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Activation of tyrosine kinase receptor signaling pathway by rasagiline facilitates neurorescue and restoration of nigrostriatal dopamine neurons in post-MPTP-induced parkinsonism

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The anti-Parkinson monoamine oxidase (MAO)-B inhibitor rasagiline (Azilect) was shown to possess neuroprotective activities, involving the induction of brain-derived- and glial cell line-derived neurotrophic factors (BDNF, GDNF). Employing conventional neurochemical techniques, transcriptomics and proteomic screening tools combined with a biology-based clustering method, we show that rasagiline, given chronically post-MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), exerts neurorescue/neurotrophic activity in mice midbrain dopamine neurons. Rasagiline induced the activation of cell signaling mediators associated with neurotrophic factors responsive-tyrosine kinase receptor (Trk) pathway including ShcC, SOS, AF6, Rin1 and Ras and the increase in the Trk-downstream effector phosphatidylinositol 3 kinase (PI3K) protein. Confirmatory Western and immunohistochemical analyses indicated activation of the substrate of PI3K, Akt and phosphorylative inactivation of glycogen synthase kinase-3β and Raf1. Thus, the activation of Ras-PI3K-Akt survival pathway may contribute to rasagiline-mediated neurorescue effect. It is interesting to determine whether a similar effect is seen in parkinsonian patients after long-term treatment with rasagiline.

Keywords: Rasagiline; Monoamine oxidase; MPTP; Neurorescue; Transcriptomics; Proteomics; Tyrosine kinase; PI3K; AKT; GDNF; BDNF

Introduction

In Parkinson's disease (PD) approximately 50–70% of the dopamine (DA)-containing neurons are already lost at the time of diagnosis (Morrish et al., 1998). Thus, the challenge has been to induce neuroprotection or neurorescue by therapeutics that would respectively prevent or stop the progressive neurodegeneration. In this respect, a recent study has been initiated to identify potential neuroprotective agents for testing in clinical trials (Ravina et al., 2003). Using specific criteria, these authors found 12 compounds to be attractive candidates, including neurotrophic factors (such as BDNF), bioenergetics (coenzyme Q) and the novel anti-Parkinson drug rasagiline (*N*-propargyl-1(*R*)-aminoindan), a second generation selective inhibitor of monoamine oxidase (MAO)-B (Youdim et al., 2001; Rascol et al., 2005). Rasagiline possesses neuroprotective activity, which has been demonstrated in various models, both *in vitro* and *in vivo*, including the neurotoxins MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine (6-OHDA) rodent models of PD (Heikkila et al., 1985; Blandini et al., 2004; Tabakman et al., 2004). Similar to other MAO-B inhibitors, the neuroprotective activity of rasagiline in the MPTP, but not the 6-OHDA model, is attributed to inhibition of MAO-B (Heikkila et al., 1984). However, rasagiline also possesses neuroprotective activity unrelated to its MAO-B inhibition since its optical *S*-isomer TVP-1022, which is a very poor MAO-A or -B inhibitor, displays similar neuroprotective effects in neuronal cell culture and in some *in vivo* models (Youdim et al., 2005). This is attributed to the propargylamine moiety since it induced the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-w and $Bclx_L$, the protein kinase C (PKC) isoforms α and ε, and reduced the expression of Bax and Bad (Bar-Am et al., 2005; Yi et al., 2005). Similarly, rasagiline has been shown to increase the levels of brain-derived- and glial cell line-derived neurotrophic factors (BDNF, GDNF) mRNAs and proteins (Maruyama et al., 2004; Weinreb et al., 2004). In addition to its neuroprotective activity, the potential neurorescue action of rasagiline was demonstrated in a progressive apoptotic model of neuronal cell death induced by long-term serum deprivation (Bar-Am et al.,

Abbreviations: BDNF, brain-derived neurotrophic factor; DA, dopamine; DAVID, Database for Visualization Annotation and Integrated Discovery; DOPAC, dihydroxyphenyl acetic acid; Fasl, Fas ligand; GDNF, glial cell line-derived neurotrophic factor; GSK, glycogen synthase kinase; HVA, homovanilic acid; JNKK, c-*jun* N-terminal kinase kinase; mGluR, metabotropic glutamate receptor; MAO, monoamine oxidase; MKK, mitogen-activated protein kinase kinase; MPP^+, 1 methyl-4 phenylpyridinium; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF, nerve growth factor; PI3K, phosphoinositol 3 kinase; PD, Parkinson's disease; PKC, protein kinase C; PLC, phospholipase C; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; Trk, tyrosine receptor kinase.

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2005). A recent controlled clinical delayed study in parkinsonian patients has shown that patients treated with rasagiline for 1 year showed less functional decline than patients whose treatment was delayed for 6 months, suggesting that the drug might possess a disease modifying property (Parkinson Study Group, 2004; Blandini, 2005), and this advantage persisted six and a half years[1].

The novel pharmacological activities of rasagiline, demonstrating neuroprotective and neurorescue activities in neuronal cell culture studies, prompted us to determine its possible neurorescue activity in post-MPTP-induced nigrostriatal dopamine neurodegeneration model in mice. Parallel detailed transcriptomics and proteomics analyses were conducted to elucidate the molecular mechanism of neuroprotective action and signaling pathways mediated by rasagiline.

