

Full-length article

Intracellular dopamine oxidation mediates rotenone-induced apoptosis in PC12 cells¹Hua-qing LIU², Xing-zu ZHU^{2,3}, En-qi WENG⁴²Department of Pharmacology, Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China; ⁴Department of Environmental Science and Technology, East China Normal University, Shanghai 200062, China**Key words**

Parkinson disease; rotenone; reserpine; deprenyl; reactive oxygen species; neurotoxicity

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Abstract

Aim: To study the role of dopamine (DA) in rotenone-induced neurotoxicity in PC12 cells. **Methods:** Cell viability was assessed by detecting the leakage of lactate dehydrogenase (LDH) into the medium. Apoptosis rate was measured by flow cytometry. Caspase-3-like activity was measured by fluorescence assay using the probe Ac-DEVD-AMC. The level of intracellular hydrogen peroxide and other peroxides in PC12 cells were quantified by loading cells with 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) in fluorescence assay. Lactic acid was measured spectrophotometrically. The DA levels in PC12 cells were determined by HPLC-ECD. **Results:** A 48-h incubation of PC12 cells with rotenone caused an apoptotic cell death and elevated intracellular reactive oxygen species (ROS) and lactic acid accumulation. Intracellular DA depletion with reserpine significantly attenuated rotenone-induced ROS accumulation and apoptotic cell death. No change was found in rotenone-induced ROS accumulation when cells were co-treated with deprenyl. Brief treatment with reserpine at the end of rotenone treatment had no effect on rotenone-induced neurotoxicity. However, when cells were first incubated with deprenyl, a monoamine oxidase-B inhibitor for 30 min then co-incubated with rotenone plus deprenyl, a brief treatment with reserpine enhanced cell injury. **Conclusion:** Rotenone-induced apoptosis in PC12 cells was mediated by intracellular dopamine oxidation.

Introduction

Parkinson disease (PD) is a chronic neurodegenerative disorder characterized by the loss of dopamine (DA) neurons in the substantia nigra, decreased striatal DA levels, and consequent extrapyramidal motor dysfunction. Although the genes responsible for a few rare familial cases of PD have been discovered^[1-3], the causes of more prevalent idiopathic PD are still unknown. Substances that are toxic to dopaminergic cells have been proposed as contributors to this neurological disease^[4,5]. MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is one of the best described neurotoxins. Evidence has shown that the neurotoxicity of MPTP depends on its active metabolite, 1-methyl-4-phenyl-pyridinium (MPP⁺), which inhibits mitochondrial complex I and depletes cellular ATP levels, resulting in cell death^[6]. Thus,

one hypothesis has focused on mitochondrial dysfunction^[7].

Rotenone, a naturally occurring, lipophilic compound from the roots of certain plants (*Derris* species), is a specific inhibitor of mitochondrial complex I and is used as the main component of many insecticides. Based on the mitochondrial dysfunction hypothesis of PD, a number of studies have evaluated the effects of rotenone on dopaminergic neurons both *in vitro* and *in vivo*^[8-15]. Treatment of mesencephalic cultures and striatal synaptosomes with rotenone caused neurotoxicity which was measured by a decreased uptake of neurotransmitters^[8,9]. Studies *in vivo* have shown that rotenone was capable of causing degeneration of dopaminergic neurons and induction of parkinsonian symptoms in animals^[10-15]. An important morphological finding in rotenone-treated rats was that the nigrostriatal DA neurons had accumulated fibrillar cytoplasmic inclusions containing

ubiquitin and alpha-synuclein, similar to Lewy bodies in idiopathic PD^[13,16]. This finding is interesting, because recent discoveries of the two causative gene products of familial PD, Parkin^[1,17,18] and alpha-synuclein^[3] indicate that failure of the ubiquitin-proteasome system might be common in both familial PD and idiopathic PD^[19]. Another reason is that rotenone is widely used as insecticide, and therefore is a real threat as an environmental substance to cause PD. Thus, elucidation of its mode of action is of high importance in understanding and potentially treating this disorder. The present study was designed to assess the neurotoxicity of rotenone on DA-producing PC12 cells and explore the possible mechanism.

Materials and methods

Reagents Dulbecco's modified Eagle's medium (DMEM) was purchased from GibcoBRL (Gaithersburg, MD, USA). Horse serum and fetal calf serum were obtained from Hyclone (Logan, UT, USA). Rotenone, reserpine, deprenyl, *N*-acetyl-*L*-cysteine (NAC), propidium iodide (PI), dihydroxybenzylamine (DHBA) and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma (St Louis, MO, USA). Caspase-3 substrate Ac-*L*-aspartic-*L*-glutamic-*L*-alanyl-*L*-aspartic acid-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was obtained from Calbiochem (La Jolla, CA, USA).

Cell culture The rat pheochromocytoma cell line PC12 cells (American Type Culture Collection, Rockville, MD, USA) were propagated in DMEM, supplemented with heat-inactivated horse serum (10%, v/v) and fetal calf serum (5%, v/v), 100 g/L streptomycin and 100 kU/L penicillin. The cultures were maintained in an incubator at 37 °C in a high humidity atmosphere of 5% CO₂. The medium was changed every 2 d and cells were passaged once a week. Twenty-four hours before addition of various reagents, the cells were seeded on 60-mm-dishes (Falcon), covered with collagen (Sigma), at a density of 1×10⁵/cm² in normal medium. Rotenone and reserpine were first dissolved in dimethyl sulfoxide (Me₂SO) and then diluted in medium to a final concentration of Me₂SO less than 0.1%. Controls for each drug condition consisted of sister cultures treated with the vehicle used to dissolve that drug. Stock solutions of deprenyl and NAC were prepared in Hanks' balanced salt solution containing 2 mmol/L HEPES.

