Research report

Increased striatal dopamine turnover following acute administration of rotenone to mice

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Abstract

Because of the potential role of mitochondrial dysfunction in nigrostriatal degeneration in Parkinson's disease, the effects of rotenone (an inhibitor of mitochondrial NADH dehydrogenase and a naturally occurring toxicant) on the levels of striatal dopamine (DA) and DA metabolites were evaluated after acute and subchronic administration to mice. Systemic acute treatment with relatively high doses of rotenone did not affect DA concentration, but caused a significant increase in both DA metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). DOPAC and HVA changes were measured at 1 day and were reversed within 1 week, paralleling the time course of rotenone-induced increase in striatal lactate levels. Subchronic administration with a relatively mild dose of rotenone did not significantly alter the striatal levels of DA and DOPAC, while it slightly reduced HVA concentration. No neurochemical signs of dopaminergic damage were seen when mice were co-exposed to rotenone and diethyldithiocarbamate, a compound known to enhance nigrostriatal injury caused by the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Also, rotenone did not cause additional injury to animals previously lesioned by MPTP. Taken together, data indicate that rotenone is not capable of causing overt dopaminergic toxicity under the testing paradigms used in this study. Rather, an increase in DA turnover, as indicated by a higher (DOPAC/HVA)/DA ratio, seems to be associated to rotenone-induced striatal energy impairment.

1. Introduction

The neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) reproduce neurochemical and pathological features of human parkinsonism such as the reduction of striatal dopamine (DA) and the loss of dopaminergic cell bodies in the substantia nigra (SN) [3,14,17,29]. Thus, studies on MPTP toxicity are likely to provide valuable insight into mechanisms of nigrostriatal degeneration in Parkinson's disease (PD). Several lines of experimental evidence suggest that MPTP-induced cell death, mediated by its toxic 1-methyl-4-phenylpyridinium metabolite (MPP⁺), involves the inhibition of complex I activity of the mitochondrial respiratory chain [23–25], with consequent ATP depletion and a loss of mitochondrial transmembrane potential [3,5,12,14,16,28]. Similar toxic events may play a role in neurodegeneration in PD since a decrease in complex I activity has been measured post mortem in both the SN and the striatum of PD patients as compared to control subjects [22,27].

Findings with the MPTP model and in PD patients raise the possibility that the nigrostriatal system may be particularly vulnerable to an impairment of mitochondrial energy metabolism. Rotenone is a 'classic' inhibitor of mitochondrial complex I and could therefore be used to further evaluate the relationship between energy deficiency and dopaminergic injury. Furthermore, since rotenoid compounds are widely used as pesticides, studies with rotenone may provide clues on environmental exposures

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that could increase the risk for PD. Particularly intriguing is the possibility that chemical interactions may play a role in nigrostriatal degeneration. The fungicide diethylthiocarbamate (DDC) has been shown to enhance dramatically MPTP-induced neurotoxicity [6,13,31]. However, whether DDC can also act synergistically with other compounds, including rotenoid derivatives, in damaging dopaminergic neurons is unknown. A limited number of studies has evaluated the effects of rotenone on dopaminergic neurons both in vitro and in vivo. Treatment of mesencephalic cultures and striatal synaptosomes with rotenone has been shown to cause neurotoxicity as measured by a decreased uptake of neurotransmitters [20,21]. Interestingly, DA uptake was significantly more affected than the uptake of serotonin, GABA or noradrenaline, thus supporting the hypothesis of a selective vulnerability of dopaminergic cells. In the in vivo setting, Heikkila and colleagues have reported a substantial depletion of striatal DA and its metabolites after the stereotaxic injection of rotenone into the median forebrain bundle of rats [11]. More recently, histological, but not biochemical, evidence of selective tissue damage has been shown in the striatum and globus pallidus of rats treated for 7–9 days with high doses of rotenone as a continuous intravenous infusion [8]. However, the possibility that nigrostriatal injury may be induced by rotenone under less severe experimental conditions and different paradigms of administration remains to be further evaluated. In the present study, changes in DA levels and metabolism were determined in the mouse striatum following a single subcutaneous injection of various doses of rotenone (acute treatment). The effects of the toxicant were also evaluated after multiple systemic administrations of a relatively mild dose (subchronic treatment). Finally, mice were exposed to both rotenone and DDC to test the hypothesis that, similar to its effect with MPTP, DDC may promote or enhance dopaminergic damage by rotenone.

2. Materials and methods

2.1. Chemicals

Rotenone and sodium diethyldithiocarbamate were purchased from Sigma (St. Louis, MO). MPTP (hydrochloride salt) was obtained from Research Biochemicals International BI (Natick, MA). All other reagents were of the highest purity available commercially.

