations of each *S*-oligonucleotide were mixed with 2 μl of transfection reagent TM-50 (Promega, Madison, WI) in medium (800 μl) with 10% fetal bovine serum for 15 min at room temperature, and then the mixture was added to cells and incubated for 24 h. On the second day, cells were incubated with new freshly prepared medium containing *S*-oligonucleotides and transfection reagent and incubated for additional 24 h. After the second incubation, the culture medium was changed again with freshly prepared medium containing *S*-oligonucleotides and transfection reagent before the routine MPP^+ experiments with or without selegiline pretreatment.

Western Blotting of Prosurvival Proteins. Cells were homogenized in cell lysis buffer that contained 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified using a Bio-Rad protein assay kit. Cell protein (20 μg) was separated by electrophoresis using a 4 to 20% gradient SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blotted with a 5% skim milk solution for 1 h at room temperature. It was then incubated overnight at 4°C with a 1/2000 dilution of designated antibody ($n > 3$). Thereafter, blots were incubated with a horseradish peroxidase-linked antibody against mouse or rabbit IgG (1:2000) for 1 h at room temperature. Membrane-bound horseradish peroxidase-labeled protein bands were monitored with chemiluminescent regents (GE Healthcare). Chemiluminescent signals were detected using X-ray film. The autoradiographic images of protein bands were analyzed and semiquantified using NIH Image software provided by Dr. Wayne Rasband (National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

Data Processing. Data are presented as mean ± S.E. of the results obtained from the average of at least three independent experiments. Results were analyzed by one-way analysis of variance, and *P* values were determined using the Student-Newman-Keuls post hoc test. Differences among means were considered statistically significant when the *P* value was less than 0.05.

Results

Effects of Selegiline on MPP^+-Induced Apoptotic Cell Death. When human SH-SY5Y cells were incubated in culture medium for 24 h as controls, less than 5% of total cells underwent apoptosis, as indicated by chromatin condensation and nuclear fragmentation revealed by the Hoechst 33258 dye-staining method. We first confirmed that when these neurotrophic cells were treated with MPP^+ (1 mM) for 24 h, the population of apoptotic cells increased from 2.5 ± 0.7 to 71.0 ± 3.6% (Table 1) because of elevated reactive oxygen species, lipid peroxidation, and oxidative stress (Andoh et al., 2003). The application of selegiline (0.03–1 μM) protected these human neuroblastoma cells from apoptotic cell death induced by MPP^+ in a concentration-dependent manner (Fig. 1, IC_{50} = ~250 nM). We further investigated the molecular mechanisms of selegiline-evoked neuroprotection, which may not be produced by its selective inhibitory action on MAO-B.

The Role of Trx mRNA Induction in Selegiline Protection against MPP^+-Induced Cytotoxicity in SH-SY5Y Cells. It is interesting that selegiline (1 μM) elevated intracellular levels of the redox protein Trx. The time course of Trx expression induced by selegiline is depicted in Fig. 2. It shows that increased levels of Trx peaked at 8 h after the incubation of cells with 1 μM selegiline. To investigate whether the endogenous Trx synthesis induced by selegiline is involved in the protection against MPP^+-induced neurotoxicity, we transfected the SH-SY5Y cells with human Trx mRNA *S*-oligonucleotides, including sense, antisense, and

TABLE 1
Effects of Trx reductase inhibition on selegiline-induced antiapoptosis
The redox cycle of endogenous Trx is sensitive to the inhibition of the selenium-containing Trx reductase by DNCB (10 μM), which was administered to SH-SY5Y cells 5 min before the 24-h incubation at 37°C in the absence or presence of both selegiline (1 μM) and MPP^+ (1 mM), the toxic metabolite of MPTP, a dopaminergic neurotoxin.

Treatments			Apoptosis
DNCB	Selegiline	MPP^+	
			%
0 μM	0 μM	0 mM	2.5 ± 0.7
0 μM	0 μM	1 mM	71.0 ± 3.6*
0 μM	1 μM	1 mM	14.2 ± 3.4**
10 μM	1 μM	1 mM	71.3 ± 6.3*
10 μM	0 μM	0 mM	5.1 ± 1.0

* $P < 0.05$ significantly increased over respective control group; $n = 6$, analysis of variance $F = 95$, *t* test.
** $P < 0.05$ significantly decreased from other MPP^+-treated groups, $n = 6$, analysis of variance $F = 95$, *t* test.