Assessment of cell viability Cell viability was assessed by detecting the leakage of lactate dehydrogenase (LDH) into the medium. Cells were treated with rotenone or other reagents, then an aliquot of medium was taken and centri-

fuged at 250×g for 5 min. Supernatant (10 mL) was added into phosphate buffer 0.1 mol/L (pH 7.0) containing sodium pyruvate 2.3 mmol/L and NADH 5 mmol/L to a total volume of 200 mL. The decrease in absorbance over time at 340 nm was monitored at 25 °C. Then the activity of LDH in medium was calculated. Cell total LDH was obtained after exposure of culture to 0.2% TritonX-100 at 37 °C for 30 min. LDH leakage was expressed as percentage of LDH in medium to total LDH.

Analysis of apoptosis by flow cytometry Apoptosis rate was measured by flow cytometry as reported previously^[19]. Briefly, PC12 cells were washed with PBS (pH 7.4), fixed in cold 70% (v/v) ethanol, and incubated under -20 °C for at least 2 h. The fixed cells were harvested by centrifugation at 250×g for 5 min. The cell pellets were resuspended in 1 mL PBS at room temperature for 10 min. After another centrifugation, the cell pellets were resuspended in 500 mL PBS containing 0.2 g/L RNase A and incubated at 37 °C for 30 min. After incubation, the cells were stained with 20 g/L PI at 4 °C for 30 min. The fluorescence of cells was measured with FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). The relative content of DNA indicated the distribution of a population of cells throughout the cell cycle. Apoptotic cells caused the appearance of a sub-diploid peak in the cell-cycle profile. The percentage of apoptotic cells was determined by using BD CellQuest software.

Assay for caspase-3 activity Caspase-3-like activity was measured as described in a previous study with modification^[20]. In brief, the PC12 cells were collected and washed with PBS (pH 7.4). After centrifugation at 250×g for 5 min, cell pellets were lysed with NP-40 (0.5%)/HEPES (10 mmol/L) (pH 7.4), containing EDTA 2 mmol/L, PMSF 0.5 mmol/L and leupeptin 5 mg/L. The lysates were centrifuged at 7500×g for 10 min. The protein concentration in the supernatant was determined with Lowry method. Then 50 μg of protein was incubated with caspase-3 substrate Ac-DEVD-AMC 50 μmol/L. The increase of fluorescence was measured every 1 min in a 30 min period using a PolarStar plate reader (BMG labtechnologies, Australia) with an excitation wavelength of 380 nm and an emission wavelength of 450 nm. The enzyme activity was expressed as fluorescent units per min per mg protein.

Measurement of reactive oxygen species production The level of intracellular hydrogen peroxide and other peroxides in PC12 cells were quantified by loading cells with 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as described previously^[19]. In brief, the PC12 cells were washed with PBS (pH 7.4), resuspended in Krebs-Ringer buffer

(HEPES 20 mmol/L, dextrose 10 mmol/L, NaCl 127 mmol/L, CaCl₂ 1 mmol/L, KCl 5.5 mmol/L, MgSO₄ 2 mmol/L, pH 7.4), and then loaded with DCFH-DA 5 mmol/L for 60 min. The cells were again washed with Krebs-Ringer buffer to remove the extracellular dye and lysed in Tris-HCl 10 mmol/L (pH 7.4) containing 0.5% Triton X-100. The fluorescence in lysates was detected using a PolarStar plate reader (BMG labtechnologies, Australia) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The fluorescence intensity was normalized based on the protein concentration of individual extract. The ROS production was expressed as percentage compared with cells without treatment.

Determination of lactic acid accumulation Lactic acid accumulation was measured as described previously^[21]. In brief, PC12 cells were homogenized with sodium phosphate 50 mmol/L (pH 6.5) and boiled for 15 min. Lactic acid content was measured spectrophotometrically at 340 nm in a buffer containing LDH and hydrazine (0.4 mol/L)/glycine (0.5 mol/L) (pH 9.0). Lactic acid accumulation was normalized based on the protein concentration of individual extract, and expressed as percentage compared with cells without treatment. Protein concentration was detected by the Lowry method.

Measurement of dopamine in PC12 cells The DA levels in PC12 cells were determined by a modification of the methods described previously^[22-24]. In brief, cells were lysed in 1% metaphosphoric acid containing EDTA 1 mmol/L. Each sample added with 20 mL of DHBA was regarded as an internal standard. After centrifugation (17 500×g for 10 min at 4 °C), the supernatant was filtered, and a 20 mL aliquot was immediately injected into the HPLC system for DHBA and DA determination. The assay was done with a BAS PM80 pump, a Waters Nova-Pak C18 column, and an electrochemical detector (BAS LC-4C). The mobile phase was citric acid 0.1 mol/L, K₂HPO₄ 0.1 mol/L, EDTA 0.1 mmol/L, 5% methanol, and sodium octylsulfate 70 mg/L (pH 3.0); the flow rate was 1.0 mL/min. The potential of the electrode was set at +0.7 V. DA level in each sample was quantified by comparing DA and DHBA peak areas with those of standard solutions containing DA and DHBA, and then normalized based on the protein concentration of each individual sample. The DA level was expressed as percentage compared with control cells without reserpine treatment.