2.2. Animals and treatments

Seven- to 8-week-old male C57BL/6 mice (20–25 g. from Simonsen Laboratories, Gilroy, CA) were maintained under a 13:11-h light/dark cycle in a temperature-controlled room with access to food and water ad libitum. All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Mice received a single subcutaneous injection of rotenone (5, 10 and 15 mg/kg) or vehicle (peanut oil) and were killed by cervical dislocation at 1, 7 or 14 days. With the highest dose of rotenone chosen for these experiments, i.e. 15 mg/kg, mice survived 24 h, but significant lethality was observed at subsequent time points. Therefore, this dose was used only to determine the most severe acute effects of rotenone at 1 day. For the subchronic regimen, mice were injected with 1/10 of the highest acute dose of rotenone (1.5 mg/kg, s.c.) three times a week (Monday, Wednesday and Friday) for 3 consecutive weeks and were killed on the third day after the last dose. In studies in which chemical interactions were evaluated, DDC (400 mg/kg, i.p.) was administered 2 h before rotenone (10 mg/kg, s.c.) or vehicle and mice were killed at 7 days. In a final set of experiments, mice received MPTP (15 mg/kg, s.c.) 7 days prior to the administration of rotenone (10 mg/kg, s.c.) and were killed after an additional 7 days. At the end of each experiment, brains were rapidly removed and cut into 1-mm-thick coronal sections on ice. The striatum was carefully excised and placed into 500 μl of ice-cold 0.4 N perchloric acid. Tissues were disrupted by sonication (10–15 s at 13 μm amplitude in an MSE Soniprep 150 sonicator) and centrifuged at 16 000×g for 12 min at 4°C. Supernatants were removed, stored at −80°C and used for DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) determinations. Pellets were resuspended in 500 μl phosphate buffer (pH 7.4) for protein analysis.

2.3. Assays

DA, DOPAC and HVA were quantified by reverse-phase HPLC coupled to electrochemical detection (EC) according to the method of Kilpatrick et al. [15]. Briefly, samples and external standards were eluted at a flow rate of 1.1 ml/min (pump model LC-600 from Shimadzu, Kyoto, Japan) using a C18 column (Brownlee, 250×4.6 mm). The mobile phase consisted of 90 mM sodium acetate/35 mM citric acid (pH 4.7), 130 μM EDTA, 230 μM 1-octanesulfonic acid and 14% methanol. The coulometric detector (ESA model 5200A, Chelmsford, MA) consisted of an analytical cell (model 5011) and a guard cell (model 5020). The guard cell and the first and second electrodes of the analytical cell were set at a potential of +435, 0 and +430 mV, respectively. The chromatograms were recorded and integrated (Shimadzu model CR 601) and values are reported either as percent of their respective controls or as ng/mg of protein.

For lactate measurements, striata were homogenized in 20 μl of a solution containing 20 mM potassium phosphate buffer (pH 6.0), 0.1 M Tris–HCl buffer (pH 8.0) and 0.5 mM dithiothreitol to achieve a final pH of 7.4 [19]. After
addition of ice-cold 8% perchloric acid (1:2 v/v), the homogenate was vortexed and centrifuged at 1500×g for 10 min at 4°C. Lactate was determined in a 10-μL aliquot of the supernatant using a colorimetric kit purchased from Sigma (St. Louis, MO).

Protein concentrations were determined by the method of Lowry et al. [18] using bovine serum albumin as a standard.

2.4. Statistics

Data were analyzed by a one-way analysis of variance (ANOVA) and, if a P value of less than 0.05 was obtained, pair-wise comparisons were performed using the Student–Newman–Keuls test.

3. Results

When mice were killed 24 h after a single subcutaneous injection of either 5, 10 or 15 mg/kg rotenone, striatal dopamine levels were the same in controls as in rotenone-treated animals. However, rotenone caused a dose-dependent increase in striatal DOPAC and HVA concentrations (Fig. 1a). At 7 days, DA remained unchanged while the increase in DA metabolites seen at 24 h was reversed. In fact, DOPAC and HVA levels were slightly reduced in mice treated with 5 or 10 mg/kg rotenone as compared to control animals (Fig. 1b). At 14 days, no significant changes in striatal DA and DA metabolites were measured in mice injected with 10 mg/kg rotenone (Table 1).

A metabolic consequence of mitochondrial electron flow blockage would be the incomplete oxidation of glucose and consequent lactate accumulation. Thus, in order to correlate systemic rotenone exposure under our experimental conditions with tissue alterations in energy metabolism, striatal lactate levels were measured at 1 and 7 days after a single subcutaneous injection of 10 mg/kg rotenone. A twofold increase in lactate was found at 1 day in rotenone-as compared to vehicle-treated mice. This effect was completely reversed at the 7 day time point (Table 2).