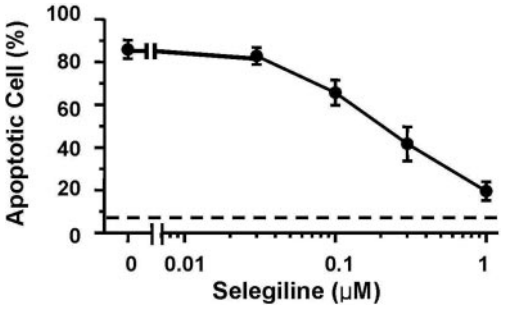

Fig. 1. Effect of selegiline on MPP^+-induced apoptosis in SH-SY5Y cells. Human SH-SY5Y cells were plated and cultured for 2 days before treatment with MPP^+ (1 mM for 24 h), the toxic MAO-B metabolite of MPTP. Several of the MPP^+-treated groups were pretreated with selegiline (0 to 1 μM, $n = 6$) 5 min before MPP^+ addition. The incubation was carried out at 37°C. The apoptotic cell nuclei were stained with fluorescent DNA dye (Hoechst 33256) and quantified as described under *Materials and Methods*. The dashed line depicts the level of apoptotic cells in the nontreated serum control groups. $P < 0.05$ significantly decreased after treatments with 0.1 to 1 μM selegiline. EC_{50} of selegiline is estimated to be approximately 250 nM.

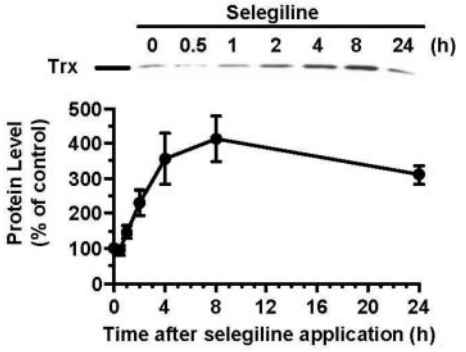

Fig. 2. Time course of Trx expression induced by selegiline. SH-SY5Y cell cultures were treated with selegiline (1 μM) for up to 24 h at 37°C. Protein in three dishes of the cell culture was harvested at different time points for detecting Trx expression by Western blotting using anti-human Trx monoclonal antibody ($n = 3$ per time point). The autoradiographic imaging data were quantified using the NIH Image software and presented as percentage of control level.

antisense mutant. Selegiline-induced expression of endogenous Trx was blocked if the SH-SY5Ycells were first transfected with antisense Trx mRNA but not with *S*-oligonucleotides of sense and antisense mutant (Fig. 3). In this particular study both the survival experiment and the antisense experiment were performed in the same cell groups. The increase in the endogenous Trx levels seems to correlate with the extent of cellular protection from MPP^+-induced apoptosis. Furthermore, the observed protective effect of selegiline was prevented by incubating with a specific inhibitor of Trx reductase, DNCB (10 μM) (Table 1). DNCB, at 10 μM or less, does not induce apoptosis in SH-SY5Y cells. These results indicate that the endogenous protective species induced by selegiline is probably Trx, which is known to play an important role in inhibiting apoptosis induced by MPP^+ (Andoh et al., 2002b; Bai et al., 2002).

Effects of Selegiline on Expression of c-Jun, Phosphorylation of MAPK/Erk1/2, and Phospho-Activation of c-Myc. In this study, we investigated whether any of these MAPK cascades and related transcription factors was involved in the selegiline-induced gene expression of Trx. Selegiline (1 μM) elevated levels of c-Jun that peaked at approximately 2 h, without altering levels of phosphorylated c-Jun (Fig. 4A). These findings suggest that selegiline may induce the transcription factor AP-1. However, selegiline did not alter protein levels of MEK1/2 and MAPK/Erk1/2. Without altering phosphorylation of MEK1/2, selegiline unexpectedly increased levels of the phosphorylated MAPK/Erk1/2, which were detected within 30 min and peaked between 2 and 4 h (Fig. 4A). Moreover, selegiline had no effect on the expression of the transcription factor c-Myc, while it increased the phospho-activation of c-Myc that peaked at 4 h after the administration of selegiline (1 μM) (Fig. 4A). These findings are in accord with a prior proposal that the elevation of Trx biosynthesis in response to preconditioning stress is mediated by enhanced biosynthesis of c-Jun and phosphor-activation of MAPK/Erk1/2 and c-Myc (Andoh et al., 2003).

