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Protection of the aged substantia nigra of the rat against oxidative damage by (−)-deprenyl

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1 We have studied the effect of (−)-deprenyl on the oxidative damage that the rat substantia nigra suffers during aging.

2 (−)-Deprenyl (2 mg kg^{-1}, three times a week) administered for two months, beginning at 22 months of age, produced a significant increase in tyrosine hydroxylase (TH) activity (2.67 ± 0.40 and 3.64 ± 0.38 nmol mg^{-1} protein h^{-1} in untreated aged rats and treated aged rats respectively, $P<0.05$) and in TH amount (0.072 ± 0.012 and 0.128 ± 0.38 absorbance 405 nm in untreated aged and treated aged rats respectively, $P<0.05$).

3 The proteins of aged rat substantia nigra showed a significant decrease of carbonyl groups in treated animals compared with saline-injected control rats (136.2 ± 21.8 and 71.5 ± 13.2 c.p.m. μg^{-1} protein in untreated aged and treated aged rats respectively, $P<0.05$).

4 The carbonyl groups measured in TH enzyme showed a statistically significant decrease (42.3%) after (−)-deprenyl treatment (471.4 ± 73.0 and 271.9 ± 50.00 c.p.m. in untreated aged and treated aged rats respectively, $P<0.001$).

5 All these results suggest that oxidative damage produced during aging is prevented by (−)-deprenyl treatment and could explain the effect of this drug in Parkinson's disease (PD) and other degenerative diseases such as Alzheimer's disease.

Keywords: Aging; deprenyl; substantia nigra; oxidative stress; tyrosine hydroxylase

Introduction

Deprenyl, an irreversible selective inhibitor of monoamine oxidase B (MAO B), has been reported as a safe therapeutic agent for a variety of neurodegenerative diseases and possibly for aging. It has several marked effects that facilitate the activity of dopaminergic neurones in the nigrostriatal system selectively (Knoll, 1993). It blocks nigrostriatal toxicity induced by 6-hydroxydopamine (6-OHDA) in rats (Zsilla *et al.*, 1986), by MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydro-pyridine) in mice (Tatton & Greenwood, 1991), and by 1-methyl-4-phenylpyridinium ion (MPP^+) in rats (Vizuete *et al.*, 1993). At the same time, multi-site studies treating Parkinson's patients with deprenyl indicated that (−)-deprenyl treatment was capable of delaying the onset of disability (The Parkinson Study Group, 1993) and possibly increasing survival of treated patients (Birkmayer *et al.*, 1985). Given in small doses over long periods, it slows down the age-related decline of performance in behavioural tests (Knoll, 1993) and extends life-span considerably in rats (Knoll, 1990). The positive effect of deprenyl on life-span has been confirmed (Milgram *et al.*, 1990; Kitani *et al.*, 1993). Furthermore, in rats, deprenyl may improve the functions of meso-limbo-cortical dopaminergic neurones, which are associated with cognitive processes (Brandeis *et al.*, 1991). Many studies have already been published on the beneficial effects of deprenyl in Alzheimer's disease (AD) (Heinonen *et al.*, 1993).

In addition, it is known that the diminution in central nervous system function that occurs with aging is accompanied by changes in various neurotransmitter systems (Agnati *et al.*, 1984). Recently, we have shown that during aging the rat substantia nigra suffers oxidative damage that results in both an increase in carbonyl groups of its total proteins and the oxidative inactivation of tyrosine hydroxylase enzyme (TH) (de la Cruz *et al.*, 1995). This is in accord with the generation of oxidants during normal metabolism which appear to play a significant role in the processes of aging (Harman, 1981). Moreover, taking into account that deprenyl treatment prevents age-related pigment changes in the substantia nigra (Knoll *et al.*, 1992), in the present work we have studied the effect of chronic treatment with (−)-deprenyl on the oxidative damage reported by us in the nigrostriatal dopaminergic system of aged rats (de la Cruz *et al.*, 1995). Twenty-two-month-old rats were treated with deprenyl (2 mg kg^{-1}) for two months until reaching 24 months of age. We studied levels of dopamine and its metabolites, the activity and content of TH, the step-limiting enzyme in the biosynthesis of dopamine, and protein marker of dopaminergic neurones. The carbonyl derivatives resulting from oxidative damage to proteins (Ahn *et al.*, 1992) were measured in homogenized proteins and TH. The neurofilaments and glial fibrillary acid protein (GFAP) were also measured. All these measurements were carried out in the substantia nigra and striatum of treated and untreated aged animals. Deprenyl seems to have a protector capacity against oxidative damage produced in the striatonigral dopaminergic system of aged rats. Deprenyl also induced TH enzyme expression in substantia nigra.

