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Effects of cytisine on hydroxyl radicals in vitro and MPTP-induced dopamine depletion in vivo

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Abstract

The potential new iron-chelator cytisine and the radical scavenger *N-tert*-butyl-α-(2-sulfophenyl) nitrone (S-PBN) were incubated in a Fenton system and hydroxyl radical formation was measured with the salicylate trapping assay. Both cytisine and S-PBN reduced hydroxyl radical formation in a concentration-dependent manner. For in vivo studies, C57BL/6 mice were injected repeatedly with cytisine (0.5 mg/kg or 2.0 mg/kg s.c.) or saline seven days before and after a single 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection (30 mg/kg s.c.). Seven days after MPTP treatment alone dopamine levels were significantly reduced to 12% of the control values ($p < 0.001$), whereas MPTP + cytisine treatment (2 mg/kg) led to more than twofold higher dopamine levels ($p < 0.01$) compared with MPTP alone. We have shown for the first time that cytisine attenuates hydroxyl radical formation in vitro and reduces MPTP-induced dopamine depletion. Thus, cytisine may be useful for the treatment of Parkinson's Disease where the chelation of iron ions could prevent neuronal cell death.

Keywords: Cytisine; Iron-chelator; Hydroxyl radical; Parkinson's disease; S-PBN; MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)

1. Introduction

Parkinson's Disease is characterized by the progressive degeneration of nigrostriatal dopaminergic neurons (Ehringer and Hornykiewicz, 1960). In spite of many decades of research the aetiology of the disease is as yet unclear. Several factors such as dysfunction of the mitochondrial respiration, generation of hydrogen peroxide by enzymatic and non-enzymatic reactions and reduced radical defence mechanisms with resulting oxidative stress, are known to contribute to the neurodegeneration of dopaminergic neurons (Cohen et al., 1997).

Some of the factors are linked and act in an additive or supra-additive way and form a vicious circle leading to degeneration of dopaminergic neurons. One of the contributing factors with predominant impact may be iron. Relatively high levels occur in the substantia nigra, which lead to an enhanced vulnerability of dopaminergic neurons. It has been reported that infusion of iron salts into this brain area can induce a degeneration of dopaminergic neurons (Sengstock et al., 1993) and decrease dopamine metabolism (Wesemann et al., 1994). The mechanism is suggested to involve an increased concentration of hydrogen peroxide in combination with excess free or low-molecular weight iron. Taken together, these might initiate the Fenton reaction resulting in increased formation of hydroxyl free radicals. Hydroxyl free radicals, by themselves, lead to several cellular damaging processes (e.g., increased lipid peroxidation and destruction of phospholipid-containing membranes). This mechanism might be particularly relevant when additional environmental toxins are involved. In this context, it is of interest that in the substantia nigra of patients with Parkinson's Disease, increased levels of iron were found (Dexter et al., 1989; Riederer et al., 1989; Sofic et al., 1991).

Several animal models can, in part, mimic the neuropathology of Parkinson's Disease. One experimentally used neurotoxin is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is converted by monoamine oxidase B to its active metabolite 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is then taken up into the dopaminergic neurons by high affinity dopamine transporters and inhibits complex 1 of the respiratory chain, in particular in the substantia nigra. Inhibition of complex 1

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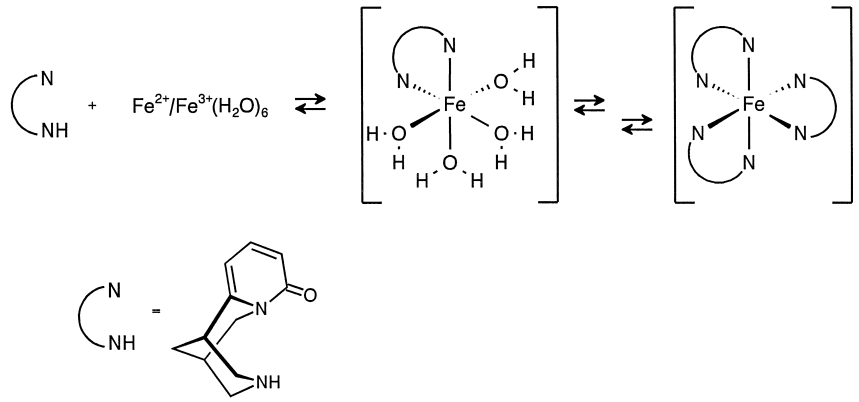

Fig. 1. Proposed reaction scheme between Fe^{2+}/Fe^{3+} and cytisine.

impairs mitochondrial electron transport and, probably through this, generating oxygen radicals (see Coyle and Puttfarken, 1993). In humans, MPTP can produce parkinsonian symptoms and a degeneration of nigrostriatal dopaminergic neurons (Langston et al., 1983).