Rotenone was also administered to mice using a subchronic regimen consisting of three weekly injections of 1.5 mg/kg for 3 consecutive weeks. A slight decrease in striatal DA and DA metabolites seemed to be caused by rotenone exposure. However, only the decrease in HVA reached statistical significance (Fig. 2).

The possibility that chemical interactions may affect rotenone neurotoxicity was investigated next. First, rotenone–DDC interactions were studied using the same paradigm of DDC administration that has been shown to enhance nigrostriatal damage by MPTP [6,13,31]. DDC was injected intraperitoneally at the dose of 400 mg/kg 2 h prior to rotenone administration (10 mg/kg, s.c.). As shown in Table 3, levels of striatal DA and DA metabolites were not different in co-exposed mice as compared to controls.

% Control

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<tbody>
<tr>
<td>DA</td>
<td>102±6</td>
</tr>
<tr>
<td>DOPAC</td>
<td>98±4</td>
</tr>
<tr>
<td>HVA</td>
<td>94±6</td>
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</table>

* Catecholamines analysis and animal treatments are described in Section 2. Mean±S.E. values of four independent determinations are shown.
caused an increase in striatal levels of DOPAC and HVA that, paralleling the time course of lactate measurements, was observed at 1 day and reversed by 1 week. Since the ratio (DOPAC + HVA)/DA is considered a marker of DA turnover (with a higher ratio indicating higher turnover), findings of this study support the conclusion that the primary consequence of acute systemic rotenone exposure is an enhancement of striatal metabolism and degradation.

Interestingly, findings obtained with rotenone are similar to the results reported in previous studies, in which other inhibitors of the mitochondrial respiratory chain were administered to rodents. For example, both sodium azide, a mitochondrial complex IV inhibitor, and 3-nitropropionic acid, a complex II blocker, did not significantly affect striatal DA, while they increased DOPAC and HVA levels when administered systemically to rats [2,4]. The extent of changes in DA metabolites with sodium azide and 3-nitropropionic acid was similar to that measured after rotenone exposure in the present investigation. Thus, it appears that a common feature of intoxication with mitochondrial poisons is an enhancement of striatal DA turnover. The mechanisms involved in this effect are currently not well established. However, under conditions of chemical hypoxia, neuronal depolarization and inhibition of energy-dependent re-uptake may lead to DA release and ultimately to an increase in neurotransmitter turnover [1,7,9,26].

The lack of evidence for dopaminergic terminal injury after acute rotenone treatment is apparently at odds with a previous report in which striatal DA was depleted following stereotaxic injection of rotenone into the rat median forebrain bundle [11]. Quite likely, however, differences in neurotoxicity may reflect differences in the degree of energy impairment caused by rotenone under various experimental conditions. In particular, direct injection of the toxicant into the brain may be expected to cause greater energy failure and more pronounced local toxic effects. Other experimental variables may underlie differences in rotenone-induced dopaminergic injury, including the duration of toxicant exposure. In the present study, mice were also subjected to a mild subchronic regimen that, similar to the acute rotenone treatment, did not produce overt neurotoxicity. In contrast, Greenamyre and colleagues have recently reported preliminary results

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Striatal lactate content (μmol l-(-)-lactate/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>0.05±0.002</td>
</tr>
<tr>
<td>Rotenone (n = 5)</td>
<td>0.10±0.02*</td>
</tr>
<tr>
<td>24 h</td>
<td>0.06±0.005</td>
</tr>
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</table>

*Lactate analysis and animal treatments are described in Section 2. Mean±S.E. values are shown. *P<0.001 as compared to control.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DA (ng/mg protein)</th>
<th>DOPAC (ng/mg protein)</th>
<th>HVA (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 15)</td>
<td>143.8±2.8</td>
<td>12.1±0.3</td>
<td>14.0±0.3</td>
</tr>
<tr>
<td>Rotenone 10 mg/kg, s.c. (n = 15)</td>
<td>126.0±4.5</td>
<td>10.5±0.9*</td>
<td>12.3±0.6*</td>
</tr>
<tr>
<td>DDC 400 mg/kg, i.p. (n = 15)</td>
<td>147.2±2.4</td>
<td>12.7±0.3</td>
<td>13.2±0.3</td>
</tr>
<tr>
<td>DDC 400 mg/kg, i.p., rotenone 10 mg/kg, s.c. (n = 5)</td>
<td>144.1±3.8</td>
<td>11.9±0.6</td>
<td>13.2±0.4</td>
</tr>
<tr>
<td>MPTP, 15 mg/kg, s.c. (n = 9)</td>
<td>75.8±5.1**</td>
<td>7.3±0.5**</td>
<td>9.7±0.3**</td>
</tr>
<tr>
<td>MPTP 15 mg/kg, s.c., rotenone 10 mg/kg, s.c. (n = 9)</td>
<td>67.1±8.0**</td>
<td>6.3±0.6**</td>
<td>8.8±0.4**</td>
</tr>
</tbody>
</table>

*Catecholamine analysis and animal treatments are described in Section 2. Mean±S.E. values are shown. *P<0.05 compared to control; **P<0.001 compared to control.

