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# Activation of mitochondrial ATP-sensitive potassium channels improves rotenone-related motor and neurochemical alterations in rats

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## Abstract

Our previous studies revealed that activation of mitochondrial ATP-sensitive potassium channels exerted protective effects on rotenone-treated rats and cultured cells. The aim of the present study is to examine the potential therapeutic effects of iptakalim, an ATP-sensitive potassium-channel opener, and diazoxide, a selective mitochondrial ATP-sensitive potassium-channel opener, on Parkinsonian symptoms in rats induced by rotenone. Rats were treated with rotenone (2.5 mg/kg s.c.) daily for 4 wk. This treatment caused a depletion of dopamine in the striatum and substantia nigra. Behaviourally, rotenone-infused rats exhibit Parkinsonian symptoms. Catalepsy was estimated by a 9-cm bar test. Treatment with L-dopa (10 mg/kg.d p.o.), iptakalim (0.75, 1.5, 3.0 mg/kg.d p.o.) and diazoxide (3.0 mg/kg.d p.o.) for 2 wk improved behavioural dysfunction and elevated dopamine contents in the striatum and substantia nigra of rotenone-treated rats. Studies also found that iptakalim and diazoxide could reduce the enzymic activities and mRNA levels of inducible nitric oxide synthase elicited by chronic administration of rotenone. All neurorestorative effects by both iptakalim and diazoxide were abolished by 5-hydroxy-decanoate, a selective mitochondrial ATP-sensitive potassium-channel blocker. Collectively, the data suggested that mitochondrial ATP-sensitive potassium channels play a key role in improving both Parkinsonian symptoms and neurochemistry alterations of rotenone model rats, and selective activation of mitochondrial ATP-sensitive potassium channels may provide a new therapeutic strategy for treatment of early Parkinson's disease.

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**Key words:** Diazoxide, iptakalim, mitochondrial ATP-sensitive potassium channels (mito-K<sub>ATP</sub>), Parkinson's disease, rotenone.

## Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown aetiology, the cardinal features of which include tremor, rigidity, slowness of movement and postural instability. Its pathological hallmarks are loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of neuronal proteinaceous cytoplasmic inclusions, commonly known as Lewy bodies (Fahn and

Przedborski, 2000). Neurodegeneration of the SNpc results in a dramatic decrease in dopamine (DA) content in the corpus striatum. Thus far, both genetic mutations and environmental factors have been considered as contributors to certain forms of this disorder (Steece-Collier et al., 2002). Recent evidence suggests that mitochondrial damage, particularly to the respiratory chain of complex I (CXI, NADH/ubiquinone oxidoreductase) resulting in oxidative and nitritative stress, underlies the pathology of PD (Dawson and Dawson, 2003; Greenamyre et al., 1999; Schapira et al., 1990; Zhang, et al., 2000). Rotenone, an inhibitor of mitochondrial NADH dehydrogenase, has been widely used as an herbicide and fish destroyer in reservoirs (Gutman et al., 1970; Horgan

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et al., 1968). A majority of studies employing rotenone have focused on the ability of this toxin to induce neuropathological effects in rats which are reminiscent of those seen in PD: loss of dopaminergic terminals in the striatum and the formation of Lewy body-like inclusions in the substantia nigra (Alam and Schmidt, 2002; Betarbet et al., 2000; Sherer et al., 2003). Behaviourally, rotenone-infused rats exhibit reduced mobility, flexed posture and, in some cases, rigidity (Sherer et al., 2003) and catalepsy (Alam and Schmidt, 2002). Therefore, the rotenone-treated rat is a suitable model to investigate new possible therapeutic targets for PD.

Current understanding of basal ganglia circuitry and the pathophysiology of PD has led to major breakthroughs in the treatment of this debilitating movement disorder. Unfortunately, there are significant problems with the current available pharmacological therapies, which mainly focus on DA replacement (L-3-4-dihydroxyphenylalanine; L-dopa) or dopaminergic agonists. There are no successful therapies to prevent cell death ('neuroprotection') and/or restore damaged neurons to a normal state ('neurorestoration'; Dawson and Dawson, 2002; Meissner et al., 2004). Consequently, considerable amounts of efforts have focused on developing novel targets for the treatment of PD.

ATP-sensitive potassium channels are located in various parts of the cells, including the surface of the plasmalemmal membrane ( $sK_{ATP}$ ) and the inner mitochondrial membrane (mito- $K_{ATP}$ , Busija et al., 2004; Inoue et al., 1991). Although the precise protein composition of mito- $K_{ATP}$  channels has not been established, a distinct pharmacology of mito- $K_{ATP}$  channels has been established: they are selectively activated by low concentrations of diazoxide (Dia) and blocked by 5-hydroxydecanoate (5-HD; Ghosh et al., 2000; Sato et al., 2000). Recently, some candidates of mito- $K_{ATP}$  channel proteins have been identified. Immunopositive colloidal gold particles were scattered over the mitochondria, suggesting that Kir6.1 was located on the inner membrane (Suzuki et al., 1997). Bajgar et al. (2001) have isolated and purified a novel mito- $K_{ATP}$  channel protein from rat brain mitochondria that exhibits ligand-binding properties similar to those of heart mito- $K_{ATP}$  channels. Accumulating data have indicated that the amount of mito- $K_{ATP}$  channels located in brain cells is at least 6-fold higher than in heart cells. There is also abundant evidence linking the activation of mito- $K_{ATP}$  channels to protection against neuronal injury or apoptosis, which conceptually supports the view that the protective effects triggered by activation of mito- $K_{ATP}$  channels may

potentially and feasibly serve therapeutic roles for PD (Horiguchi et al., 2003; Kis et al., 2003; Liu et al., 2002; Rajapakse et al., 2002; Tai et al., 2003; Teshima et al., 2003).