Cytisine is a drug which has agonistic actions at nicotinic cholinoceptors (see Lefkowitz et al., 1996). In addition, its structure (Fig. 1) suggests that it might form complexes with metals such as iron and may thus interfere with the Fenton reaction leading to a decrease of hydroxyl free radical production. Therefore, the following aspects were investigated: (i) the possible reduction of hydroxyl free radical levels in a tissue-free in vitro Fenton system; and (ii) possible neuroprotective effects of the drug against MPTP-induced dopamine depletion in vivo.

2. Materials and methods

2.1. In vitro studies

2.1.1. Radical generating system

Hydroxyl free radicals were generated by incubation of a mixture of 10 μM $FeSO_4$ and 10 μM H_2O_2 in 2 ml of KH_2PO_4–KOH buffer adjusted to pH 7.4 for 15 min of 37°C. For determination of hydroxyl free radicals 20 μM salicylic acid was also present in the solution. For evaluation of the radical attenuating effects of cytisine or *N-tert*-butyl-α-(2-sulfophenyl) nitrone (S-PBN), they were co-incubated with salicylic acid in the Fenton system described above.

2.1.2. Determination of hydroxyl radical levels

Hydroxyl free radical levels were determined according to the salicylate trapping method (Floyd et al., 1984) with slight modifications. Immediately after the 15 min incubation period, a 90-μl aliquot of the reaction mixture was acidified with perchloric acid (10 μl, 0.4 M). These solutions were directly administered via an injector (Rheodyne model 9125, 20 μl sample loop) into the high-performance liquid chromatography system (HPLC). The two main products, namely 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid, were quantified as a result of the reaction of hydroxyl radicals with salicylic acid.

2.1.3. HPLC measurement

We developed a HPLC method to separate and quantify the two main products of the in vitro reaction of salicylic acid with hydroxyl radicals simultaneously with dopamine and its metabolites 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) (Ferger et al., 1998).

Aliquots of the samples were analysed by a HPLC system with a two channel electrochemical detector (BAS LS 4C, Bioanalytical Systems, West Lafayette, USA). The detector potential was set at +750 mV using a glassy carbon electrode versus an Ag/AgCl reference electrode. The mobile phase contained 0.14 g octane sulfonic acid sodium salt as an ion-pair reagent, 0.1 g disodium EDTA and 6 ml triethylamine in 1 l of Millipore Q® water. Concentrated phosphoric acid was used to adjust the pH to 2.8. Before 35 ml of acetonitrile was added, the solution was filtered through a 0.45 μm nylon filter. An on-line degasser (CMA 260, Carnegie Medicin, Sweden) was used to degas the mobile phase before an inert HPLC pump (CMA 250, Carnegie Medicin, Sweden) delivered the eluent at a rate of 0.6 ml/min into the column. A reversed-phase column (125 × 3 mm with pre-column 5 × 3 mm) filled with Nucleosil 120-3 C18 (Macherey-Nagel, Düren, Germany) was used for separation. Data were recorded by a two channel integrator (Model 1022x, Perkin-Elmer, USA). In order to calculate the concentrations of 2,3- and 2,5-dihydroxybenzoic acid, dopamine, DOPAC and HVA the area under the curve of the recorded peaks was determined and calibrated with external standards.

2.2. In vivo studies

The experimental protocols were approved by the appropriate institutional governmental agency (Regierungspräsident Gießen, Germany).

2.2.1. Animals

Adult male C57BL/6 mice weighing 23–25 g and about 3 to 4 months old (Charles River, Sulzfeld, Germany) were housed in groups of 5 under standardized conditions (temperature 23 ± 2°C, relative humidity 55 ± 5%, 12 h light-dark-cycle (lights on 7:00 h–7:00 h) with free access to a standard diet (Altromin®, Fa. Altromin, Lage, Germany) and tap water. The experimental protocol consisted of 6 groups each with 7–10 animals.

2.2.2. Experimental groups

Group Sal + Sal: Mice received injections of saline twice daily for a period of 14 days and an injection of saline (instead of MPTP) on day 8 ($n = 7$).

Group Cyt (0.5) + Sal: Mice received injections of cytisine (0.5 mg/kg) twice daily for a period of 14 days and an injection of saline (instead of MPTP) on day 8 ($n = 10$).