Methods

Animals, treatment and dissection

Male Wistar rats, 22 months old, were used for this study. They were divided into two groups. Control animals were given s.c. injections of physiological saline solution three times a week for a period of two months. Experimental animals were injected s.c. with (−)-deprenyl (2 mg kg^{-1}) dissolved in saline

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solution three times a week for the same period. The day following the last injection, animals were decapitated between 10 h 00 min and 11 h 00 min and the brains removed and placed on an ice-cold plate. The substantia nigra and striatum were dissected according to the atlas of Paxinos & Watson (1986). A cut was made through the corpus callosum and the cortex was peeled back, exposing the striatum which was carefully removed. The mesencephalon was divided into two parts with a cut from the ventral side perpendicular to the long axis of the mesencephalon, exactly at the caudal border eminence. The two substantia nigrae were then easily identified and freely dissected. The ventral tegmental area (A 10) was not included. The total time for tissue isolation was less than 3 min. The rats did not show brain tumours (these pathological lesions can affect forebrain cell functions; Lamour *et al.*, 1987). After dissection the brain parts were immediately frozen in liquid N_2 and stored at −80°C until assay.

(−)-Deprenyl was purchased from Research Biochemicals Inc., Natick, MA, U.S.A. Extra Avidine peroxidase conjugate and 2,2-azino-*bis*(3 ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-tyrosine hydroxylase monoclonal antibody, anti mouse-Ig-biotin conjugate, anti-neurofilament 68 kDa and anti-glial fibrillary acidic protein (GFAP) were purchased from Boehringer Mannheim.

Measurement of dopamine and its metabolites

Analyses were performed by h.p.l.c. with electrochemical detection as described by Castaño *et al.* (1993).

The brain tissues were homogenized in 0.1 M perchloric acid containing 1 mM sodium bisulphite by ultrasonic disintegration over ice with a Labsonic 1510. Samples were centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was then filtered through a 0.2 μm filter and injected onto h.p.l.c.

The pellet was resuspended in carbonate-bicarbonate buffer pH 9.0. These samples were used to measure the content of proteins and TH. Protein concentration of samples was determined as described by Lowry *et al.* (1951).

Tyrosine hydroxylase activity assay

TH activity was measured *in vitro* as described previously (Reinhard *et al.*, 1986) with some modifications. Briefly, samples were homogenized (5 w/v) in 50 mM Tris buffer containing 0.2 mM dithiothreitol (DTT) and 8% sucrose. An aliquot was further diluted 1:60 with 30 mM Tris-acetic acid containing 0.1% Triton X-100 and incubated with 2.5 nmol of tyrosine-HCl (containing 0.4 μCi $nmol^{-1}$ of L-[ring-3,5-^{3}H]-tyrosine), 50 nmol of the cofactor 6(**R**)-L-*erythro*-5,6,7,8-tetrahydrobiopterin, 5,000 units of catalase and 5 nM DTT in 100 mM potassium phosphate, pH 6.0. The released [^{3}H]-OH was separated by an aqueous slurry of activated charcoal, and the radioactivity was determined by liquid scintillation counting.

Quantification of tyrosine hydroxylase

Concentration of TH from substantia nigra and striatum was measured by an enzyme-linked immunoabsorbant assay (ELISA). This was performed as previously described (Mogi *et al.*, 1987; Wolf *et al.*, 1991) with modifications. Antigen was diluted in carbonate-bicarbonate buffer pH 9.0 (100 μg protein ml^{-1}). The wells of the NUNC microtitre plate were coated in triplicate with 100 μl/well and incubated overnight at 4°C. Blanks without the first antibody and negative controls with liver homogenates (100 μg protein) were included in triplicate on each plate. Unbound antigen was removed by inverting the plate and washing well with PBS-Tween; this process was repeated three times. Unbound sites were blocked with 200 μl of 3% (w/v) bovine serum (BSA) in PBS-Tween and left at room temperature for 1 h. The wells were rinsed with PBS-Tween five times. After rinsing, 100 μl anti-TH solution diluted 1/100 in PBS-Tween was added to each well and incubated for 1 h at room temperature. The plate was then washed as before and incubated for 1 h at room temperature with biotinylated sheep anti-mouse IgG (1/500 in PBS-Tween). Subsequently, the plate was washed again and 100 μl of Avidin-peroxidase conjugate in appropriate dilution (1/100) was added to each well. Following 1 h of incubation at room temperature, 100 μl substrate solution (10 mg ABTS dissolved in 100 ml citrate buffer, pH 5.8, containing 0.02% hydrogen peroxide) was distributed into each well. The reaction was allowed to develop for 60 min at room temperature, in the dark. The absorbance was measured at 405 nm with a plate reader (Titertek multiskan II).