Iptakalim (Ipt) is a  $K_{ATP}$  channel opener used as an antihypertensive drug (Wang, 1998, 2003). Interestingly, our recent research has revealed that Ipt could open mito- $K_{ATP}$  channels thereby exhibiting significant neuroprotection – not only in promoting behavioural recovery but also in protecting neurons against necrosis and apoptosis in different animal models of stroke and rotenone-induced Parkinsonian symptoms in rats, as well as cell cultures (Wang et al., 2004; Yang et al., 2004). The aims of this study are to study the possible treatment of Ipt and Dia on Parkinsonian symptoms in rats induced by rotenone and investigate the neurorestorative role(s) of mito- $K_{ATP}$  channel openers on neurodegenerative diseases. Behavioural measures sensitive to varying degrees of catalepsy were used (Schallert et al., 2000). DA and related neurotransmitters in the striatum and substantia nigra tissues were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). The activities and mRNA levels of nitric oxide synthase (NOS) were measured as described below.

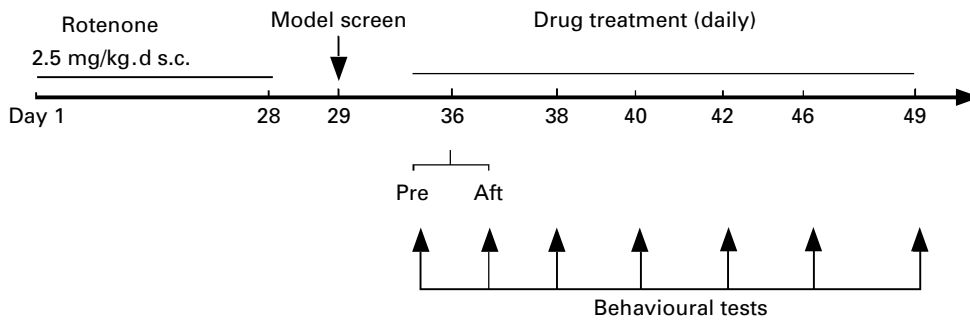
## Methods

### Materials

Rotenone, Dia, L-dopa and all the solvents for HPLC-ECD were obtained from Sigma (St. Louis, MO, USA). Sodium 5-HD was purchased from Valeant Pharmaceuticals International (Costa Mesa, CA, USA). All PCR primers were purchased from Sangon (Shanghai, China). All reagents for RT-PCR were purchased from Promega (Madison, WI, USA).

### Animals and treatment

A total of 150 adult male Sprague–Dawley rats (Shanghai, China) aged 7 wk were chosen for the experiments. At the beginning of the experiments, they weighed 220–240 g. They were kept under standard laboratory conditions (six housed per large animal cage): free access to standard laboratory food and tap water, constant room temperature of 22 °C, 50–60% humidity with a natural day–night cycle, etc. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and approved by IACUC (Institutional



**Figure 1.** Experimental design.

Animal Care and Use Committee of Nanjing Medical University).

Dimethyl sulphoxide (DMSO)/PEG (1/1) was used as vehicle for rotenone. Dia was dissolved in 100% DMSO as vehicle and diluted with sterile saline to a concentration of 1 mg/ml (0.2% DMSO) for administration to rats. Ipt and 5-HD, a selective mito- $K_{ATP}$  channel blocker, was dissolved in saline. L-dopa methyl ester was dissolved in physiological saline containing 0.2% ascorbic acid. Benserazide hydrochloride (Sigma; 1/4 dose of L-dopa), a peripheral dopa-decarboxylase inhibitor, was also dissolved in physiological saline.

Rats were injected subcutaneously (s.c.) with 2.5 mg/kg rotenone/d at 08:00, 14:00 and 20:00 hours respectively, for 28 d. The injection volume was 0.1 ml/100 g body weight. Control rats were treated with an equal volume of rotenone solvent. A catalepsy test was used to screen the PD model 4 wk after systemic rotenone treatment. The rats were placed with both forepaws on bars 9 cm above and parallel from the base and were in a half-rearing position. Latency time of the removal of the paw was recorded. The criterion for a successful model was defined as latency time  $\geq 15$  s. Sixty-four successful PD model rats were selected and divided into eight groups according to the latency time. The number of rats in every group was eight. The mean of latency time of each group remains proximate. The groups were outlined below.

