Parkinsonian Rotenone Mouse Model: Reevaluation of Long-Term Administration of Rotenone in C57BL/6 Mice

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Chronic systemic exposure of Lewis rats to rotenone produced many features of Parkinson’s disease (PD), including nigrostriatal dopamine (DA) neurodegeneration and the formation of cytoplasmic inclusions in nigral DA neurons. We also reported that chronic oral administration of rotenone at 30 mg/kg for 28 d caused specific nigrostriatal DA neurodegeneration in C57BL/6 mice. To establish a PD model more suitable for evaluating nigrostriatal DA neurodegeneration, the present study has been designed to assess the neurotoxicity of rotenone after daily oral administration at 30 or 100 mg/kg for 56 d in C57BL/6 mice. The survival rate of rotenone-treated mice at 30 mg/kg did not change from 28 to 56 d, although the survival rate of rotenone-treated mice at 30 mg/kg was decreased to about 70% within one week. The survival rate of the rotenone-treated mice at 100 mg/kg was suddenly decreased after 28 d, and finally to about 15% at 56 d. Rotenone at 30 mg/kg, but not 100 mg/kg, for 28 d caused a significant loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra. Rotenone at 100 mg/kg caused a highly variable loss of TH-positive neurons among individual mice. Rotenone at 30 mg/kg for 56 d caused a significant loss of TH-positive neurons and behavioral impairment. In addition, α-synuclein immunoreactivity was increased in surviving TH-positive neurons in a time-dependent manner. Thus, this protocol for chronic administration of rotenone at 30 mg/kg for 56 d is more useful for understanding the mechanism of DA neurodegeneration.

Key words Parkinson’s disease; dopamine; α-synuclein; rotenone

In Parkinson’s disease (PD), although the mechanisms of neurodegeneration are not fully understood, oxidative stress, excessive free-radical formation, environmental toxins, and/or endogenous neurotoxins may be involved in the massive loss of nigrostriatal dopamine (DA) neurons. Although the pathophysiological mechanisms of the selective loss of DA neurons are still unclear, it has been suggested that reduced activity of complex I of the mitochondrial respiratory chain in the substantia nigra may be involved in the pathophysiology of PD. These findings in PD patients raise the possibility that the nigrostriatal system may be particularly vulnerable to impairment of the mitochondrial respiratory chain. Rotenone, an complex I inhibitor, could therefore be used to further evaluate the relationship between mitochondrial dysfunction and dopaminergic injury.

Betarbet et al. demonstrated that in rats, especially Lewis rats, chronic systemic exposure to rotenone through jugular vein cannulation reproduced many features of PD, including nigrostriatal DA neurodegeneration and the formation of cytoplasmic inclusions in nigral DA neurons that were reminiscent of Lewy bodies (LBs). Furthermore, rotenone-treated rats showed abnormal behaviors. The same group demonstrated that these features of PD can be created by chronic systemic exposure to rotenone following the implantation of subcutaneous osmotic pumps, and suggested that this new protocol for chronic rotenone administration is a substantial improvement in terms of simplicity. However, the success rate of the model was about 50%. On the other hand, previous publications demonstrated acute or subchronic administration of rotenone was not able to cause the neuropathological characteristics of PD in mice. Recently, we reported that chronic oral administration of rotenone caused specific nigrostriatal DA neurodegeneration in C57BL/6 mice. Using this model, we investigated neuroprotective effects of chemical chaperone, D2/D3-receptor agonist and nicotinic acetylcholine receptor stimulation against rotenone-induced DA neurodegeneration. In addition, marked α-synuclein immunoreactivity was detected in some surviving tyrosine hydroxylase (TH)-positive neurons in rotenone-treated mice. The previous experimental condition of rotenone mouse model may resemble early PD symptoms, rather than atypical parkinsonism. Thus, to establish a more suitable model for PD, the present study was designed to assess the neurotoxicity of rotenone after daily oral administration at 30 or 100 mg/kg for 56 d in C57BL/6 mice.

MATERIALS AND METHODS

Animals and Oral Administration of Rotenone Eight-week-old male C57BL/6N mice (20—25 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were acclimated to and maintained at 23 °C under a 12-h light/dark cycle. Mice were housed in standard laboratory cages and had free access to food and water throughout the study period. All animal experiments were carried out in accordance with the ‘National Institutes of Health Guide for the Care and Use of Laboratory Animals,’ and the protocols were approved by the ‘Committee for Animal Research at Kyoto Pharmaceutical University.’ Rotenone (Sigma, St. Louis, MO, U.S.A.) was administered orally once a day at doses of 30 or 100 mg/kg for 56 d. Rotenone was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC, Nacalai Tesque, Kyoto, Japan) with Tween-20. Subsequently, the suspension was administered orally once a day at a volume of 5 ml/kg.