Statistics Data for cell viability, caspase-3 activity, ROS production and lactic acid accumulation were presented as mean±SD of four independent experiments. Data for dopamine levels in cells are means±SD of three experiments performed in triplicate. Statistical analysis was performed by

applying the Student's *t*-test and one-way ANOVA.

Results

Oxidative stress is involved in rotenone-induced neurotoxicity When PC12 cells were treated with 12.5 and 25 nmol/L rotenone for 48 h, LDH leakage increased from 7.8%±1.1% to 19.7%±1.1% and 30.1%±3.6% ($P<0.01$ vs control), respectively (Figure 1A). The rotenone-induced cell injury was associated with caspase-3 activation (Figure 1B), supporting the notion that an apoptotic cell death mechanism was involved in rotenone-induced neurotoxicity. Rotenone treatment induced a concentration-dependent increase of ROS levels within PC12 cells (Figure 1C). Rotenone treatment also induced an increase in lactic acid accumulation in PC12 cells (Figure 1D), suggesting that both oxidative stress and mitochondrial dysfunction were responsible for cell injury. The effects of rotenone were attenuated by 2.5 and 5 mmol/L NAC co-treatment (Figure 2A-2C), further supporting the notion that oxidative stress was involved in rotenone neurotoxicity. In addition, 2.5 and 5 mmol/L NAC also attenuated rotenone-induced increase in lactic acid accumulation (Figure 2D).

Rotenone-induced ROS accumulation is mediated by DA oxidation To test whether manipulation of DA levels would attenuate rotenone-induced cell death, PC12 cells were first depleted of intracellular DA using reserpine and then treated with rotenone. Reserpine (1 and 5 μmol/L) treatment for 3 h induced 66% and 87% depletions of DA respectively as compared with the control (Figure 3). The rotenone-induced elevation of LDH leakage and caspase-3 activation were attenuated in reserpine-pretreated PC12 cells (Figure 4A-4B), suggesting that DA played an important role in rotenone-induced cell injury. The rotenone-induced intracellular accumulation of ROS was also attenuated by this manipulation (Figure 4C). The results suggest that DA oxidation is involved in the production of ROS. In addition, lactic acid accumulation assay indicated that the rotenone-induced energy crisis was partially alleviated if PC12 cells were pretreated with reserpine (Figure 4D).

Analyzed quantitatively by flow cytometry, the apoptotic rates increased significantly in PC12 cells treated with rotenone (12.5 and 25 nmol/L) for 48 h (Figure 5A). The co-treatment with NAC 2.5 and 5 mmol/L or pre-treatment with reserpine 1 and 5 mmol/L for 3 h also decreased the rotenone-induced apoptotic rate (Figure 5B and 5C).

DA auto-oxidation is responsible for rotenone-induced neurotoxicity To test whether DA deamination or auto-oxidation was more responsible for rotenone-induced neuro-