Group A: Vehicle control rats + 0.2% DMSO in saline [per oral (p.o.)];

Group B: Rotenone model rats + 0.2% DMSO in saline (p.o.);

Group C: Rotenone model rats + 0.75 mg/kg.d Ipt (p.o.);

Group D: Rotenone model rats + 1.5 mg/kg.d Ipt (p.o.);

Group E: Rotenone model rats + 3.0 mg/kg.d Ipt (p.o.);

Group F: Rotenone model rats + 10 mg/kg.d L-dopa (p.o.);

Group G: Rotenone model rats + 3.0 mg/kg.d Dia (p.o.);

Group H: Rotenone model rats + 5.0 5-HD mg/kg.d (p.o.) + 3.0 mg/kg.d Ipt (p.o.);

Group I: Rotenone model rats + 5.0 5-HD mg/kg.d (p.o.) + 3.0 mg/kg.d Dia (p.o.).

On day 36, Ipt, Dia or L-dopa combined with benserazide hydrochloride (1/4 dose of L-dopa) was administered by gavage (p.o.) on a daily basis for 14 d. Benserazide hydrochloride was administered 30 min before treatment with L-dopa. The volume was 1 ml/100 g body weight for all drugs. Behavioural tests were performed 60 min after drugs administration.

### Behavioural tests

The catalepsy test was chosen for the assessment of the effects of drugs on rotenone-induced Parkinsonian symptoms and was performed as illustrated in Figure 1. All experiments were arranged between 09:00 and 14:00 hours, always in the same context and at standard conditions. The maximum prolonged latency time was 180 s (Alam and Schmidt, 2002, 2004).

### Neurochemistry procedure

One week after the behavioural test, animals were sacrificed and their brains were rapidly removed (within 25–40 s) and placed in ice-chilled 0.9% NaCl solution for 1 min. Tissue samples of the striatum and substantia nigra were taken bilaterally and immediately weighed and stored in liquid nitrogen until assay.

Frozen tissue was sonicated in 0.1 M HClO<sub>4</sub> containing 0.5% Na<sub>2</sub>EDTA and 0.1% Na<sub>2</sub>SB<sub>3</sub>OB<sub>3</sub> (30 µl for every 10 mg tissue) and centrifuged at 12000 g for 20 min at 4 °C. The supernatant was centrifuged again for another 10 min under the same conditions. Sample supernatant was analysed directly for DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), noradrenalin (NE), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA, 5-HT's metabolite) by reverse-phase HPLC coupled to an electrochemical detector (HPLC-ECD).

HPLC-ECD for monoamine neurotransmitters consisted of BAS LC-4C, a reversed-phase C<sub>18</sub> column (Ultrasphere ODS 4.6 mm × 250 mm, 5 µm; GL Sciences Inc., Tokyo, Japan), chromatograph interface DA-5, and solvent delivery system. The mobile phase consisted of 0.1 M citrate, 75 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM EDTA, 1.0 mM 1-heptanesulphonic acid, 10% methanol (pH 3.9). Chromatograms were analysed with the aid of a chromatographic data system (BAS; Yang et al., 2004). Sample results were expressed as pg/mg of tissue.

#### *NOS activity assay*

Nitric oxide (NO) is synthesized from L-arginine by the enzyme NOS (Knowles and Moncada, 1994). Three main types of the synthase (NOS) enzyme are associated with differing roles: endothelial NOS (eNOS), which is expressed in endothelial cells; inducible NOS (iNOS), which is not expressed in the CNS except in the inflammatory state after lipopolysaccharide or cytokine induction in glial cells; and neuronal NOS (nNOS), which is localized in neurons (Heneka and Feinstein, 2001; Vincent and Kimura, 1992). In this study, NOS activities in the striatum and substantia nigra were determined as described previously (Zhu et al., 2002). Briefly, samples of striatum and substantia nigra were homogenized and NOS activities in supernatants were measured spectrophotometrically using a commercially available kit (Juli Bioengineering Co., Nanjing, China). Ca<sup>2+</sup>-independent NOS activity was measured by adding EGTA (3 mM) to chelate-free Ca<sup>2+</sup> from the reaction mixture. Ca<sup>2+</sup>-dependent NOS activity was computed by subtracting Ca<sup>2+</sup>-independent NOS activity from total NOS activity. NOS activity is expressed as picomoles of NO formed in 1 min by 1 mg of protein. Calcium-dependent activity in cytosolic samples is considered to represent nNOS because eNOS is primarily associated with membranes (Johansson et al., 2002). Protein concentration in the samples was determined by the Bradford method (Bradford, 1976).

#### *RNA extraction and reverse transcription-PCR (RT-PCR)*

Total RNA was extracted from the samples of striatum and substantia nigra using Trizol reagent according to the manufacturer's instructions (Sigma). The PCR primers were designed to amplify the iNOS product (230 bp). The composition of iNOS was selected from the sequence (GenBank accession no: NM\_012611). The sense primer for iNOS was 5'-CTGCATGGAA-CAGTATAAGGCAAAC-3', and the antisense primer was 5'-CAGACAGTTTCTGGTTCGATGTCATGA-3'. For assessment of the cDNA quality, a pair of GAPDH primers was designed (GenBank accession no: BC059110). The forward primer was 5'-TGGTGCC-AAAAGGGTCATCTCC-3' and the reverse primer was 5'-GCCAGCCCCAGCATCAAAGGTG -3' to amplify a 559-bp product. PCR conditions for iNOS were 35 cycles of denaturation at 95 °C for 45 s, annealing at 63 °C for 45 s, and extension at 72 °C for 45 s. PCR conditions for GAPDH were 25 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s. Following the last cycle, the final extension was performed at 72 °C for 10 min for all PCR analyses. The amplified products were separated by electrophoresis in 1 × Tris-acetate EDTA buffer with a 2% agarose gel containing 0.1 µg/ml of ethidium bromide (4 µl). The DNA bands were visualized and analysed by JD-801 Gel Electrophoresis Image analytic system (Jiangsu, China). The ratio of NOS mRNA and GAPDH mRNA was calculated.