4. Discussion

In the present study, the effects of energy metabolism impairment on striatal dopaminergic terminals were assessed after exposure of mice to rotenone, a known inhibitor of mitochondrial complex I. Administration of relatively high doses of rotenone using an acute systemic model caused metabolic changes consistent with a shift from aerobic to anaerobic glucose utilization, as indicated by increased striatal lactate levels. These changes were present immediately after rotenone exposure at 1 day, but were reversed within the following week. Rotenone-induced energy impairment did not seem to be accompanied by overt damage to the dopaminergic terminals since DA concentration was not significantly decreased at any of the time points examined (1 day to 3 weeks). Rather, rotenone

![Graph showing striatal DA metabolism at 3 weeks following the subchronic administration of rotenone (1.5 mg/kg s.c., total number of doses=9)](image)

The mechanisms involved in this effect are currently not well established. However, under conditions of chemical hypoxia, neuronal depolarization and inhibition of energy-dependent re-uptake may lead to DA release and ultimately to an increase in neurotransmitter turnover [1,7,9,26].

The lack of evidence for dopaminergic terminal injury after acute rotenone treatment is apparently at odds with a previous report in which striatal DA was depleted following stereotaxic injection of rotenone into the rat median forebrain bundle [11]. Quite likely, however, differences in neurotoxicity may reflect differences in the degree of energy impairment caused by rotenone under various experimental conditions. In particular, direct injection of the toxicant into the brain may be expected to cause greater energy failure and more pronounced local toxic effects. Other experimental variables may underlie differences in rotenone-induced dopaminergic injury, including the duration of toxicant exposure. In the present study, mice were also subjected to a mild subchronic regimen that, similar to the acute rotenone treatment, did not produce overt neurotoxicity. In contrast, Greenamyre and colleagues have recently reported preliminary results
showing that continuous intravenous infusion of rotenone caused highly selective dopaminergic lesions that began in the striatum and progressed retrogradely to the SN [10]. Ferrante et al. have also found histological evidence of selective damage in the striatum and globus pallidus of rats when rotenone was administered via intravenous infusion [8]. In these studies in which neurotoxicity was observed, rotenone was administered at larger daily doses [8] and for a longer period of time [10] than in the present work, suggesting that frank dopaminergic injury may only be a consequence of robust and prolonged rotenone treatment.

Chemical interactions represent a relatively unexplored mechanism by which exposure to toxic agents may contribute to nigrostriatal damage. A dramatic example of synergistic interaction leading to enhanced dopaminergic degeneration is that between MPTP and the fungicide DDC [6,13,31]. Since rotenone and the toxic metabolite of MPTP, MPP+, share the property of inhibiting mitochondrial complex I, the possibility that interactions between rotenone and DDC may induce neurotoxicity was assessed as part of this investigation. Data reveal that striatal DA levels are unaffected by co-administration of mice with rotenone and DDC, suggesting that DDC does not interfere with energy metabolism and that its ability to enhance MPTP toxicity involves mechanisms other than MPP+-induced complex I inhibition. Another hypothesis tested in this study is that a pre-existing lesion of the nigrostriatal system may make it more vulnerable to chemical hypoxia caused by rotenone. However, even mice pre-treated with MPTP, in which approximately 50% of striatal DA was depleted, did not show signs of increased dopaminergic damage after subsequent exposure to rotenone.

In summary, our data indicate that the effects of rotenone on dopaminergic striatal terminals are dependent upon the route and schedule of its administration. After systemic exposure, rotenone acutely increases DA turnover, reproducing a feature that seems to be common to other mitochondrial poisons. This effect, on the other hand, does not necessarily lead to dopaminergic injury that may occur only after prolonged exposure to relatively high doses of the toxicant. Even though co-administration of rotenone with DDC or MPTP did not cause overt damage under the conditions used in this study, these data to not rule out the possibility that synergistic interactions may contribute to rotenone neurotoxicity under different experimental paradigms and with other potential dopaminergic toxicants [30].

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