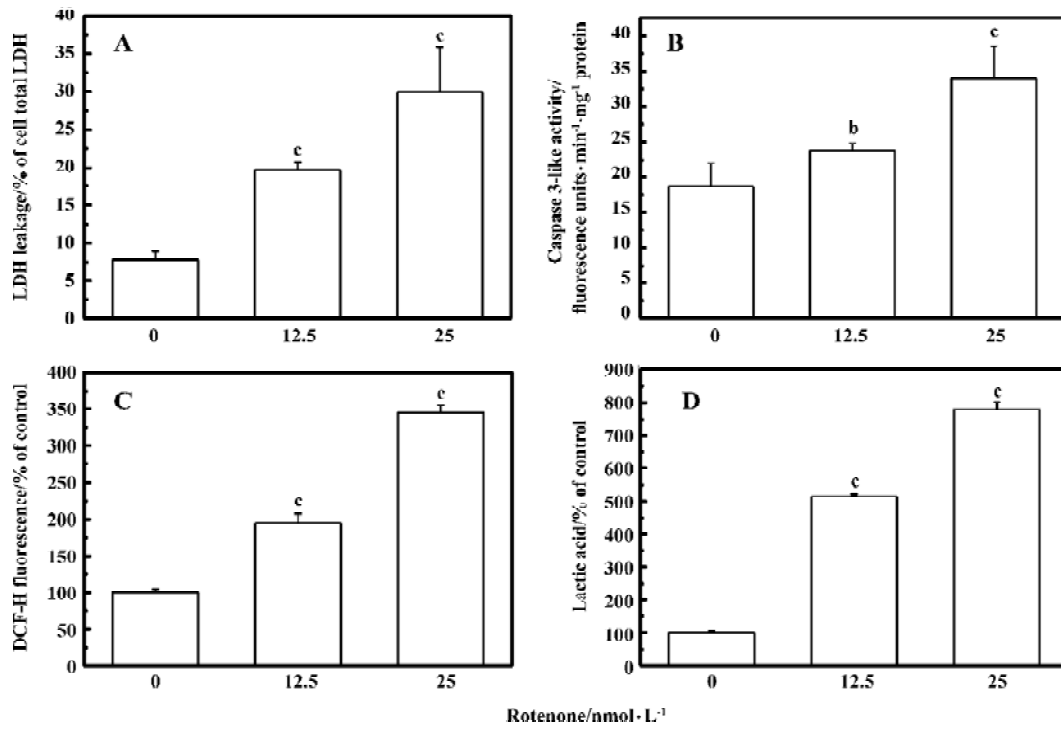


Figure 1. Effects of rotenone on PC12 cells. The leakage of LDH was expressed as percentage of LDH in medium to total LDH (A). Caspase-3-like activity was expressed as fluorescence units per min per mg protein (B). ROS production was expressed as percentage compared with control group (C). Lactic acid content expressed as percentage compared with control group (D). $n=4$. Mean±SD. ^b $P<0.05$, ^c $P<0.01$ vs control.

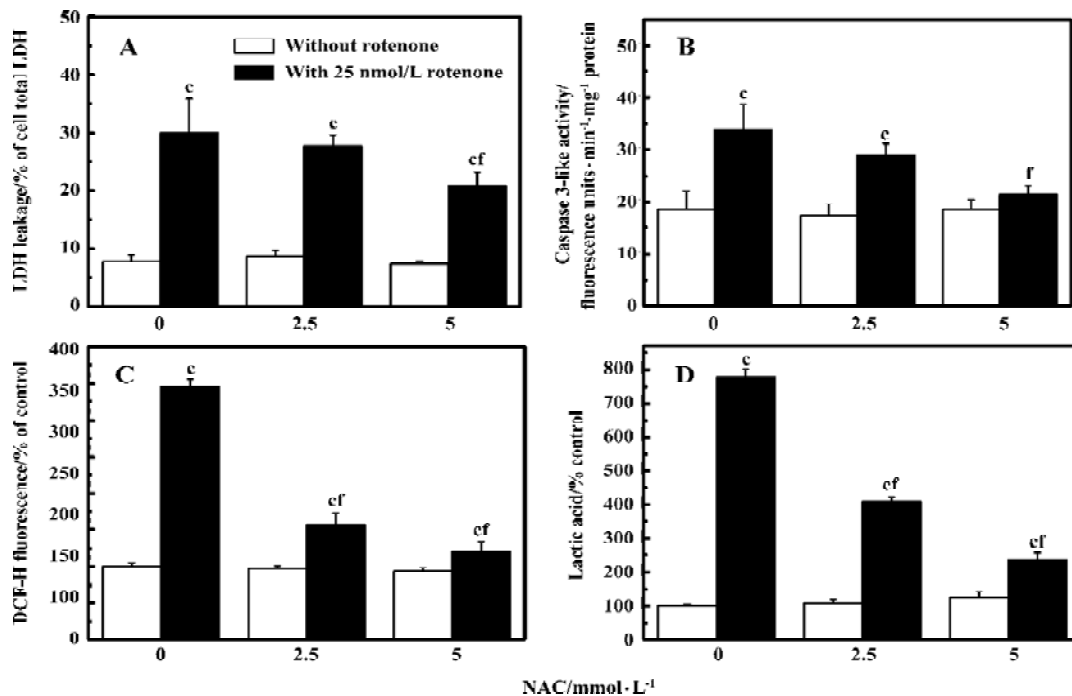


Figure 2. Effect of *N*-acetyl-*L*-cysteine on rotenone-induced PC12 cell injury. Cell viability (A), caspase-3-like activity (B), ROS production (C), and lactic acid accumulation (D) were assessed respectively as described in Figure 1. $n=4$. Mean±SD. ^c $P<0.01$ vs control. ^f $P<0.01$ vs 25 nmol/L rotenone treatment in the absence of NAC.