#### *Statistical analysis*

All data are expressed as mean ± S.E.M. Statistical analysis was carried out using STATA 7.0 software (Stata Corporation, College Station, TX, USA). The catalepsy data of different groups were compared by the non-parametric (multifactorial Kruskal-Wallis test, rank transformation and one-way ANOVA followed by Scheffé multiple-comparison test). The neurochemical data were analysed by one-way ANOVA followed by Newman-Keuls test. A *p* value <0.05 and *p* <0.01 was taken as levels of statistical significance (Alam and Schmidt, 2004).

### **Results**

#### *Behavioural tests*

##### *Catalepsy*

Rotenone-treated animals showed prolonged latency time compared to the vehicle-treated control group

**Table 1.** Effects of iptakalim, diazoxide and L-dopa on catalepsy [latency time (s)] of rotenone-treated rats

Groups (mg/kg)	Day						
	36-Pre	36-Aft	38	40	42	46	49
(A) Veh + Sal	1.7 ± 0.6	1.9 ± 0.7	2.3 ± 0.5	1.8 ± 0.6	1.8 ± 0.6	1.9 ± 0.5	2.0 ± 0.4
(B) Rot + Sal	28.5 ± 6.3	29.2 ± 9.5	34.1 ± 10.6	41.3 ± 18.0	39.6 ± 17.5	45.4 ± 15.0	46.5 ± 10.3
(C) Rot + 0.75 Ipt	27.3 ± 5.3	28.3 ± 6.9	25.6 ± 6.8	21.4 ± 8.1##	20.4 ± 8.6###	22.9 ± 6.1###	22.6 ± 6.2##
(D) Rot + 1.5 Ipt	28.0 ± 6.1	27.4 ± 3.5	17.8 ± 5.0#	13.8 ± 3.5##	12.0 ± 4.4###	13.0 ± 4.6###	15.9 ± 4.2##
(E) Rot + 3.0 Ipt	29.3 ± 4.5	28.4 ± 7.8	25.3 ± 8.0	19.4 ± 5.3##	20.2 ± 6.5###	21.3 ± 6.2###	22.1 ± 6.0###
(F) Rot + 10 L-dopa	27.4 ± 6.1	28.1 ± 10.3	13.8 ± 3.1###	12.0 ± 4.1##	11.4 ± 3.7###	14.6 ± 4.7###	17.8 ± 4.7##
(G) Rot + 3.0 Dia	28.6 ± 6.7	26.6 ± 8.8	22.5 ± 7.9	18.9 ± 5.1##	17.9 ± 6.3###	19.8 ± 4.6###	20.9 ± 2.5##
(H) Rot + 5.0 5-HD + 3.0 Ipt	28.0 ± 8.2	29.3 ± 7.8	33.5 ± 9.6	33.6 ± 8.6 <sup>+</sup>	41 ± 9.5 <sup>++</sup>	41.6 ± 11.9 <sup>+</sup>	42.8 ± 10.9 <sup>++</sup>
(I) Rot + 5.0 5-HD + 3.0 Dia	27.3 ± 5.5	28.7 ± 6.6	31.6 ± 8.5	36.1 ± 6.7††	39.8 ± 6.9††	37.9 ± 12.6†	40.3 ± 11.4†

Veh, Vehicle; Sal, saline; Rot, rotenone; Ipt, iptakalim; Dia, diazoxide; 5-HD, 5-hydroxydecanoate.

Successful Parkinson's disease model rats were grouped and treated with 0.2% DMSO in saline, L-dopa (10.0 mg/kg . d p.o.), Ipt (0.75, 1.5, 3.0 mg/kg . d p.o.) or Dia (3.0 mg/kg . d p.o.) with or without 5-HD (3.0 mg/kg . d, p.o.) respectively. Catalepsy tests were performed 60 min after drug administration. Data are expressed as latency time of the removal of the paw (s) and given as mean ± S.E.M. ( $n=8$ ).  $p$  represents statistical analysis: #  $p<0.05$ , ##  $p<0.01$  vs. group B;  $^+p<0.05$ ,  $^{++}p<0.01$  vs. group E and †  $p<0.05$ , ††  $p<0.01$  vs. group G (multifactorial Kruskal-Wallis test, rank transformation and one-way ANOVA followed by Scheffé multiple comparison test).