Group Cyt (2.0) + Sal: Mice received injections of cytisine (2.0 mg/kg) twice daily for a period of 14 days and an injection of saline (instead of MPTP) on day 8 ($n = 10$).

Group Sal + MPTP: Mice received injections of saline twice daily for a period of 14 days and an injection of MPTP (30 mg/kg) on day 8 ($n = 7$).

Group Cyt (0.5) + MPTP: Mice received injections of cytisine (0.5 mg/kg) twice daily for a period of 14 days and an injection of MPTP 30 mg/kg on day 8 ($n = 10$).

Group Cyt (2.0) + MPTP: Mice received injections of cytisine (2.0 mg/kg) twice daily for a period of 14 days and an injection of MPTP (30 mg/kg) on day 8 ($n = 10$).

On day 8, in addition to the single MPTP injection all mice received an enhanced protocol of cytisine administration: beginning 30 min prior to MPTP injection and then 30, 90, 210, 330 and 450 min after MPTP injection for a total of 6 injections of cytisine. In control groups saline was administered instead of MPTP and cytisine (see details given above).

2.2.3. Determination of locomotor activity

Locomotor activity of all the mice was measured one day after a single MPTP or saline injection. The results are expressed as % change from the control group (Sal + Sal) which was regarded as 100%.

Locomotor activity measurements were performed in 50 × 50 × 35 cm activity cages connected to an IBM computer which automatically counted the interruptions of the 8 horizontal photobeams placed 2 cm above the bottom at a sampling rate of approximately 50 Hz. Immediately after injection of cytisine or saline, the mice were placed into separate activity boxes (one per box) in a randomized manner. After 5 min exploration time the locomotor activity was monitored for 60 min.

2.2.4. Determination of monoamines and metabolites

Seven days after MPTP or saline administration mice were sacrificed by cervical dislocation. The brains were rapidly removed and immediately placed on an ice-cooled plate for dissection of the neostriatum. Immediately after dissection the neostriatum was placed in a 1.5 ml tube containing ice-cooled perchloric acid (500 μl, 0.4 M) homogenised for 1 min at 20 000 rpm (Ultra Turrax model T5, Bachofer Reutlingen, Germany) and centrifuged for 15 min at 5000 rpm and 4°C (Hermle model Z 252 MK, Gosheim, Germany). Aliquots of the supernatant were passed through a 0.2 μm filter, then directly analysed with HPLC in combination with electrochemical detection or frozen at −70°C until analysis. The concentrations are given as ng/mg wet tissue weight.

All data were analysed using analysis of variance (ANOVA) with a subsequent Duncan's multiple comparison test. $P < 0.05$ was considered as statistically significant.

2.3. Drugs and chemicals

MPTP-HCl was purchased from Research Biochemicals International (RBI) Cologne, Germany. *N-tert*-butyl-α-(2-sulfophenyl) nitrone, dopamine HCl, DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), salicylic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and 1-octane sulfonic acid sodium salt were obtained from Sigma Chemie, Munich, Germany, HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Cytisine was isolated from the fruits of *Laburnum anagyroides* and *L. watereri* and purified by column chromatography. All of the standards were of the highest purity commercially available. The standards for HPLC were dissolved in 0.1 M perchloric acid. Cytisine and MPTP were dissolved in saline. All doses or concentrations are expressed in terms of the free base. Cytisine, MPTP and saline were administered subcutaneously into the dorsal neck in a volume of 10 ml/kg body weight.

3. Results

3.1. Effects of cytisine and S-PBN on the hydroxyl free radicals produced by the Fenton reaction in vitro

Incubation of salicylic acid alone in the radical generating system resulted in the appearance of the two main products 2,3-and 2,5-dihydroxybenzoic acid (1.4 μM and 1.7 μM, respectively) reflecting an increased hydroxyl radical formation. In control experiments in presence of salicylic acid but without iron ions or H_2O_2, neither of the two products were detectable under our conditions.

Increasing concentrations of cytisine or the S-PBN (10 μM-200 μM) decreased the levels of 2,3- and 2,5-dihydroxybenzoic acid in a concentration-dependent manner (Fig. 2). At a concentration of 10 μM cytisine significantly reduced the levels of 2,5-dihydroxybenzoic acid, at 50 μM also of 2,3-dihydroxybenzoic acid. The highest concentration of cytisine (200 μM) reduced the increase of 2,3- and