Role of Protein Kinase A and C in Phospho-Activation of MAPK/Erk1/2 and the Expression of Trx Induced by Selegiline. It is known that phosphorylated MEK elicits the activation or phosphorylation of Erk1/2 members of MAPK. However, the levels of MEK and phosphorylated MEK did not change after the application of selegiline (1 μM) in the human SH-SY5Y cells (Fig. 4A). In our attempt to identify which protein kinase (i.e., PKA and PKC) is responsible for the phosphorylation of MAPK/Erk1/2, we investigated the effects of specific protein kinase inhibitors, H-89 (K_i for PKA, 48 nM; K_i for PKC, 32 μM) and bisindolylmaleimide (BIM) (K_i for PKC, 10 nM; K_i for PKA, 2 μM) that are highly selective inhibitors of protein kinase A and C, respectively. Preincubation of SH-SY5Y cells with H-89 (4 μM) but not BIM (1 μM) inhibited selegiline-induced MAPK/Erk1/2 phosphorylation by approximately 60% (Fig. 4B). In contrast, BIM—a protein kinase C inhibitor—failed to alter the basal protein levels of the phosphorylated MAPK/Erk1/2. Together, these results indicate that selegiline activates a PKA-depen-

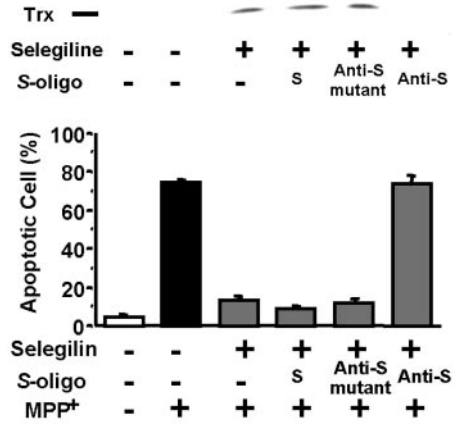

Fig. 3. Effects of sense, antisense, and antisense mutant phosphothionate oligonucleotides of human Trx on the expression of Trx and the suppression of MPP^+-induced apoptosis produced by selegiline. SH-SY5Y cells were transfected with *S*-oligonucleotide probes against human Trx mRNA, including sense (S), antisense (Anti-S), and antisense mutant (Anti-S mutant) probes before the exposure of these human neurotrophic cells to MPP^+ neurotoxin (1 mM for 24 h). Selegiline (1 μM for 24 h) was applied immediately before MPP^+ in some experimental groups to induce Trx and to inhibit MPP^+-induced apoptosis. Cells were harvested and analyzed for the expression of endogenous Trx using Western blotting analysis (top) and apoptosis (n = 6).