**Table 2.** Effects of iptakalim, diazoxide and L-dopa on monoamine levels in the striatum of rotenone-treated rats

Groups (mg/kg)	DA	DOPAC	HVA	NE	5-HT	5-HIAA
(A) Veh + Sal	7141 ± 422	1673 ± 314	429 ± 73	3141 ± 330	468 ± 66	434 ± 55
(B) Rot + Sal	2920 ± 347**	1294 ± 225*	333 ± 52**	1776 ± 446**	239 ± 57**	275 ± 40**
(C) Rot + 0.75 Ipt	4505 ± 641***	1640 ± 294#	403 ± 71#	2564 ± 538***	395 ± 64###	436 ± 69###
(D) Rot + 1.5 Ipt	6745 ± 574###	1602 ± 285#	414 ± 69#	3110 ± 398###	410 ± 70###	439 ± 71###
(E) Rot + 3.0 Ipt	7014 ± 870###	1729 ± 219###	400 ± 63#	3004 ± 546###	345 ± 70###	420 ± 74###
(F) Rot + 10 L-dopa	7462 ± 794###	2318 ± 664***	472 ± 108 ##	2857 ± 315###	293 ± 70**	328 ± 72
(G) Rot + 3.0 Dia	6104 ± 724***	1660 ± 360#	424 ± 69 ##	2755 ± 347###	411 ± 48##	439 ± 66##
(H) Rot + 5.0 5-HD + 3.0 Ipt	3586 ± 476*** <sup>++</sup>	1274 ± 211*** <sup>++</sup>	345 ± 40* <sup>+</sup>	1915 ± 167*** <sup>++</sup>	262 ± 39*** <sup>+</sup>	308 ± 38*** <sup>+</sup>
(I) Rot + 5.0 5-HD + 3.0 Dia	3324 ± 451***††	1230 ± 251***†	337 ± 68***††	2028 ± 270***††	278 ± 37***††	317 ± 35***††

DA, Dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; NE, noradrenalin; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; Veh, vehicle; Sal, saline; Rot, rotenone; Ipt, iptakalim; Dia, diazoxide; 5-HD, 5-hydroxydecanoate. Data are expressed as picograms per milligram (pg/mg) of fresh brain weight and given as mean ± S.E.M. ( $n=8$ ).  $p$  represents statistical analysis: \*  $p<0.05$ , \*\*  $p<0.01$  vs. group A; #  $p<0.05$ , ##  $p<0.01$  vs. group B;  $^+p<0.05$ ,  $^{++}p<0.01$  vs. group E; †  $p<0.05$ , ††  $p<0.01$  vs. group G (one-way ANOVA followed by the Newman-Keuls test).

using the bar test. On day 1 of treatment with L-dopa, there was no significant difference in the descent latency compared to model rats with saline (group B). The L-dopa administration group (group F) showed a significant reversal in the descent latency compared to the model group (group B) on days 38, 40, 42, 46, 49 ( $p<0.01$ , Table 1). PD model rats treated with Ipt and Dia exhibited a significant decrease in latency time similar to rats administered L-dopa, especially in group D (Ipt 1.5 mg/kg . d p.o.) and group G (Dia 3.0 mg/kg . d p.o.), which was more significant than that observed in group C (Ipt 0.75 mg/kg . d p.o.)

and group E (Ipt 3.0 mg/kg . d p.o.). In groups H and I, the latency time of the rats pre-administered 5-HD (5.0 mg/kg . d p.o.), a selective mito- $K_{ATP}$  channel blocker, showed no significant difference compared with the model rats (group B,  $p>0.05$ ).

### Neurochemical changes

In the second experiment, levels of DA and its metabolites (DOPAC and HVA), NE, 5-HT and its metabolite (5-HIAA) were measured in the substantia nigra (Table 2) and striatum for each group of animals

**Table 3.** Effects of iptakalim, diazoxide and L-dopa on monoamine levels in the substantia nigra of rotenone-treated rats

Groups (mg/kg)	DA	DOPAC	HVA	NE	5-HT	5-HIAA
(A) Veh + Sal	372 ± 34	151 ± 28	74 ± 7	149 ± 14	502 ± 40	539 ± 74
(B) Rot + Sal	199 ± 28**	102 ± 19**	40 ± 7**	81 ± 14**	355 ± 67**	404 ± 56**
(C) Rot + Ipt 0.75	260 ± 30***#	137 ± 23##	52 ± 6***#	104 ± 12***#	415 ± 7*	438 ± 64*
(D) Rot + Ipt 1.5	312 ± 28##	153 ± 23##	64 ± 9##	134 ± 19##	467 ± 65##	496 ± 46##
(E) Rot + Ipt 3.0	347 ± 28##	163 ± 11##	69 ± 6##	139 ± 11##	486 ± 39##	500 ± 25##
(F) Rot + L-dopa 10	281 ± 63***#	198 ± 25***#	76 ± 8##	152 ± 16##	362 ± 62**	390 ± 66**
(G) Rot + Dia 3.0	376 ± 61##	159 ± 23##	71 ± 10##	142 ± 21##	467 ± 54##	496 ± 46##
(H) Rot + 5-HD 5.0 + Ipt 3.0	238 ± 25***++	104 ± 22***++	48 ± 5***++	95 ± 10***++	400 ± 40***++	418 ± 40***++
(I) Rot + 5-HD 5.0 + Dia 3.0	212 ± 31***††	110 ± 36***††	42 ± 6***††	84 ± 12***††	370 ± 47***††	384 ± 38***††

DA, Dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; NE, noradrenalin; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; Veh, vehicle; Sal, saline; Rot, rotenone; Ipt, iptakalim; Dia, diazoxide; 5-HD, 5-hydroxydecanoate. Data are expressed as picograms per milligram (pg/mg) of fresh brain weight and given as mean ± S.E.M. ( $n=8$ ).  $p$  represents statistical analysis: \*  $p<0.05$ , \*\*  $p<0.01$  vs. group A; #  $p<0.05$ , ##  $p<0.01$  vs. group B; +  $p<0.05$ , ++  $p<0.01$  vs. group E; †  $p<0.05$ , ††  $p<0.01$  vs. group G (one-way ANOVA followed by the Newman-Keuls test).