Materials and methods

Animals and treatments

All procedures were carried out in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Ethics Committee of the Technion, Haifa, Israel. Male C57/BL6 mice (20–22 g; Harlan, Israel) were housed under a 12 h light interval. Mice were acclimatized for 5 days before any treatment.

Five groups of mice ($n=6$–10) were assigned for the neurorescue study. MPTP (24 mg/kg, i.p, per day) was administrated for 4 days, followed by a further 4 days resting period (day 8) to allow for the full conversion of MPTP to its active metabolite, MPP^+ (Levites et al., 2001). The groups of mice sacrificed at day 8 (MPTP day 8), was enrolled for evaluation of spontaneous recovery following the lesion. At day 8, either rasagiline mesylate (0.05 mg/kg, MPTP/rasagiline) or water (MPTP day 18) was administered orally for 10 days, reaching a total treatment period of 18 days. The last 2 groups consisted of mice receiving rasagiline only (0.05 mg/kg, orally) and water (orally) as controls.

All mice were sacrificed by cervical dislocation. Isolated striata of mice were biochemically analyzed for catecholamine content by HPLC, while ventral midbrains were used for global gene/protein expression analysis. Midbrains were punctured under dissecting microscope (Olympus, Melvile, NY, USA), within coronal slice between bregma 2.75 and 3.1 (A Sterotaxic Atlas of the Mouse Forebrain, Public Health Service, Maryland, USA, 1975). The midbrain of one hemisphere was immediately immersed in liquid nitrogen for protein extraction, while the other one was processed for RNA.

cDNA and protein expression studies

RNA forms the midbrain of control and rasagiline groups was analyzed for global transcript expression, using Atlas mouse 1.2 cDNA arrays (Clontech, Palo Alto, CA, USA), which included 1185 genes (see Supplementary data for detailed description). Total protein from the midbrain of control, rasagiline and MPTP mice groups was processed for global protein expression by the PowerBlot facility (Clontech, Lexington, KY, USA), determining the expression level of 715 different mice proteins (see Supplementary data for detailed description). Full gene and protein expression data have been deposited in NCBIs Gene Expression Omnibus (GEO) and is accessible through GEO Series accession numbers GSE1856 and GSE1857, respectively.

Neurochemical analysis

In order to determine the correlation between MAO-B inhibition and neurorescue, mice received 0.05 and 0.1 mg/kg rasagiline with or without pretreatment with MPTP ($n=15$). Striata were rapidly dissected and tyrosine hydroxylase (TH) activity, dopamine (DA) and its metabolites, homovanilic acid (HVA) and dihydroxyphenyl acetic acid (DOPAC) levels were analyzed using electrochemical (EC) coupled HPLC, as was previously described (Sagi et al., 2003). Striatal monoamine oxidase (MAO) activity was determined using C^{14}-phenylethylamine as a substrate for MAO-B as previously described (Grunblatt et al., 2001).

Immunohistochemistry

Six mice per group were deeply anesthetized with Ketamine hydrochloride 100 mg/ml (Font-dodge, Iowa, USA) plus Xylazine 20 mg/ml (VMD, Arendonk, Belgium) and were transcardially perfused with PBS for 2 min followed by 90 ml of 4% (vol/0.1 M PBS vol) paraformaldehyde for 9 min. Brains were post-fixed in 4% (vol/0.1 M PBS vol) paraformaldehyde (48 h, 2–8°C) and incubated in 70% ethanol followed by subsequent dehydration steps as described (Huang et al., 2005), see Supplementary data for detailed description.

Assessment of neuronal immunolabeling

Assessment of neuronal loss in the substantia nigra (SN) pars compacta (SNpc) was determined by serial section analysis of the area encompassed between −3.08 and −3.20 from bregma since the reduction of TH-positive neurons in response to MPTP treatment is most prominent at medial levels of the SNc, around the medial terminal nucleus, as shown previously (Hayley et al., 2004). A total of 6–10 mice per treatment group were employed. For each TH labeled section, adjacent tissue was stained for cresyl violet in order to confirm the vitality of these cells. Quantification of TH, as well as all other antibody staining, was calculated with AnalySIS 4.0 imaging software (Soft imaging system, Lakewood, CO, USA), using an AX70 microscope system with a DP70 camera (Olympus, Melvile, NY, USA) by a technician unaware of the experimental conditions. For each animal, estimates of the total TH-positive stained and cresyl violet-stained neurons in the SNpc were calculated on each section by measuring the hematoxylin-positive nuclei number and the average diameter of the TH-positive neuron nucleus for correction of the total number of neurons per section according to the formula $N=n\ (t/(t+d))$, where N=average total number of cells, n=number of cells counted, t=section thickness and d=cell nucleus diameter (Abercrombie, 1946). No significant differences in cell nucleus diameter were found among brains.

Statistical analysis

Differences in all biochemical measures were evaluated by one-way ANOVA, followed by a *post hoc* student *t*-test, whereas differences in immunochemical staining were processed with the non-parametric Kruskal–Wallis analysis of variance (ANOVA) followed by the Mann–Whitney *U*-test. Values of $p<0.05$ were considered significant.

[1] The abstract entitled "Early rasagiline therapy shows long-term benefit for Parkinson's disease" was presented by the Parkinson Study group in the 9th International Congress of Parkinson's disease and Movement Disorder, New Orleans, 2005.