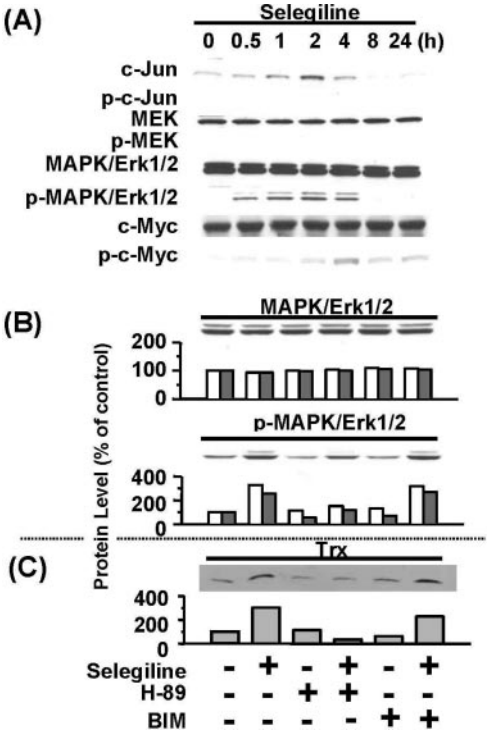

Fig. 4. Effects of selegiline on protein kinase-mediated phosphorylation of c-Jun, MAPK/Erk1/2, and c-Myc. A, SH-SY5Y cells were treated with Selegiline (1 μM) for up to 24 h at 37°C; cells were harvested at different time points for determining the expression and phospho-activation of c-Jun, MEK1/2, MAPK/Erk1/2, and c-Myc by Western blotting procedures. These data represent a typical result of the experiment that was repeated three times to measure the indicated proteins and phosphorylated-c-Jun (p-c-Jun), MEK1/2, MAPK/Erk1/2, phosphorylated-MAPK/Erk1/2 (p-MAPK/Erk1/2), c-Myc, and phosphorylated-c-Myc (p-c-Myc). Because MEK1/2 was not altered by selegiline treatment, we used selective protein kinase inhibitors to determine which protein kinase mediated the phosphoactivation of MAPK/Erk 1/2. In other experiments (B and C), SH-SY5Y cells were pretreated with the PKA inhibitor H-89 (4 μM) and the PKC inhibitor BIM (1 μM) 1 h before the administration of selegiline (1 μM). Open and hatched columns in B show the data of p42 Erk 1 and p44 Erk 2, respectively. C, results of the expression of Trx. The autoradiographic data were quantified using NIH Image software and presented as percentage of nontreated control. This experiment was repeated three times and every result showed the same tendency.

dent pathway that leads to phospho-activation of MAKP/Erk1/2. Moreover, protein kinase A inhibitor, but not protein kinase C inhibitor, inhibited the expression of Trx induced by selegiline (Fig. 4C).

Trx Prevents MPP^+-Induced Lipid Peroxidation and Cell Death. A recent in vivo study indicated that induction of Trx and related pro-survival proteins (superoxide dismutase, catalase, and GSH peroxidase) protect midbrain nigral neurons from MPTP-induced neurotoxicity (Kojima et al., 1999). Our previous in vivo data also indicate that selegiline protects A9 dopaminergic nigral neurons from oxidative injury caused by MPP^+, the toxic metabolite of MPTP (Wu et al., 1995). It has been shown to increase levels of reactive oxygen species, such as reactive hydroxyl radicals, which can react with polyunsaturated fatty acids to generate peroxyl lipid radicals, and the related toxic species, malondialdehyde and 4-hydroxy-2,3-nonenal (Chiueh et al., 1994; Rauhala et al., 1998). Malondialdehyde reacts with amino acids to form a fluorescent complex that is a reliable marker for lipid peroxidation. The application of MPP^+ (1 mM) causes a 2.7-fold increase in malondialdehyde adducts over controls; exogenously administered Trx (1 μM) significantly suppressed the formation of malondialdehyde (Fig. 5A) and apoptosis (Fig. 5B) caused by MPP^+ ($p < 0.05$).

Selegiline Induces Trx Expression that Prevents MPP^+-Induced Cell Death in Primary Cultures of Mouse Dopaminergic Neurons. In the above study, human neuroblastoma SH-SY5Y cells were used. The expected neuroprotective effect of selegiline was also confirmed in mouse midbrain dopaminergic primary neuronal cultures (Fig. 6). Selegiline (1 μM) induced the expression of Trx; this endogenous Trx expression was inhibited accordingly by pretreatment of antisense but not sense and antisense mutant *S*-oligonucleotides against mouse Trx mRNA. Furthermore, increases in endogenous Trx levels seem to correlate with the extent of cellular protection from MPP^+-induced apoptosis in primary neuronal cultures of mouse midbrain dopaminergic neurons. These findings are in accord with prior reports that selegiline protects nigrostriatal dopaminergic neurons from oxidative injury caused by MPP^+ in both in vitro preparations (Mytilineou and Cohen, 1985; Vizuete et al., 1993) and in vivo (Wu et al., 1995).