(Table 3). We found that chronic rotenone administration reduced DA content of the striatum by ~60%, NE content by nearly 40%, and 5-HT and its metabolite (5-HIAA) by almost 25%, similar to the reduction observed in DA metabolites (such as DOPAC, and the final metabolite, HVA). L-dopa administration (10 mg/kg.d p.o.) elevated the levels of DA and NE, but had no effect on the levels of 5-HT and 5-HIAA. Ipt (0.75 mg/kg.d p.o.) significantly elevated the content of DA in the striatum and substantia nigra compared to the model rats (group B). In groups D, E, F and G, neurotransmitter levels of rotenone model rats treated with Ipt (1.5, 3.0 mg/kg.d p.o.), Dia (3.0 mg/kg.d p.o.) and L-dopa (10 mg/kg.d p.o.) for 2 wk showed no significant differences with the vehicle control rats (group A). In groups H and I, 5-HD (5.0 mg/kg.d p.o.) abolished the effects of Ipt and Dia.

#### Identification of NOS activities and mRNA levels in the striatum and substantia nigra

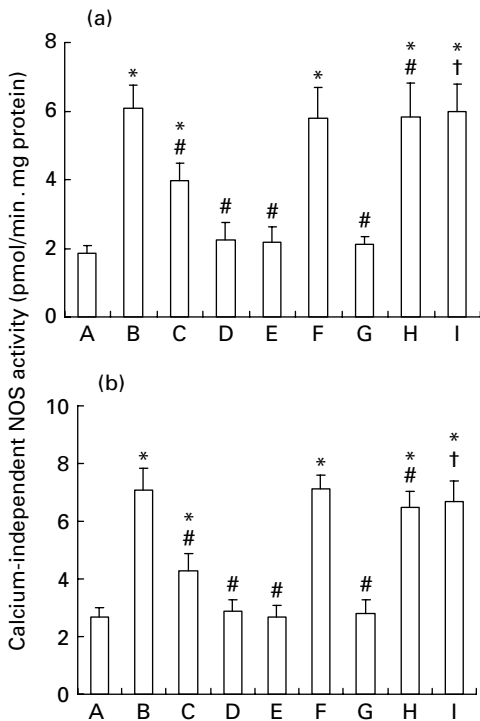
In this study, we measured NOS activities and mRNA levels in both the striatum and substantia nigra. The iNOS activities and mRNA levels in rotenone-treated rats were several times higher compared to vehicle control rats (Figures 2 and 3). Except for group C, there was no significant difference between the vehicle control group and Ipt- or Dia-treated groups. Ipt (0.75 mg/kg.d, p.o.) also significantly reduced iNOS activities and mRNA levels in group C compared to group B (model rats). 5-HD abolished the effects of Ipt and Dia. L-dopa had no effect on NOS activity and mRNA levels in both brain regions.

nNOS activities of the striatum and substantia nigra of the model rats (group B) showed no difference with the rats administered Ipt, Dia or L-dopa respectively, although they were significantly higher than that of the vehicle control rats (group A). The mRNA levels in both brain regions also showed no difference among each group (data not shown).

#### Discussion

Rotenone induced depletion of ATP and overproduction of reactive oxide species (ROS) by inhibiting CXI. The toxicity of rotenone in the nigro-striatal dopaminergic system depends on the drug regimen because acute, high doses are toxic to virtually any cell of an organism, but a low dose given chronically can be tolerated by most cells, except dopaminergic neurons (Alam and Schmidt, 2002; Betarbet et al., 2000; Fahn and Przedborski, 2000; Ferrante et al., 1997; Sherer et al., 2003). In the present study, rats were chronically administered 2.5 mg/kg.d rotenone for 4 wk and exhibited Parkinsonian symptoms. Accompanying a 60% decrease in striatal DA levels, the ratios of DOPAC/DA and HVA/DA increased. These results suggest an increased turnover and release of DA as a compensatory mechanism in surviving neurons exists after partial injury of the nigro-striatal system (Hsieh et al., 2002; Thiffault et al., 2000). Compared to normal rats, rotenone-treated rats displayed an increase in cataleptic behaviour and a decrease in locomotor activity, which could be reversed by administration of L-dopa, also supporting the view that rotenone-induced neurodegeneration