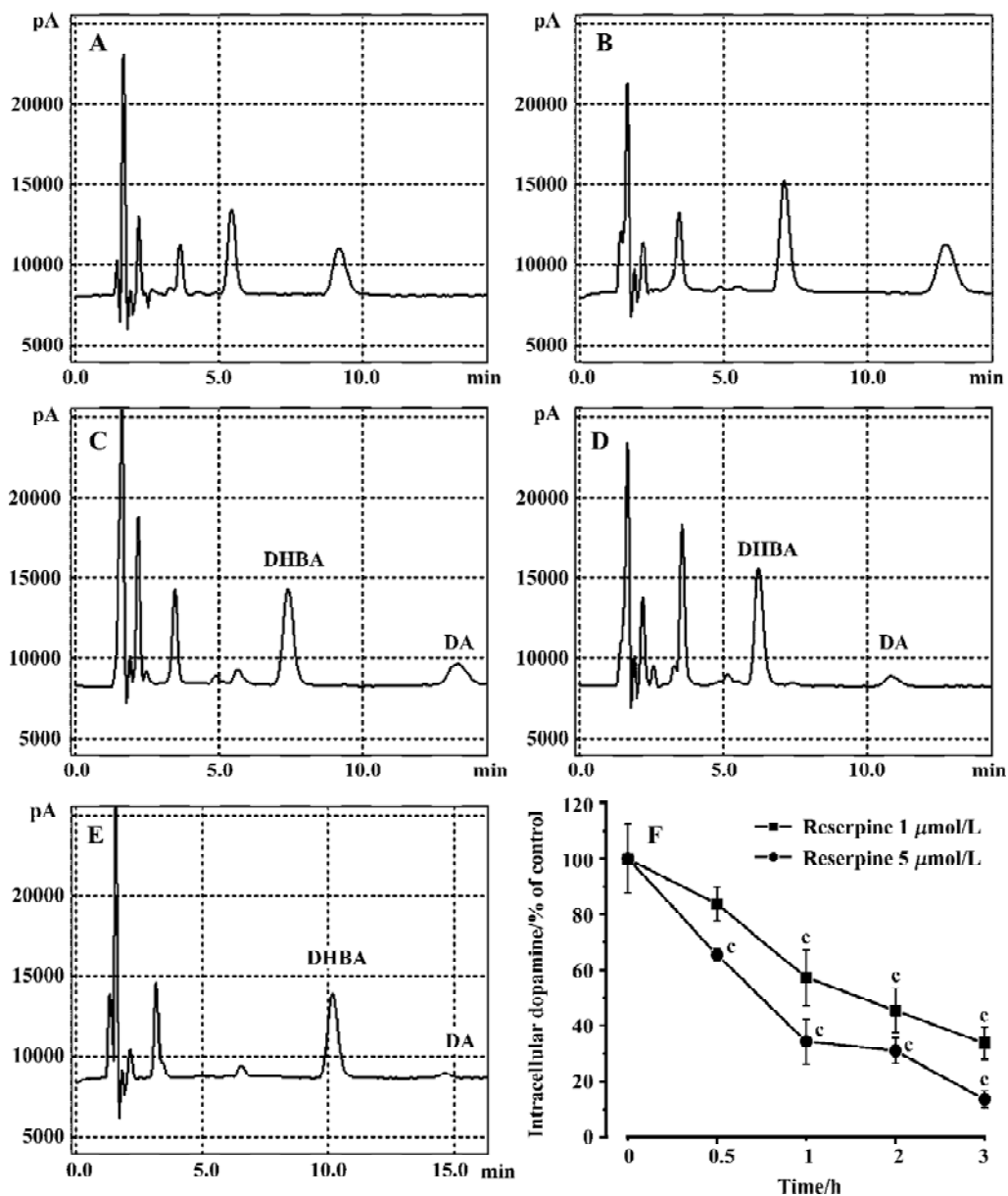


Figure 3. Reserpine-induced intracellular dopamine depletion in PC12 cells. Representative of HPLC-EC of lysate from control cells (A) and cells treated with reserpine 5 μmol/L for 30 min (B), 1 h (C), 2 h (D), and 3 h (E). (F): Mean±SD of three experiments performed in triplicate. **P*<0.01 vs control.

toxicity in PC12 cells, deprenyl, a monoamine oxidase-B (MAO-B) inhibitor, was used. The treatment with deprenyl at concentration between 25 to 200 μmol/L for 48 h had no significant effects on the LDH leakage in PC12 cells (Figure 6). When PC12 cells were first treated with 100 μmol/L deprenyl for 30 min and then co-treated with deprenyl 100 μmol/L plus rotenone 25 nmol/L for 48 h, no significant difference was found in the LDH leakage, caspase-3 activa-

tion or ROS production if compared with PC12 cells treated with rotenone 25 nmol/L alone (Table 1). The results suggest that the oxidative deamination of DA catalyzed by MAO-B was not a major source of ROS in the present studies.

Vesicular monoamine transporter is crucial for cell survival when there is mitochondrial dysfunction plus enhanced DA auto-oxidation To examine whether vesicular monoamine transporter (VMAT) was involved in roten-