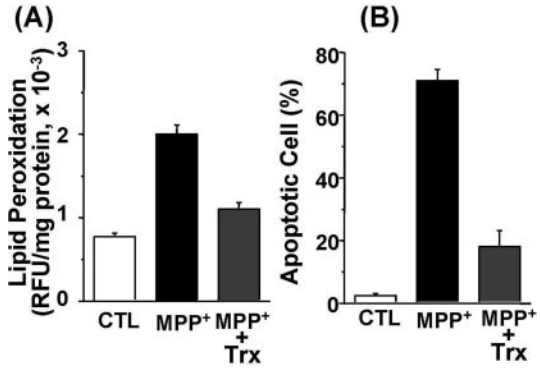

Fig. 5. Effect of exogenously administered Trx on lipid peroxidation (A) and apoptosis (B). SH-SY5Y cells were incubated with the oxidized protein Trx (1 μM), which is membrane-permeable and can be reduced intracellularly to its active form by Trx reductase (see Table 1). Lipid peroxidation (A) and apoptotic cell death induced by MPP^+ (1 mM) (B) were also determined before and after the preincubation of SH-SY5Y with Trx (1 μM) 10 min before MPP^+ application. ($n = 4$).

Discussion

Mytilineou and Cohen (1985) proposed a direct neuroprotective action of selegiline against MPP^+-induced neurotoxicity in midbrain dopamine cell cultures. However, this proposal was supplanted by a more prominent hypothesis that selegiline protects against MPTP-induced nigral degeneration simply through its known inhibitory action on MAO-B, which participates in converting MPTP to its toxic metabolite MPP^+ (Cohen et al., 1984; Chiueh, 1988; Heikkila et al., 1991). A decade later, this direct neuroprotection hypothesis of selegiline was substantiated by in vivo studies showing that selegiline indeed prevents MPP^+-induced neurotoxicity (Vizuete et al., 1993; Wu et al., 1995). However, some of the in vivo results suggest that selegiline protects and rescues A9 nigral neurons against oxidative stress and degeneration caused by MPP^+ via mechanisms independent of its capacity to inhibit MAO-B enzymatic activity. Prior studies also indicated that selegiline may induce antioxidative and neurotrophic genes for enhancing neuronal survival in vivo (Carrillo et al., 1991; Vizuete et al., 1993; Kitani et al., 1994; Semkova et al., 1996; Tatton et al., 1996; Revuelta et al., 1997; Kunikowska et al., 2002). Nevertheless, it should be noted that selegiline rescues only moderately affected midbrain dopamine neurons in vivo (Wu et al., 1995). The clinical efficacy of selegiline may be hampered by the fact that it may be too late for any drug to rescue severely damaged brain neurons.

The present in vitro results obtained from both neuroblasts and primary neuronal cultures imply that Trx may play a pivotal role in the induction by selegiline of antixoidative proteins (e.g., MnSOD) and antiapoptotic proteins (e.g., Bcl-2) for promoting cell vitality and survival. PKA rather than PKC may be involved in the phospho-activation of MAPK and c-Myc for the induction of Trx by selegiline at or below micromolar concentrations. Experimental data obtained from the use of human Trx antisense *S*-oligonucleo-

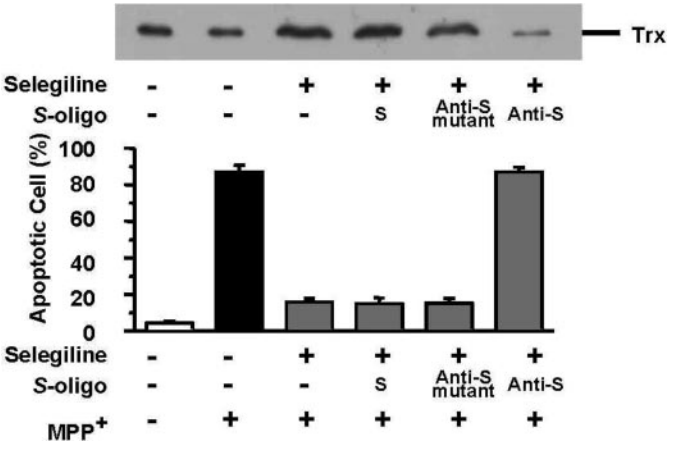

Fig. 6. Effects of sense, antisense, and antisense mutant phosphothionate oligonucleotides of mouse Trx on the expression of Trx (A) and the suppression of MPP^+-induced apoptosis produced by selegiline (B) in primary cultures of mouse midbrain dopaminergic neurons. Primary neuronal cultures of mouse midbrain dopaminergic neurons were transfected with *S*-oligonucleotide probes against mouse Trx mRNA, including sense (S), antisense (Anti-S), and antisense mutant (Anti-S mutant) probes before the exposure of these human neurotrophic cells to MPP^+ neurotoxin (1 mM for 24 h). Selegiline (1 μM for 24 h) was applied immediately before MPP^+ in some experimental groups to induce Trx and to inhibit MPP^+-induced apoptosis. Cells were harvested and analyzed for the expression of endogenous Trx using Western blotting analysis (top) and apoptosis ($n = 6$).