Quantification of neurofilaments and glial fibrillary acidic protein

These determinations were carried out as described above, using anti-neurofilament 68 kDa and anti-GFAP monoclonal antibodies.

Determination of carbonyl groups of proteins in homogenates

Carbonyl groups were determined by measurement of incorporation of tritium (^{3}H) after reduction with sodium boro [^{3}H]-hydride as described by Lenz *et al.* (1989).

Determination of carbonyl groups of tyrosine hydroxylase enzyme

The procedure used four rats in each experimental group. Each substantia nigra was homogenized in RIPA buffer (10 mM Tris/HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 1% deoxycholic acid and 0.1% sodium dodecyl sulphate (SDS) plus protease inhibitors: leupeptin, 0.5 mg l^{-1}, pepstatin, 0.7 mg l^{-1} and phenylmethyl-sulphonyl fluoride (PMSF), 40 mg l^{-1}) using a Labsonic 1510 homogenizer. The homogenates were centrifuged at 12,000 *g* in a microfuge and an aliquot of the supernatant (150 μg protein) was labelled by treatment with radioactive sodium borohydride as described by Lenz *et al.* (1989). After incubation for 30 min at 37°C, samples were dialyzed in a Microdialyzer system 500 (Pierce) using a 3,500 mol.wt. cut-off dialysis membrane. Monoclonal antibody against TH was incubated with protein A sepharose CL-4B (Sigma Chemical Co.) for 4 h at 4°C and the complex was washed three times in RIPA buffer. An aliquot of the radiolabelled and dialyzed sample (10 μg protein) was incubated with the complex protein A-antibody overnight at 4°C. The samples were centrifuged in a microfuge and the immunoprecipitate was washed three times with RIPA buffer. Then, 0.5 ml of Protosol (New England Nuclear) was added to the samples. After heating at 60°C overnight, 17 μl of glacial acetic acid was added. Radioactivity incorporated into TH was determined using 10 ml of Formula-989 LSC cocktail (New England Nuclear).

Statistical analyses

All results are the mean ± s.d. from four determinations. Student's *t* test was used. When the *P* value was <0.05 (*) or <0.01 (**), the difference was considered significant.

Results

Effects of (−)-deprenyl treatment on concentrations of dopamine and its metabolites in striatum and substantia nigra

Table 1 shows the levels of dopamine and its metabolites in the striatum and substantia nigra of aged rats treated and untreated with (−)-deprenyl. The deprenyl treatment produced changes in the catecholamine content: there was a significant

increase in dopamine content (21 and 23%) along with a significant decrease of 3,4-dihydroxyphenylacetic acid (DOPAC) (−73 and −55%) in striatum and substantia nigra respectively.

The treatment also produced a significant increase in 3-methoxytyramine (3-MT) (40% in striatum and 140% in substantia nigra), along with a significant decrease in homovanillic acid (HVA) (−65%) in striatum, without change in substantia nigra.

Effects of (−)-deprenyl treatment on the activity and the amount of the TH enzyme in striatum and substantia nigra

The activity of TH was measured *in vitro*. The (−)-deprenyl treatment produced changes in TH enzyme (Table 2), the TH activity increasing in substantia nigra (40%) without significant changes in striatum. This increase of TH activity is in accord with the increase in TH amount. The enzyme amount did not change in striatum but a significant increase was found in substantia nigra (70%).

Effects of (−)-deprenyl treatment on carbonyl groups in homogenates and in TH enzyme in striatum and substantia nigra

The carbonyl groups of proteins have been widely used as a measurement of proteins modified by oxidation. The carbonyl groups in the homogenate of striatum did not change following deprenyl treatment; however, the carbonyl groups in the homogenate of substantia nigra decreased significantly (−48%) (Table 3), reaching a concentration of carbonyl groups similar to that found in substantia nigra of young animals (de la Cruz *et al.*, 1995). The carbonyl groups of the specific proteins of TH enzyme from substantia nigra also decreased (−43%) after deprenyl treatment, reaching a concentration similar to that found in young animals (de la Crux *et al.*, 1995).