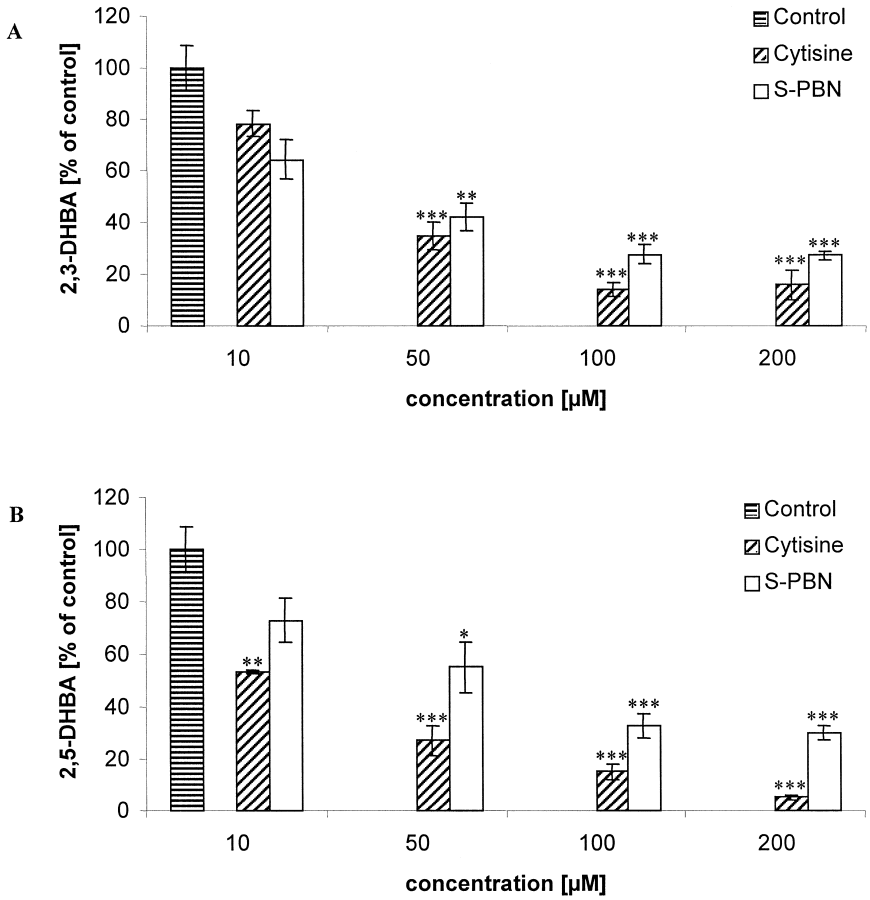

Fig. 2. Effects of cytisine, S-PBN and saline controls on hydroxyl free radical levels in an in vitro Fenton system. 2,3-dihydroxybenzoic acid values (2,3-DHBA) (A) and 2,5-dihydroxybenzoic acid (2,5-DHBA) values (B) indicate the alterations on hydroxyl free radical levels. Data are mean ± S.E.M. (bars) ($n = 4$ samples). $*\ P < 0.05$, $**\ P < 0.01$, $***\ P < 0.001$ by ANOVA with subsequent Duncan's test compared with saline controls. For further information see Section 2.