tides and Trx reductase inhibitor indicate that selegiline-induced cytoprotection against MPP^+ is probably mediated by elevated expression of Trx genes and proteins. A similar conclusion is drawn by recent studies of the induction of Trx gene (Kojima et al., 1999; Andoh et al., 2002). These new findings support a notion that MAO-B inhibition plays no role in the mechanism underlying selegiline-induced neuroprotective and/or neurorescue properties (Mytilineou and Cohen, 1985; Vizuete et al., 1993; Wu et al., 1995; Tatton et al., 1996). This new selegiline hypothesis of a Trx-mediated neuroprotective mechanism may explain why, in addition to its inhibition of neurotoxicity produced by MPTP, selegiline also protects against brain injury caused by 6-hydroxydopamine, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine, methyl-β-acetoxyethyl-2-chloroethylamine, 5,6-dihydroxyserotonin, 3,4-methylenedioxymethamphetamine, and MPP^+ (Mytilineou and Cohen, 1985; Vizuete et al., 1993; Wu et al., 1995; Tatton et al., 1996; Ebadi et al., 2002).

Earlier studies indicate that selegiline may induce several cytoprotective genes, such as NGF and GDNF, from non-neuronal cells such as astroglia cells; GDNF is known to protect nigral neurons against MPTP-induced neurotoxicity (Kordower et al., 2000). The present results indicate that selegiline's neuroprotective action may be acting, at least in part, through the induction of Trx and MnSOD, but not Cu/ZnSOD and catalase, in SH-SY5Y neuroblastoma cells that can differentiate into dopaminergic, serotonergic, and cholinergic neurons after the incubation with phorbol ester. Our results also provide critical evidence demonstrating that selegiline induced a redox protein Trx, which has recently been shown to mediate preconditioning-induced hormesis (Andoh et al., 2003). Moreover, this notion is also supported by the fact that Trx-dependent neuroprotective effects of selegiline against MPP^+ were reduced by approximately 80% after the transfection of cells with Trx antisense but not sense and antisense mutant *S*-oligonucleotides.

In preconditioned cells, Trx mediates the induction of Bcl-2 and MnSOD leading to a compensatory increase in cellular defense against oxidative stress caused by either serum withdrawal or MPP^+ (Andoh et al., 2002). It is also known that selegiline induces the biosynthesis of Bcl-2 (Tatton et al., 1996) and MnSOD (Kunikowska et al., 2002). Our results further suggest that selegiline rapidly induces elevation of Trx, subsequently followed by the increases in both Bcl-2 and MnSOD levels (Andoh et al., 2002b). Overexpression of Bcl-2 increases resistance against MPTP (Yang et al., 1998). The induction of Bcl-2 by Trx leads to the suppression of the release of mitochondrial cytochrome *c* and the protection of SH-SY5Y cells from MPP^+-induced apoptosis (Andoh et al., 2002, 2003). Moreover, increased expression of MnSOD is known to enhance neuronal resistance to oxidative brain injury produced by MPTP/MPP^+ in both in vitro and in vivo preparations (Andrews et al., 1996; Andoh et al., 2002b). Increased expression of mitochondrial MnSOD leads to removal of reactive superoxide radical anions, minimizing the generation of cytotoxic peroxynitrite and terminating lipid peroxidation-induced chain reactions. In fact, the induction of Trx among other antioxidative proteins increases the resistance of brain dopamine neurons against oxidative insults caused by MPTP in mice (Kojima et al., 1999). These findings suggest that Trx-mediated expression of both MnSOD and Bcl-2 by selegiline plays a pivotal role in protecting mitochondria and nuclei against lipid peroxidation and DNA damage/apoptosis caused by MPTP/MPP^+ and perhaps other neurotoxins as well. A possible molecular mechanism underlying the induction of the Trx gene and proteins by selegiline is summarized in Fig. 7. The phospho-activation of the 44- and 42-kDa MAP kinases or Erk1/2 may contribute to selegiline-induced neuroprotection against MPP^+-evoked neurotoxicity, because it has been suggested that Erk1/2 phosphorylation can activate a dual survival mechanism. In fact, selegiline increased the expression of c-Jun within 30 min; expression peaked at 2 h and thus could lead to increased levels of the redox-sensitive transcription factor AP-1. It is noteworthy that levels of phosphorylated MAPK/Erk1/2 were induced by selegiline, appearing within 30 min and lasting for more than 4 h after treatment. The inhibition of PKA but not PKC blocked approximately 60% of the phosphorylation of MAPK/Erk1/2 induced by selegiline; these results suggest that activation of MAPK/Erk1/2 by selegiline may involve the PKA-mediated signaling pathway, whereas the preconditioning-induced hormetic pathway is mediated by PKG (Andoh et al., 2003). Selegiline-activated MAPK/Erk1/2 can then be translocated into the nucleus to regulate transcription factors such as c-Myc (Davis, 1995). Activation of either c-Myc or c-Jun has been proposed to modulate stress-induced gene induction. The PKG-mediated phospho-activation of c-Myc by phosphorylated Erk1/2 is known to induce the *Trx* gene in preconditioned SH-SY5Y cells (Andoh et al., 2003). As discussed above, selegiline-induced MAPK cascades seem to involve the PKA pathway; however, the mechanistic nature of this selegiline-induced gene induction remains to be elucidated. In perspective, selegiline may become a lead drug that can open a new research field of chemical preconditioning to mimic nonlethal oxidative stress-induced adaptation for awaking genes and their proteins in modulating cellular functioning (Chiueh and Andoh, 2002). In addition to human neuroblastoma cells, selegiline also protected the MPP^+-in-