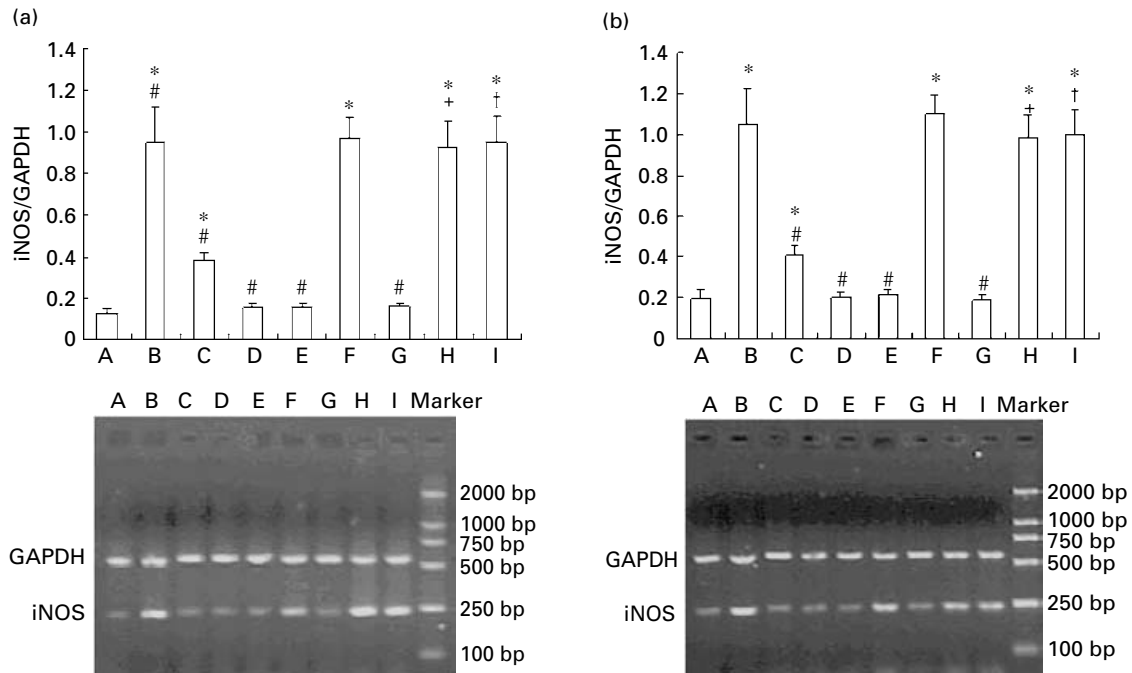
**Figure 2.** iNOS activity in the striatum (a) and substantia nigra (b). Data are presented as mean  $\pm$  S.E.M. ( $n=5$ ).  $p$  represents statistical analysis: \*  $p < 0.01$  vs. lane A; #  $p < 0.01$  vs. lane B; +  $p < 0.01$  vs. lane E; †  $p < 0.01$  vs. lane G (one-way ANOVA followed by the Newman-Keuls test). Lane A, vehicle control rats + 0.2% DMSO in saline (p.o.); lane B, rotenone model rats + 0.2% DMSO in saline (p.o.); lane C, rotenone model rats + 0.75 mg/kg. d Ipt (p.o.); lane D, rotenone model rats + 1.5 mg/kg. d Ipt (p.o.); lane E, rotenone model rats + 3.0 mg/kg. d Ipt (p.o.); lane F, rotenone model rats + 10 mg/kg. d L-dopa (p.o.); lane G, rotenone model rats + 3.0 mg/kg. d Dia (p.o.); lane H, rotenone model rats + 5.0 mg/kg. d 5-HD + 3.0 mg/kg. d Ipt (p.o.); lane I, rotenone model rats + 5.0 mg/kg. d 5-HD + 3.0 mg/kg. d Dia (p.o.).

in rats represents a valid animal model of PD (Alam and Schmidt, 2002, 2004).

Interestingly, Dia and Ipt could also improve the behavioural alterations and restored DA deletion in the nigro-striatal system of rotenone-treated rats, which could be abolished by pretreatment with 5-HD, a selective mito- $K_{ATP}$  channel blocker. As a result, it is clear that activation of mito- $K_{ATP}$  channels was involved in the neurorestorative effects of Dia and Ipt. The exact mechanism of how mito- $K_{ATP}$  channels are involved in neuroprotection and neurorestoration remains unknown. We supposed that dopaminergic neurons and glial mechanisms were both involved in the neurorestorative effects produced by mito- $K_{ATP}$  channel activation.