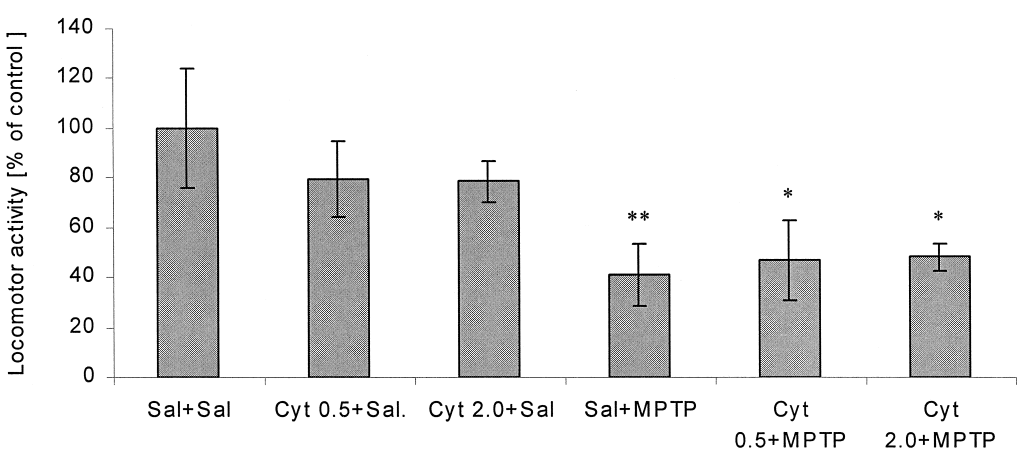

Fig. 3. Effects of saline + saline (Sal + Sal), cytisine (0.5 mg/kg) + saline (Cyt 0.5 + Sal), cytisine (2.0 mg/kg) + saline (Cyt 2.0 + Sal), saline + MPTP (Sal + MPTP), cytisine (0.5 mg/kg) + MPTP (Cyt 0.5 + MPTP), cytisine (2.0 mg/kg) + MPTP (Cyt 2.0 + MPTP) on locomotor activity in a 60 min test session. The injections were performed s.c. The locomotor activity of the Sal + Sal group was regarded as 100%. Data are mean ± S.E.M. (bars) ($n = 7$–10 mice). $*\ P < 0.05$, $**\ P < 0.01$ by ANOVA with subsequent Duncan's test compared with saline controls.

2,5-dihydroxybenzoic acid about 20% or less than 10% of the control values, respectively. Overall, the potent radical scavenger S-PBN was in equivalent concentrations slightly less effective (at concentrations of 50–200 μM) than cytisine and reduced the increase of 2,3- and 2,5-dihydroxybenzoic acid about 30% of the control values, in concentrations of 100 or 200 μM.

This demonstrates the probable reduction of hydroxyl free radical levels by cytisine.

3.2. Effects of cytisine on locomotor activity

The locomotor activity was measured one day after MPTP or saline treatment. Fig. 3 summarizes the results of