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body weight. 0.5% CMC was administered orally to control mice as a vehicle. Motor activity was measured using the rota-rod treadmill (accelerating model 7750, Ugo Basile, Varese, Italy), and the accelerating rotor mode was used (10 speeds from 4 to 40 rpm for 5 min). The interval from when mice mounted the rod to when it fell off was recorded as the performance time.

**Tissue Preparation and Immunohistochemistry** After the behavior test, treated mice were perfused through the aorta with 50 ml of 10 mM phosphate-buffered saline (PBS), followed by 150 ml of a cold fixative consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB), under deep anesthesia with pentobarbital (100 mg/kg, intraperitoneally (i.p.)). After perfusion, the brain was quickly removed and postfixed for 2 d with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4 °C. The cryo-protected brain blocks were cut into 60 μm slices on a cryostat. Brain slices were incubated with primary antibodies: mouse monoclonal antibodies against TH (diluted 1:10000, Sigma, St. Louis, MO, U.S.A.), glial fibrillary acidic protein (GFAP; 1:3000, Chemicon, Temecula, CA, U.S.A.) and phosphorylated tau (AT8; 1:3000, Innogenetics, Gent, Belgium); rabbit polyclonal antibody against ionized calcium-binding adapter molecular 1 (Iba1; 1:5000, Wako, Osaka, Japan) for 3 d at 4 °C. After several washes, these slices were incubated with biotinylated antibodies against mouse or rabbit immunoglobulin G (IgG) (1:2000; Vector Laboratories, Burlingame, CA, U.S.A.) as appropriate for 2 h at room temperature. These slices were then incubated with avidin peroxidase (Vectastatin ABC Elite kit, 1:4000; Vector Laboratories, Burlingame, CA, U.S.A.) and fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (1:500), and fluorescence was observed using a laser scanning confocal microscope (LSM510, Carl Zeiss, Jena, Germany).

**Stereological Analysis of DA Neurons** TH-positive neurons in the substantia nigra pars compacta (SNpc) were estimated using Stereo Investigator software (MBF Bioscience, Williston, VT, U.S.A.) and stereologic principles, as described previously. The posterior midbrain, which yielded a dark blue color, was decreased by about 40% within one week. Furthermore, the survival rate of rotenone-treated mice at 30 mg/kg was decreased by about 30% within one week (Fig. 1B). From then on until 56 d, the survival rate of rotenone-treated mice at 30 mg/kg was not changed similarly to previous our results. On the other hand, the survival rate of rotenone-treated mice at 100 mg/kg was decreased by about 40% within one week. The survival rate of rotenone-treated mice at 100 mg/kg was suddenly decreased after 28 d, and finally to about 15% by 56 d (Fig. 1B).

**Effect of Rotenone at 30 mg/kg** As shown in representative photomicrographs, rotenone at 30 mg/kg reduced the number of TH-positive neurons in the SNpc (Figs. 2A—C). In the stereological analysis, rotenone at 30 mg/kg caused a significant loss of TH-positive neurons in a time-dependent manner (Fig. 2F). In addition, these mice exhibited a marked reduction in endurance time on the rota-rod in a time-dependent manner (Fig. 3). Although TH-positive neurons were

**RESULTS**

**The Survival Rate of Rotenone-Treated Mice** Firstly, we investigated body weight and survival rate of rotenone-treated mice. We confirmed that rotenone-treated mice at 30 or 100 mg/kg for 56 d did not display difference in weight change in comparison with vehicle-treated mice (Fig. 1A). The survival rate of vehicle-treated mice did not change during the experimental period (Fig. 1B). The survival rate of rotenone-treated mice at 30 mg/kg was decreased by about 30% within one week (Fig. 1B). From then on until 56 d, the survival rate of rotenone-treated mice at 30 mg/kg was not changed similarly to previous our results. On the other hand, the survival rate of rotenone-treated mice at 100 mg/kg was decreased by about 40% within one week. Furthermore, the survival rate of rotenone-treated mice at 100 mg/kg was suddenly decreased after 28 d, and finally to about 15% by 56 d (Fig. 1B).