First, in the rotenone model, the free toxin concentration is in the range of 20–30 nM. This corresponds to the degree of CXI inhibition found in PD (Schapira et al., 1998) but might not substantially impair ATP synthesis in brain mitochondria. Liss believed that the  $sK_{ATP}$  channel is differentially expressed in dopaminergic neurons and directly senses CXI inhibition. It is, however, sufficient to activate SUR1/Kir6.2-containing  $sK_{ATP}$  channels in dopaminergic neurons. The protection afforded by  $sK_{ATP}$  activation is largely due to energy preservation and reduction of  $Ca^{2+}$  influx (Yamada et al., 2001), while excessive opening of  $sK_{ATP}$  channels in neurons may have deleterious consequences: resulting in chronic hyperpolarization and reduction of excitability, reducing expression of activity-dependent genes that promote survival, such as neurotrophins and causing fatal consequences for the dopaminergic neuron (Liss and Roeper, 2001; Yamada et al., 2001). Moreover, recent studies indicated that NO played an important role in rotenone-induced selective nigro-striatal neurodegeneration (Bashkatova et al., 2004; He et al., 2003). NO can inhibit mitochondrial complexes I, II, and IV in the respiratory chain, activate or initiate DNA strand breakage, lipid peroxidation and protein oxidation by increasing the generation of toxic radicals such as hydroxyl radicals and peroxynitrite (Brown and Borutaite, 2002; Ebadi and Sharma, 2003). Peroxynitrite could deactivate tyrosine hydroxylase, the initial and rate-limiting enzyme in the biosynthesis of DA (Ara et al., 1998; Beckman et al., 1993; Blanchard-Fillion et al., 2001). Therefore, the injury and inactivation of tyrosine hydroxylase caused by dopaminergic neurons may be responsible for the decline in DA synthesis and release (Abreu et al., 2000; Ischiropoulos et al., 1995). All these events have been postulated to be involved in PD. Previous studies suggested that low doses of rotenone induced apoptosis instead of necrosis in dopaminergic neurons (Fiskum et al., 2003; Greenamyre et al., 1999). This should be the result of chronic  $sK_{ATP}$  activity of dopaminergic neurons and the attack of NO. We also found that the behavioural dysfunction of the model rats without any treatment became worse although rotenone withdrew, which also supported the view that apoptosis occurred (Table 1). So rats treated with 2.5 mg/kg. d rotenone for 4 wk in our study mimic early PD.

Under the physiological conditions, mito- $K_{ATP}$  channel openers could not only preserve mitochondrial function by regulating intracellular signal transduction, mitochondrial volume and calcium homeostasis, but also exert anti-apoptotic effects



**Figure 3.** iNOS mRNA levels in the striatum (a) and substantia nigra (b). Data are presented as mean  $\pm$  S.E.M. ( $n=5$ ).  $p$  represents statistical analysis: \*  $p<0.01$  vs. lane A; #  $p<0.01$  vs. lane B; +  $p<0.01$  vs. lane E; †  $p<0.01$  vs. lane G (one-way ANOVA followed by the Newman-Keuls test). Lane A, vehicle control rats + 0.2% DMSO in saline (p.o.); lane B, rotenone model rats + 0.2% DMSO in saline (p.o.); lane C, rotenone model rats + 0.75 mg/kg. d Ipt (p.o.); lane D, rotenone model rats + 1.5 mg/kg. d Ipt (p.o.); lane E, rotenone model rats + 3.0 mg/kg. d Ipt (p.o.); lane F, rotenone model rats + 10 mg/kg. d L-dopa (p.o.); lane G, rotenone model rats + 3.0 mg/kg. d Dia (p.o.); lane H, rotenone model rats + 5.0 mg/kg. d 5-HD + 3.0 mg/kg. d Ipt (p.o.); lane I, rotenone model rats + 5.0 mg/kg. d 5-HD + 3.0 mg/kg. d Dia (p.o.).

by affecting cytochrome c release and caspase activation and translocation of apoptosis-related cytosolic Bax to mitochondria (Busija et al., 2004; Horiguchi et al., 2003; Kis et al., 2003; Liu et al., 2002; Mattson and Liu, 2003; Rajapakse et al., 2002; Teshima et al., 2003). We, therefore, supposed that Ipt and Dia might restore the sick dopaminergic neurons and preserve DA synthesis and release, thereby improving the behavioural dysfunction by opening mito- $K_{ATP}$  channels although further study needs to be done to investigate the exact mechanism.

Was there a non-dopaminergic neuron mechanism underlining the protections of mito- $K_{ATP}$  openers? In this study, there was a robust increase in both NOS activity (including iNOS and nNOS) and iNOS mRNA levels in the striatum and substantia nigra of rats challenged with rotenone, which were associated with behavioural abnormalities and decrease of DA contents in those regions respectively. The increase of iNOS activities might be due to the elevated mRNA levels. Interestingly, Ipt and Dia reduced the activity of iNOS, but had no effect on the elevated activity of nNOS (data not shown) after chronic

rotenone treatment, suggesting that NO from iNOS expressed in glial cells appears to be a key mediator of rotenone-induced neuronal death. Similar results were also found in previous studies (Brown and Bal-Price, 2003; Sparrow, 1994–1995). The results suggested that mito- $K_{ATP}$  channels of glial cells might be another important target for the restorative effect of Ipt and Dia against rotenone toxicity. We also believed that mito- $K_{ATP}$  openers exert similar restorative effects on other neurons in the brain challenged by rotenone. However, the endogenous mechanism of how mito- $K_{ATP}$  channels regulate iNOS mRNA levels is unknown. Further work needs to be done to address whether selective iNOS inhibitors can exert protective effects against rotenone-induced neuronal injury in rats and decide the exact function of mito- $K_{ATP}$  channels in glial cells.

In this study, we demonstrate, for the first time, that rotenone-induced Parkinsonian symptoms (e.g. catalepsy) in rats are reversed by Ipt and Dia, and that mito- $K_{ATP}$  channel openers may therapeutically be used for early PD. The regulation of dopaminergic neurons apoptosis and glial iNOS activity might

be a neurorestorative target of mito- $K_{ATP}$  channel openers against rotenone toxicity.

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## Statement of Interest

None.

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