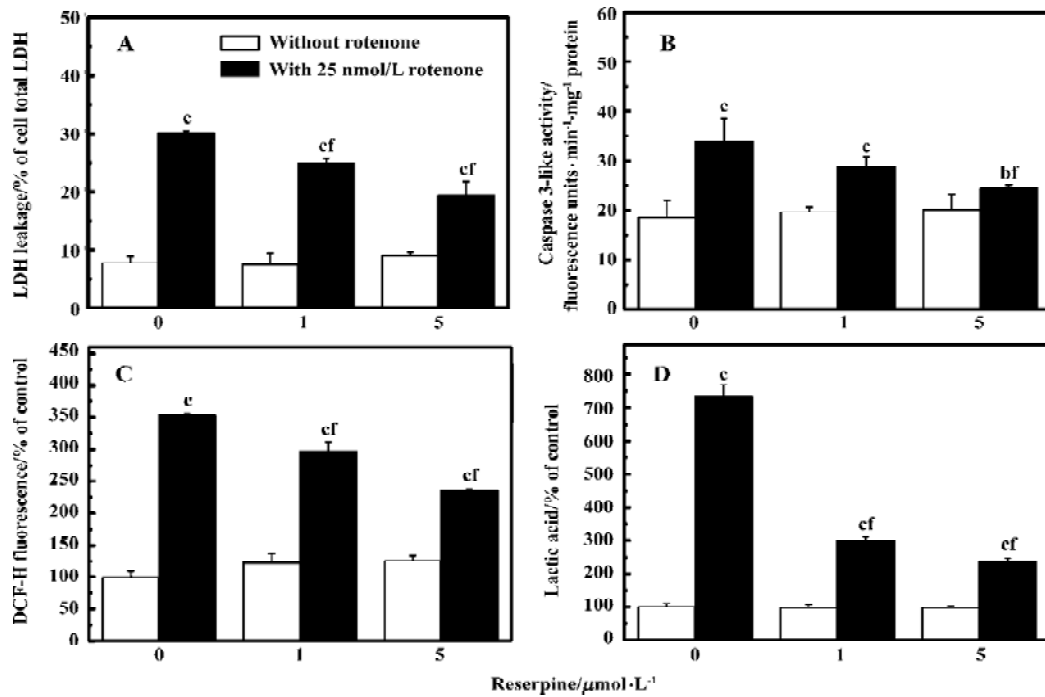


Figure 4. Effect of reserpine on rotenone-induced PC12 cell injury. LDH leakage (A), caspase-3-like activity (B), ROS production (C), and lactic acid accumulation (D) were measured respectively as described in Figure 1. $n=4$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control. ^f $P<0.01$ vs 25 nmol/L rotenone treatment in the absence of reserpine.

Table 1. Effects of brief reserpine treatment before the completion of 48 h deprenyl plus rotenone treatment on LDH leakage, caspase-3-like activity, and ROS production. Mean \pm SD from four independent experiments. ^c $P<0.01$ vs control. ^f $P<0.01$ vs 100 μ mol/L deprenyl plus 25 nmol/L rotenone treatment.

	LDH leakage/ % of cell total LDH	Caspase-3-like activity/ fluorescence unit \cdot min ⁻¹ \cdot mg ⁻¹ protein	DCF-H fluorescence/ % of control
Control	7.83 \pm 1.10	18.60 \pm 3.41	100.00 \pm 5.09
Rotenone (25 nmol/L) ¹	30.12 \pm 0.34 ^c	33.87 \pm 4.70 ^c	351.77 \pm 4.27 ^c
Reserpine (1 mmol/L) ²	7.54 \pm 2.01	19.56 \pm 1.08	122.78 \pm 13.98
Reserpine (5 mmol/L) ³	9.08 \pm 0.59	20.12 \pm 3.10	125.92 \pm 8.54
Rotenone (25 nmol/L)+Reserpine (1 mmol/L) ⁴	32.47 \pm 2.01 ^c	34.46 \pm 2.31 ^c	360.23 \pm 5.87 ^c
Rotenone (25 nmol/L)+Reserpine (5 mmol/L) ⁵	33.01 \pm 1.79 ^c	34.89 \pm 0.79 ^c	369.49 \pm 7.81 ^c
Deprenyl (100 μ mol/L)+Rotenone (25 nmol/L) ⁶	31.27 \pm 2.22 ^c	34.02 \pm 1.14 ^c	357.39 \pm 7.83 ^c
Deprenyl (100 μ mol/L)+Rotenone (25 nmol/L)+Reserpine (1 mmol/L) ⁷	44.68 \pm 3.49 ^f	38.78 \pm 2.02 ^f	378.45 \pm 2.08 ^f
Deprenyl (100 μ mol/L)+Rotenone (25 nmol/L)+Reserpine (5 mmol/L) ⁸	51.37 \pm 2.62 ^f	42.54 \pm 1.84 ^f	435.72 \pm 5.47 ^f

¹ PC12 cells were treated with 25 nmol/L rotenone for 48 h.

² PC12 cells were treated with 1 mmol/L reserpine for 30 min.

³ PC12 cells were treated with 5 mmol/L reserpine for 30 min.

⁴ PC12 cells were treated with 25 nmol/L rotenone for 48 h and then with 1 mmol/L reserpine before the end of 48 h rotenone treatment.

⁵ PC12 cells were treated with 25 nmol/L rotenone for 48 h and then with 5 mmol/L reserpine before the end of 48 h rotenone treatment.

⁶ PC12 cells were pretreated with 100 μ mol/L deprenyl for 30 min and then with 100 μ mol/L deprenyl plus 25 nmol/L rotenone for 48 h.

⁷ PC12 cells were pretreated with 100 μ mol/L deprenyl for 30 min and then with 100 μ mol/L deprenyl plus 25 nmol/L rotenone for 48 h. Reserpine (1 mmol/L) was added 30 min before the end of 48 h deprenyl plus rotenone treatment.

⁸ PC12 cells were pretreated with 100 μ mol/L deprenyl for 30 min and then with 100 μ mol/L deprenyl plus 25 nmol/L rotenone for 48 h. Reserpine (5 mmol/L) was added 30 min before the end of 48 h deprenyl plus rotenone treatment.