![Fig. 1. Effects of Rotenone on the Survival Rate and Body Weight](image)

Rotenone (suspended in 0.5% CMC) was orally administered to C57BL/6 mice at 30 or 100 mg/kg (each group, n=16). 0.5% CMC was administered orally to control mice as vehicle (n=9). (A) Body weight. (B) The survival rate.
decreased by oral administration of rotenone, α-synuclein immunoreactivity was detected in some surviving TH-positive neurons in the SNpc of rotenone-treated mice (Fig. 4). Interestingly, α-synuclein immunoreactivity was increased in the surviving TH-positive neurons in the SNpc of rotenone-treated mice at 30 mg/kg for 56 d in comparison with those for 28 d. On the other hand, activated astrocytes and microglia, and tau phosphorylation were not observed in the mouse model (Fig. 5).

**DISCUSSION**

Several epidemiologic studies have suggested that some pesticides and environmental toxins, inhibitors of mitochondrial complex I, are involved in the pathogenesis of PD. Rotenone is a lipophilic compound that easily crosses biological membranes. Following systemic administration, rotenone
evenly distributes throughout the body and gains access to all cells. Previous studies have suggested that the optimal dose for inducing the pathology of PD was 2—3 mg/kg per day using osmotic pump in Lewis rats. These rats were often characterized by obvious weight loss. In the current study, rotenone-treated C57BL/6 mice even at 100 mg/kg per os (p.o.) tested gained weight similar to vehicle-treated mice. However, the survival rate of rotenone-treated mice at 100 mg/kg was decreased to about 10% by 56 d. Rotenone-treated mice at 100 mg/kg exhibited a marked reduction in endurance time on the rota-rod in comparison with those at 30 mg/kg. Previous studies have shown that widespread inhibition of mitochondrial complex I and peripheral toxicity rather than nigrostriatal DA neurodegeneration may be responsible for reduced locomotion. These results suggest that peripheral toxicity may markedly appear in rotenone-treated mice at 100 mg/kg in comparison with those at 10 mg/kg.

The role of mitochondria in PD has been accentuated by the observation that 1-methyl-4-phenylpyridium ion (MPP\textsuperscript{+}), the metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and an inhibitor of complex I, causes a parkinsonian syndrome. Microglial activation was induced by acute MPTP treatment (high dose), but not by chronic MPTP treatment (low dose), in C57BL/6 mice. Additionally, an adeno-associated virus (AAV)-derived Apaf-1 DN inhibitor prevented chronic MPTP toxicity. However, the AAV-derived Apaf-1 DN did not inhibit DA neuronal death in acute MPTP toxicity. Therefore, the primary cascade activated by MPTP neurotoxicity may be different between mice that received acute and chronic MPTP treatment.

In the present study, slow and chronic rotenone neurotoxicity did not induce the activation of glial cells. It may be considered that DA neurons are relatively more susceptible to rotenone cytotoxicity than glial cells because of chronic exposure to oxidative stress by DA metabolites and disruption of the microtubule-based transporter of DA vesicles. In addition, tauopathy could not be detected in this mouse model. Thus, the slow and chronic nature of rotenone neurotoxicity may lead to moderate neurodegeneration in relatively limited nigrostriatal dopaminergic system of C57BL/6 mice even after 56 d.

Genetic studies led to the discovery of a small percentage of familial PD cases linked directly to genetic mutations, as well as gene duplications and triplications. The first gene associated with PD was \(\alpha\)-synuclein (PARK1). Furthermore, duplication and triplications of \(\alpha\)-synuclein are linked to an early onset familial PD (PARK4). These genetic studies suggest that changes in \(\alpha\)-synuclein protein levels may represent a gain of toxic function. In the present study, the number of \(\alpha\)-synuclein and TH double-positive cells were increased in a time-dependent manner. These results suggest that chronic exposure of rotenone for 56 d may be useful tool to examine the relation between \(\alpha\)-synuclein and the mechanism of DA neurodegeneration.

In the present study, we used C57BL/6 mice. This C57BL/6 strain has been frequently used for MPTP-treated mice, and PD-related transgenic or knockout mice. Several genetic mice models of PD have been generated, but these models did not reproduce the full pathology and progression seen in PD. Therefore, chronic exposure of rotenone in genetic mouse model of PD may allow investigations of possible interactions between environmental and genetic factors. Rotenone mice model may provide an interesting animal model for preclinical examinations of neuroprotective strategies.

In conclusion, chronic oral administration of rotenone at 30 mg/kg for 56 d selectively induced nigrostriatal DA neurodegeneration and motor deficits, and increased the cytoplasmic accumulation of \(\alpha\)-synuclein in surviving DA neurons. These results suggest that this rotenone mouse model may be useful for understanding the mechanism of DA neurodegeneration in PD.

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