Effects of (−)-deprenyl treatment on the neurofilaments 68 kDa and GFAP in striatum and substantia nigra

The effects of (−)-deprenyl treatment in aged rats on the neurofilaments 68 kDa and in GFAP have been studied. The deprenyl treatment did not produce any change in the levels of neurofilaments 68 kDa in either of the structures studied (data not shown). However, this treatment produced changes in the GFAP in both structures. There was a significant increase of GFAP in the striatum of aged rats treated with deprenyl (0.729 ± 0.030 and 0.804 ± 0.040 in untreated aged rats and aged rats treated with deprenyl respectively, $P < 0.05$). In contrast, there was a significant decrease of GFAP in substantia nigra of aged rats treated with deprenyl (0.772 ± 0.016 and 0.755 ± 0.006 in untreated aged rats and aged rats treated with deprenyl, respectively, $P < 0.005$. The results are expressed as absorbance at 405 nm).

Discussion

In order to show the basic mechanisms involved in the inhibition of MAO enzymes, leading to a reduced conversion of dopamine to DOPAC that results in increased dopamine levels and decreased DOPAC, we studied the changes in catecholamine content produced by deprenyl treatment in aged rats. There was a significant increase in dopamine content along with a significant decrease of DOPAC in striatum and substantia nigra. These results are in agreement with previous findings (Heinonen *et al.*, 1993).

Table 3 Measurement of carbonyl groups in aged rats after treatment with (−)-deprenyl

	Striatum homogenates	*Substantia nigra homogenates*	*Substantia nigra TH enzyme*
Controls	141.96±28.1	136.2±21.8	471.4±73.0
Deprenyl	143.48±16.8	71.5±13.2**	271.9±50.0**

Measurements of carbonyl groups in protein homogenates of substantia nigra and striatum are expressed as c.p.m. μg^{-1} protein. The carbonyl groups of TH enzyme of substantia nigra are expressed as c.p.m. All data are mean±s.d. from four determinations. Statistical significance: Student's *t* test: $*P < 0.05$; $**P < 0.01$. Deprenyl compared with controls.

Table 1 Levels of dopamine and its metabolites in aged rats after treatment with (−)-deprenyl

	Striatum		*Substantia nigra*	
	Controls	*Deprenyl*	*Controls*	*Deprenyl*
Dopamine	4356±306	5304±107**	241.3±5.5	298.3±23.5*
DOPAC	1745±164	485±63**	71.73±3.9	32.8±0.4**
3-MT	293.0±36	411.5±30**	19.70±1.6	47.33±2.3**
HVA	469.3±59	175.6±28**	18.4±1.5	19.3±1.3

All results are expressed as ng g^{-1} wet tissue as means±s.d. from four determinations. Statistical significance: Student's *t* test: $*P < 0.05$; $**P < 0.01$; deprenyl compared with controls.

Table 2 Tyrosine hydroxylase (TH) activity and content in aged rats after treatment with (−)-deprenyl

	TH activity (nmol mg^{-1} protein h^{-1})		*TH content* (absorbance 405 nm)	
	Controls	*Deprenyl*	*Controls*	*Deprenyl*
Striatum	6.51±0.64	6.59±1.24	0.184±0.021	0.196±0.006
Substantia nigra	2.67±0.40	3.64±0.38*	0.072±0.012	0.128±0.013**

TH activity was measured *in vitro* and TH content was obtained by measuring absorbance at 405 nm using a monoclonal antibody to TH. All data are mean±s.d. from four determinations. Statistical significance: Student's *t* test: $*P < 0.05$; $**P < 0.01$; deprenyl compared with controls.

The treatment with deprenyl also produced a significant increase in 3-MT, along with a substantial decrease in HVA, in striatum. Taking into account that the highest dose in rats which blocks MAO B activity but leaves MAO A relatively unaffected is about 0.25–0.5 mg kg^{-1} (Knoll, 1978), such results could be produced by a nonselective inhibition by deprenyl of MAO A activity. Some metabolic differences have been found between substantia nigra and striatum. It thus seems surprising that although the higher 3-MT increase was found in substantia nigra, HVA did not change in this structure but decreased significantly in striatum.

The deprenyl treatment also produced an increase in TH activity along with an increase in TH amount in substantia nigra without significant changes in striatum. The substantia nigra showed a significant decrease in carbonyl groups in both homogenate and TH enzyme after deprenyl treatment.