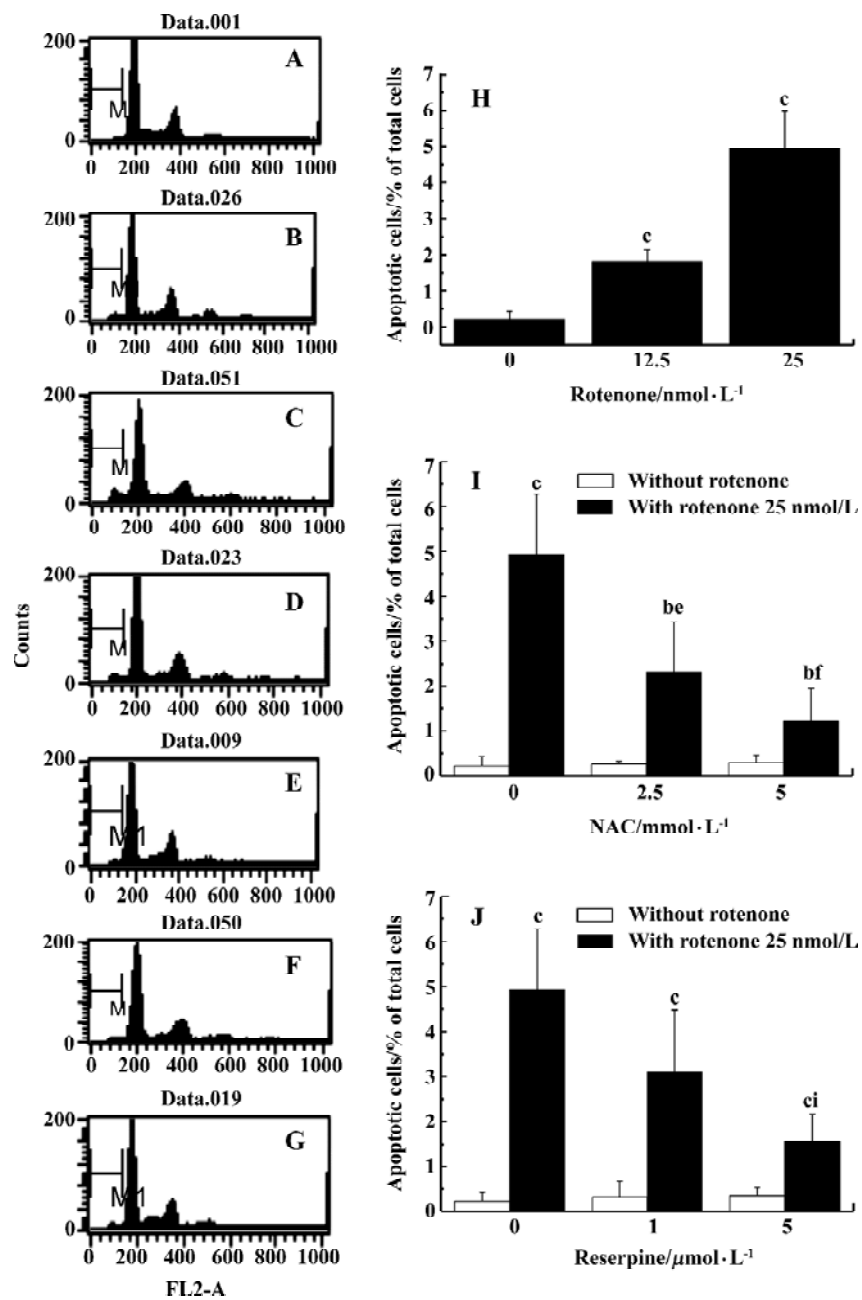


Figure 5. Rotenone-induced increased apoptotic rate in PC12 cells. Representative of flow cytometry of control cells (A), and cells treated with 12.5 nmol/L rotenone (B) or 25 nmol/L rotenone for 48 h (C). (D): Representative of flow cytometry of PC12 cells were incubated with 25 nmol/L rotenone plus 2.5 mmol/L NAC, or 25 nmol/L rotenone plus 5 mmol/L NAC (E); cells were treated with 1 mmol/L reserpine for 3 h and then with 25 nmol/L rotenone for 48 h (F), or 5 mmol/L reserpine for 3 h and then with 25 nmol/L rotenone for 48 h (G). Mean±SD of four independent experiments for each treatment group. ^b*P*<0.05, ^c*P*<0.01 vs control. ^e*P*<0.05, ^f*P*<0.01 vs 25 nmol/L rotenone treatment in the absence of NAC. ⁱ*P*<0.01 vs 25 nmol/L rotenone treatment in the absence of reserpine.

one-induced neurotoxicity in PC12 cells, 1 or 5 μmol/L reserpine was added 30 min before the end of 48 h-rotenone treatment. No change was found in rotenone-induced neurotoxicity with the brief reserpine treatment. However, when PC12 cells were pretreated with deprenyl 100 μmol/L for 30

min and then with deprenyl 100 μmol/L plus rotenone 25 nmol/L for 48 h, the 30-min incubation with reserpine 1 or 5 μmol/L before the end of 48-h treatment with deprenyl plus rotenone induced significant increases in LDH leakage, caspase-3 activation, and ROS (Table 1).