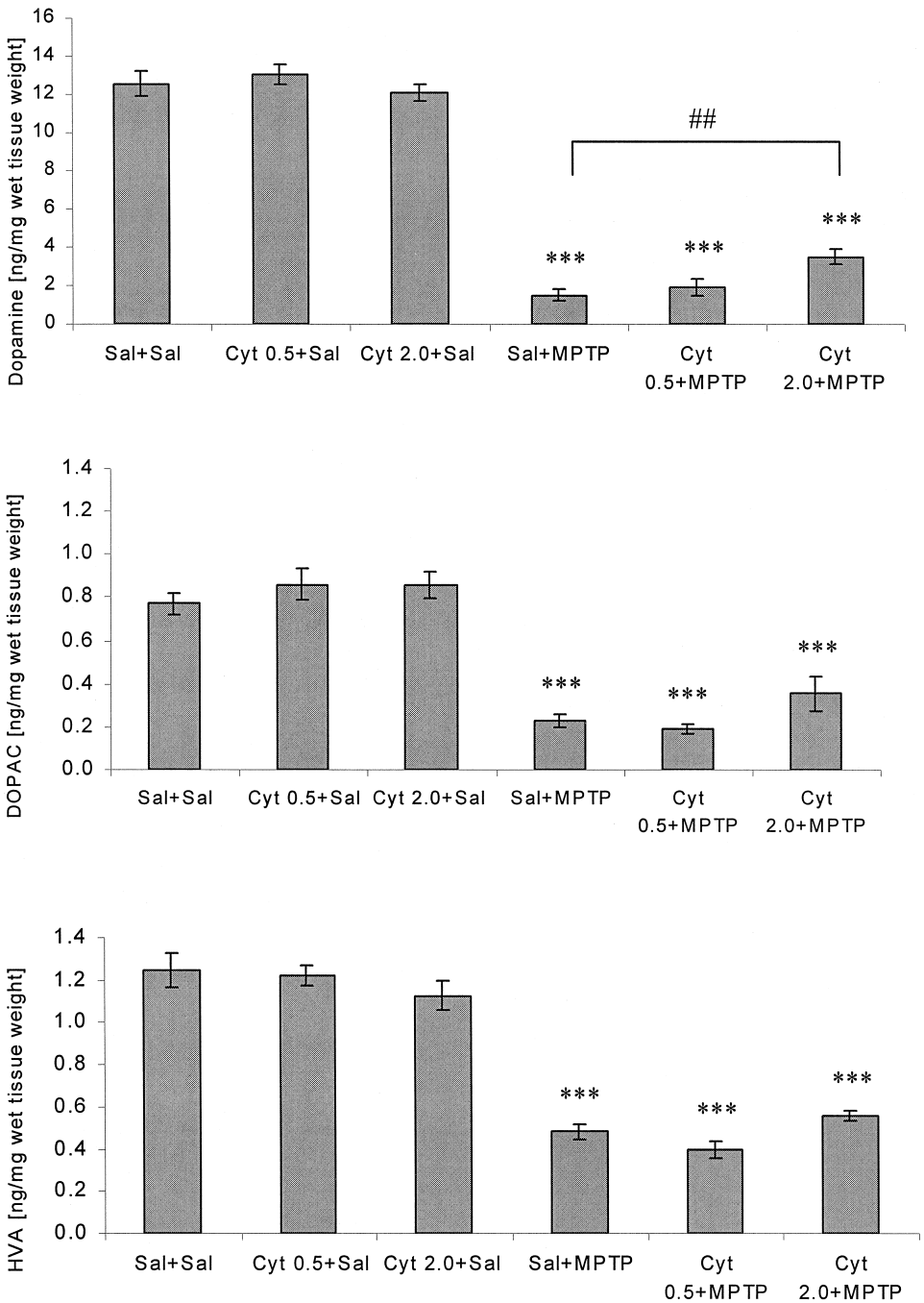

Fig. 4. Effects of saline + saline (Sal + Sal), cytisine (0.5 mg/kg) + saline (Cyt 0.5 + Sal), cytisine (2.0 mg/kg) + saline (Cyt 2.0 + Sal), saline + MPTP (Sal + MPTP), cytisine (0.5 mg/kg) + MPTP (Cyt 0.5 + MPTP), cytisine (2.0 mg/kg) + MPTP (Cyt 2.0 + MPTP) on striatal dopamine (upper part), DOPAC (middle part) and HVA (lower part). The injections were performed s.c. Data are mean ± S.E.M. (bars) ($n = 7–10$ mice). $***P < 0.001$ by ANOVA with subsequent Duncan's test MPTP treated groups compared with their corresponding non-MPTP treated controls and $^{\#\#}P < 0.01$ Cyt + MPTP treated groups compared within the Sal + MPTP treated animals.

the locomotor activity test. MPTP treatment led to a significant depression of locomotor activity to 41, 46.7 and 47.8% of the saline control in the saline, cytisine (0.5 mg/kg) and cytisine (2.0 mg/kg) MPTP treated groups, respectively. The MPTP-treated cytisine groups did not significantly differ from the saline group. In the non-MPTP treated cytisine groups the locomotor activity was also not significantly altered compared with the saline group.

3.3. Effects of cytisine on the MPTP-induced degeneration of dopaminergic neurons

Cytisine treatment alone (Cyt + Sal; 0.5 or 2.0 mg/kg) did not significantly alter the striatal concentrations of dopamine (Fig. 4, upper part) or its metabolites DOPAC (Fig. 4, middle part) or HVA (Fig. 4, lower part). MPTP treatment alone (Sal + MPTP) produced a very strong decrease in dopamine concentration to 12% of the saline treated control (Sal + Sal). The MPTP-induced depletion of dopamine was significantly less pronounced in the Cyt (2.0) + MPTP group (28% of Sal + Sal values) in comparison with Sal + MPTP group levels, whereas the lower dose (0.5 mg/kg) of cytisine treatment did not significantly affect the depletion of dopamine (15.5% of Sal + Sal values) in comparison with Sal + MPTP group levels. The concentrations of DOPAC and HVA were also decreased by MPTP administration alone, although the falls in the