These results demonstrate that deprenyl treatment produces an induction of TH along with a protection against oxidative damage during the aging process in substantia nigra. Deprenyl treatment prevents age-related pigment changes in the substantia nigra (Knoll *et al.*, 1992). Rinne *et al.* (1991) showed that the number of medial nigral neurones was greater, and the number of Lewy bodies per neurone was smaller, in those PD patients who had been treated with deprenyl in combination with levodopa compared with those who were treated with levodopa alone. This result could be important, since all of these patients were in the final stage of the disease and severely disabled, and thus had a considerable loss of nigral neurones. It should be noted, however, that Ingram *et al.* (1993) did not find any beneficial effects in aged mice chronically treated with deprenyl.

The protective effect of deprenyl against oxidative stress could be produced by its inhibitory action on MAO B (Cohen & Spina, 1989). However, some evidence indicates that the inhibition of MAO A could also be important (for review see Cesura & Pletscher, 1992). In brain, MAO may play an essential role in oxidative stress which, by altering the redox state of neuronal and glial cells, leads to cell damage and tissue degeneration. Several investigators have shown increased MAO activity (particularly of MAO B) in certain brain areas of elderly people and in neurological disorders (Strolin Benedetti & Dostert, 1989). MAO B is highly expressed in astrocytes in or around senile plaques in patients with senile dementia of the Alzheimer type (Nakamura *et al.*, 1990). The protective effect of deprenyl against oxidative stress could also be produced by the induction of superoxide dismutase (SOD) and catalase (CAT) –enzymes involved in the scavenging of free radicals– in the nigrostriatal system (Carrillo *et al.*, 1994). Deprenyl increases SOD and CAT activities (but not that of glutathione peroxidase) in the rat in some brain regions, including striatum and substantia nigra (Carrillo *et al.*, 1994). Furthermore, the action of deprenyl could also include its inhibitory effect upon the dopamine uptake system (Knoll, 1993). The component inhibitors of the dopamine uptake system produce an induction of the TH enzyme (Vrana *et al.*, 1993). Another possibility is that deprenyl produces its protective effect by activating glial cells, with the consequent production of neurotrophic factors. To test this hypothesis we studied the neurofilaments 68 kDa and GFAP after deprenyl treatment. Our results are in agreement with those of Biagini *et al.* (1993) who reported that deprenyl increases GFAP immunoreactivity selectively in activated astrocytes in rat brain; they suggested that the drug is effective only on astrocytes which are activated by the insult. Biagini *et al.* (1993) showed that deprenyl treatment potentiates both the lesion-induced increase of GFAP and the basic fibroblast growth factor (bFGF). However, Li *et al.* (1993) reported the reduction of GFAP mRNA abundance induced by deprenyl in C6 glioma cells. The decrease of GFAP found by us in substantia nigra could be due to protection given by deprenyl against oxidative stress in this structure produced by aging, since such oxidative damage would induce the formation of GFAP.

These results demonstrate that deprenyl treatment (2 mg kg^{-1}) for two months in aged rats produces a strong protection against the oxidative stress that occurs during aging, along with an induction of the TH enzyme, the TH amount reaching similar levels to that in young animals (de la Cruz *et al.*, 1995). The overall results could explain some pharmacological effects of deprenyl. It potentiates and prolongs the efficacy of levodopa in the treatment of PD (Heinonen & Rinne, 1989). Deprenyl, when used alone, can prolong the time during which a patient is able to manage without levodopa in the early phase of PD (Tertrud & Langston, 1989). Deprenyl also potentiates the action of endogenous dopamine, thereby improving the function of nigrostriatal dopaminergic neurones and alleviating the disability of Parkinson patients. Principally, deprenyl may be responsible for delaying the onset of disability of PD by an increase of dopamine as a result of MAO inhibition, by an induction of TH that also increases the dopamine levels, and in addition by protection against oxidative stress, oxidative damage being one of the most widely accepted recent theories for the aetiology of PD.

Conclusions

Deprenyl treatment (2 mg kg^{-1}) for two months in aged rats produces a strong protection against the oxidative stress that occurs during aging, along with an induction of the TH enzyme in substantia nigra - the amounts of TH and carbonyl groups reaching similar levels to those of young animals (de la Cruz *et al.*, 1995). These effects could be produced by the inhibitory action of deprenyl on the MAO system. These results can explain some of the actions of deprenyl described in its treatment of PD and probably of other neurodegenerative diseases such as AD and in aging. Moreover, these results suggest that deprenyl treatment could be used in other neurodegenerative diseases produced by oxidative damage.

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