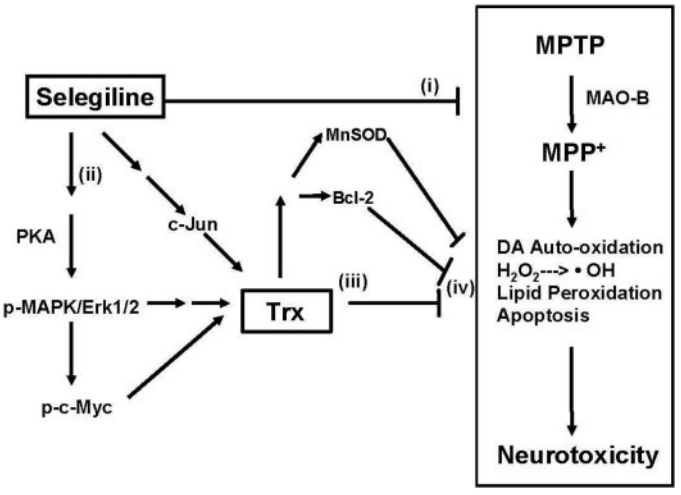

Fig. 7. Schematic diagram of proposed Trx-mediated cytoprotective mechanism(s) of selegiline. i, inhibition of MAO-B. At relatively higher concentrations, selegiline inhibits MAO-B and prevents the conversion of MPTP to the toxic metabolite, MPP^+. ii, signal cascades and gene induction. Selegiline induces Trx transcription at or below 1 μM concentrations via its c-Myc, AP-1, and PEA3 binding sites. iii, Trx-mediated antioxidative and antiapoptotic mechanisms. Selegiline induces MnSOD and Bcl-2. iv, neuroprotection. Selegiline protects against oxidative stress and apoptosis caused by MPTP and MPP^+ in both in vitro and in vivo preparations.

duced cell death in primary neuronal cultures of mouse dopaminergic neuron through the expression of Trx (Fig. 6); our new finding supports an early notion that selegiline rescues midbrain dopamine neurons in mice via a MAO-independent mechanism (Wu et al., 1995). Therefore, the modification of selegiline structure by retaining its capacity of induction of survival genes/proteins may lead to new pharmaceutical lead compounds targeting neuroprotective therapeutics. The development of new drugs that induce multiple prosurvival genes and proteins for enhancing cellular defense against progressive neurodegenerative brain disorders may be a key contribution to the rescue and repair of brain neurons after prolonged oxidative injury.

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