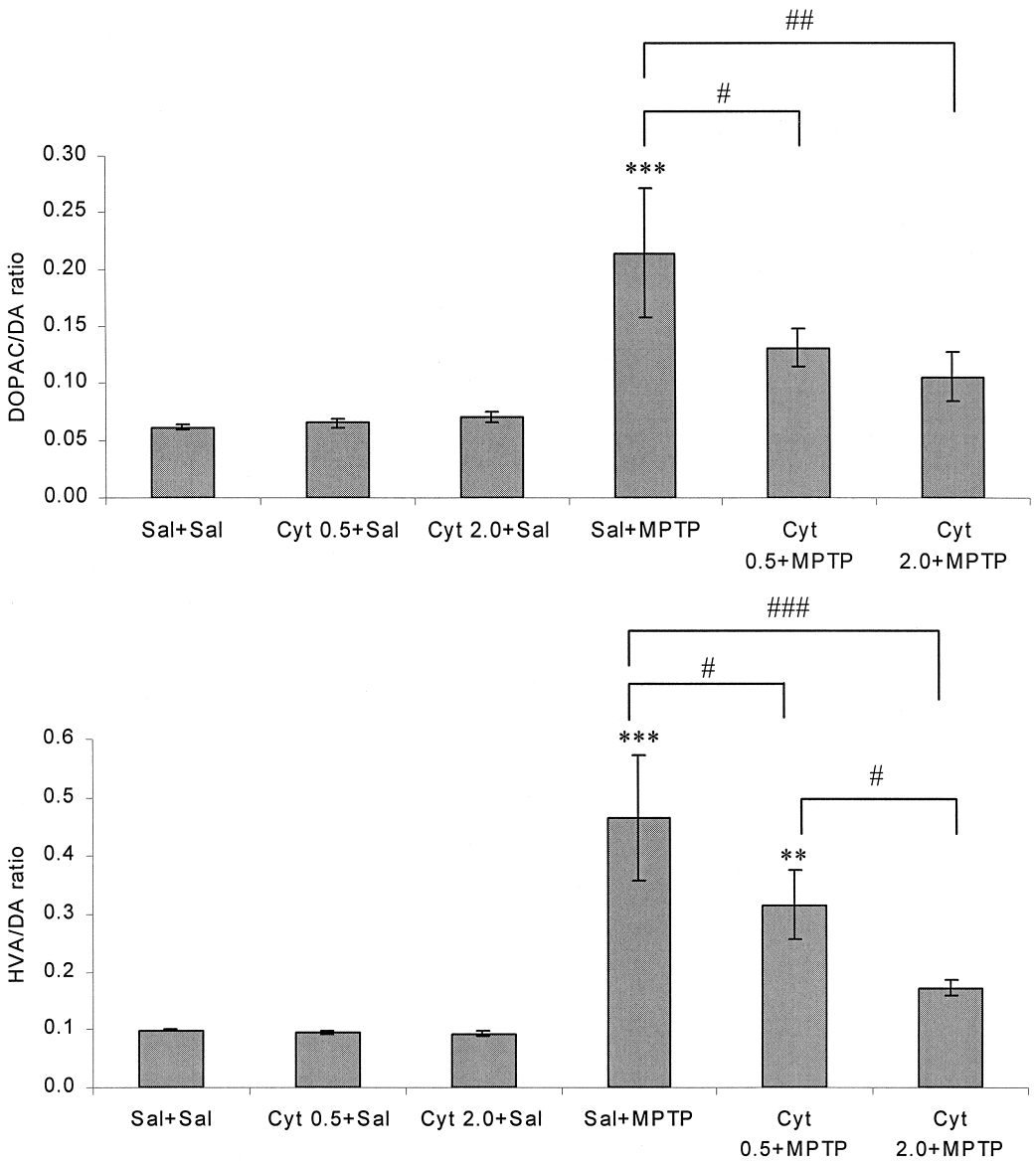

Fig. 5. Effects of saline + saline (Sal + Sal), cytisine (0.5 mg/kg) + saline (Cyt 0.5 + Sal), cytisine (2.0 mg/kg) + saline (Cyt 2.0 + Sal), saline + MPTP (Sal + MPTP), cytisine (0.5 mg/kg) + MPTP (Cyt 0.5 + MPTP), cytisine (2.0 mg/kg) + MPTP (Cyt 2.0 + MPTP) on striatal dopamine turnover indicated as DOPAC/dopamine ratio (upper part) and HVA/dopamine ratio (lower part). The injections were performed s.c. Data are mean ± S.E.M. (bars) ($n = 7–10$ mice). $**P < 0.01$, $***P < 0.001$ by ANOVA with subsequent Duncan's test MPTP treated groups compared with their corresponding non-MPTP treated controls and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ Cyt + MPTP treated groups compared within the Sal + MPTP treated animals.

concentrations of these metabolites were somewhat less pronounced than those of dopamine (29.8 and 38.6% of Sal + Sal values, respectively).

Cytisine failed to significantly alter the MPTP-induced depletion of dopamine metabolites at either concentration tested here.

The ratios DOPAC/dopamine and HVA/dopamine are regarded as indicators of dopamine turnover.

As shown in Fig. 5 (upper and lower part) cytisine treatment alone did not affect the ratios. MPTP treatment alone significantly increased the DOPAC/dopamine and HVA/dopamine ratios and these increases were significantly reduced by both dosages of cytisine.

4. Discussion

The present study is, to our knowledge, the first to demonstrate a reduction of hydroxyl radical production by cytisine in vitro and is also the first in vivo study which addresses the ability of cytisine to protect against MPTP-induced toxicity.

In vivo cytisine partially prevented the MPTP-induced decrease in striatal dopamine concentration and the increase in dopamine turnover. The increase in dopamine turnover is a compensatory mechanism which acts to normalize the impaired dopaminergic neurotransmission. Thus a reduction of the compensatory increase in dopamine turnover seems to be an important indicator that dopaminergic neurons are being, in part, rescued from neurodegeneration. The in vitro findings observed in the Fenton system seem to support the neuroprotective effects of cytisine in the in vivo experiments. The decrease of hydroxyl free radical levels by cytisine found in vitro might be explained either by a direct inhibition of the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$$

or by mechanisms removing previously formed hydroxyl radicals.

Preliminary in vitro experiments measuring UV absorbance spectra of cytisine and of cytisine–iron complexes (Seitz, unpublished results) suggest that cytisine may form direct complexes with iron, so that cytisine might by this mechanism interfere with the Fenton reaction. A proposed reaction scheme for this complex formation is shown in Fig. 1. However, alternative mechanisms cannot be excluded.

It is suggested that iron is involved in the pathogenesis of neurodegenerative disorders such as Parkinson's Disease. This assumption is supported by the finding of Ben-Shachar and Youdim (1991) that intranigral iron injection induces behavioural and biochemical 'parkinsonism' in rats. Furthermore, its accumulation in the substantia nigra and concomitant induction of oxidative stress in melanised dopaminergic neurons might be a factor contributing to Parkinson's Disease (Youdim et al., 1993). In patients with Parkinson's Disease, intranigral increases in iron, mainly in iron (III), were found in the zona compacta of the substantia nigra (Riederer et al., 1989; Sofic et al., 1991). Experimental models of neurodegeneration also show alterations in substantia nigra iron concentrations. The increased iron concentration in the substantia nigra of animals treated with 6-hydroxydopamine (Monteiro and Winterbourn, 1989; Oestreicher et al., 1994) or MPTP (Temlett et al., 1994; Mochizuki et al., 1994) seems to be related to a disturbed iron homeostasis which might correspond to the situation in patients with Parkinson's Disease. The significance of a disturbed iron distribution implies a mechanism behind the protective effects of iron chelators in Parkinsonian animal models. The iron chelator desferrioxamine was found to be protective against the neurotoxic effects of 6-hydroxydopamine in rats (Ben-Shachar et al., 1991). Furthermore, Santiago et al. (1997) recently reported that local perfusion with desferrioxamine protects against MPP^+ induced neurotoxicity in a microdialysis study. Synergistic interactions between MPTP (or MPP^+) on the one hand and iron on the other, seem to be of particular importance. Iron can, at least in mouse astrocytes, stimulate conversion of MPTP to MPP^+ (Di Monte et al., 1995). Equally, Mochizuki et al. (1994) found an excessive iron accumulation in the substantia nigra of monkeys after pretreatment with MPTP.

There is a body of evidence that the dopaminergic neurotoxicity is in part mediated by an increased iron release from ferritin via mechanisms such as a decrease in pH, an increase of free radicals or by an elevation of brain iron levels resulting from a damaged blood-brain barrier (Gerlach et al., 1994; Olanow and Youdim, 1996). Youdim et al. (1993) reported that the biochemical changes obtained in Parkinsonian substantia nigra are similar to those obtained in tissue with iron overload (siderosis).

In patients with Parkinson's Disease, the iron uptake mechanisms are obviously altered (Logroscino et al., 1997), and this is in part due to an overexpression of lactoferrin receptors in the substantia nigra (Faucheux et al., 1995). Furthermore, a reduction of ferritin levels in patients with Parkinson's Disease leads to diminished intracellular storage and an elevated concentration of free iron (Dexter et al., 1990). Taken together, excessive iron accumulation in the brain is postulated to be a potential risk for neurodegeneration in Parkinson's Disease (Lan and Jiang, 1997).

In their review Gassen and Youdim (1997) reiterated the cerebro- and oculotoxicity effects of the iron chelator desferrioxamine and postulated the potential role of new iron chelators which overcome the disadvantages of desferrioxamine. They emphasised that the development of new bioactive iron chelating agents to counteract free radical formation could be an important new approach for developing neuroprotective treatments for Parkinson's Disease and related neurological disorders.

In contrast to desferrioxamine, cytisine is able to cross the blood brain barrier and it was found that a subcutaneous cytisine application rapidly leads to substantial brain concentrations (Reavill et al., 1990).

In the present experiments, cytisine neither altered the locomotor activity nor showed other behavioural signs of acute toxicity in the dosages used. This seems to be somewhat surprising, since it is well known that cytisine can induce intoxication in children, who ingested cytisine-rich fruits of *L. anagyroides*. Furthermore, cytisine alone did not alter dopamine turnover as a neurochemical parameter.

5. Conclusion

Cytisine or related drugs are promising substances to studying neuroprotective effects on nigrostriatal dopaminergic neurons. Centrally acting iron chelators could be of therapeutic value in Parkinson's Disease if adverse side effects can be excluded.

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