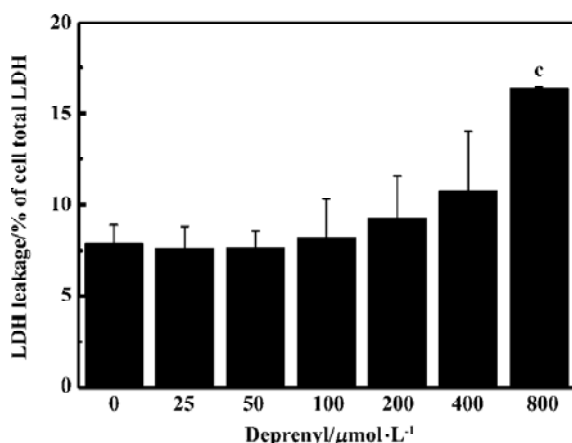


Figure 6. Effects of deprenyl on PC12 cells. Mean \pm SD values of four independent experiments for each treatment group. ^c $P < 0.01$ vs control.

Discussion

In the present study, the mechanisms of rotenone-induced neurotoxicity in DA-producing PC12 cells was investigated. We found that rotenone induced an apoptotic type of cell death. This result is similar to the results reported by Bal-Price and Brown^[25]. They found that a 24-h incubation of PC12 cells with nitric oxide donors or rotenone, in the presence of glucose, induced apoptosis of PC12 cells as determined by nuclear morphology and caspase-3 activation. We found that rotenone induced apoptosis in PC12 cells accompanied with an elevated ROS production. Given that mitochondria is a major cellular source of ROS^[26] and that rotenone is a kind of complex I inhibitor, it could be hypothesized that rotenone-induced ROS might be originated from mitochondria. Indeed, high concentrations of rotenone have been reported to induce superoxide production *in vitro* and *in vivo*^[27-30]. In isolated nerve terminals, Sipos *et al*^[31] found that inactivation of complex I to a small extent (16%) resulted in a significant increase in ROS formation. However, other studies have suggested converse results^[32-34]. Lotharius and O'Malley^[35] found that low doses (5-50 nmol/L) of rotenone killed dopaminergic neurons with a similar time course and morphology to MPP⁺. However, no increase was found in intracellular superoxide levels after the dopaminergic neurons were treated with rotenone for 0.5, 1, 3, and 6 h. Because the classic complex I inhibitor rotenone did not induce ROS, they concluded that MPP⁺-induced superoxide did not arise from blockade of electron transport. In the present study, however, we found a significant increase of ROS production from PC12 cells after rotenone treatment for 48 h. While DCFH-DA was used as a probe to measure the redox state of a cell, the results were validated carefully.

We found that the rotenone-induced ROS accumulation and cell death were attenuated by an antioxidant, NAC. This result supports the notion that an oxidative stress mechanism is involved in rotenone-induced neurotoxicity in PC12 cells.

If toxic agents were involved in the pathogenesis of PD, this should likely reflect a multifactorial etiology in which the effects of environmental insults are compounded by predisposing genetic traits, age-related changes and interactions with endogenous elements (i.e. factors inherent to the nigrostriatal tissue). The presence of dopamine within nigrostriatal neurons may itself constitute a risk factor that enhances their vulnerability to toxic events, such as increased production of oxidizing species. To test whether manipulation of DA levels would attenuate rotenone-induced cell death, cells were depleted of intracellular DA using reserpine prior to rotenone treatment. In the present studies, we demonstrated that reserpine induced a concentration- and time-dependent DA depletion in PC12 cells. When PC12 cells were treated with 1 and 5 mmol/L reserpine for 3 h, 66% and 87% decreases in DA levels were found respectively, as compared with the control (Figure 3). Our results were similar to those reported by Brautigam *et al*^[36] who reported more than 50% depletion of intracellular DA in PC12 cells after reserpine incubation. Our finding that DA depletion significantly attenuated rotenone-induced ROS production and cell death suggests that DA plays an important role in rotenone-induced ROS production and cell death. Since DA in the cytoplasm can be readily autoxidized^[37] or deaminated^[38] by MAO-B to produce ROS^[39], deprenyl, a MAO-B inhibitor, was used to test whether MAO-B inhibition could protect PC12 cells from rotenone-induced ROS accumulation and cell death. Although it has been shown that MAO-B inhibition by pretreatment with deprenyl significantly reduces cellular DOPAC formation in PC12 cells^[40], it had no effect on the rotenone-induced ROS accumulation and cell death in our studies, suggesting that the oxidative deamination of DA catalyzed by MAO-B was not the main source of ROS in rotenone-treated PC12 cells and that DA auto-oxidation is responsible for rotenone-induced oxidative stress.

The presence of dopamine within nigrostriatal neurons can itself constitute a risk factor that enhances their vulnerability to toxic agents. For example, rotenone-induced dendrite loss was severe in the substantia nigra, whereas noncatecholamine neurons, such as those in the perifornical nucleus, were more resistant^[41]. Other factors might also play roles in the vulnerability to toxic agents since some dopamine neurons in hypothalamic A11 and the ventral tegmental area were spared when treated with rotenone. By *in*

situ hybridization, a weaker expression of VMAT was found in the rat substantia nigra than in the ventral tegmental area^[42]. If VMAT effectively protects the cell against oxidative stress induced by dopamine, the weak expression of VMAT in substantia nigra might account for its particular vulnerability to toxic agents.

No change was found in the rotenone-induced neurotoxicity. However, when PC12 cells were pretreated with deprenyl and then with deprenyl plus rotenone for 48 h, significant increases in LDH leakage, caspase-3 activation, and ROS production were found. The results suggest that VMAT activity might be important for cell survival if there is mitochondrial dysfunction. In other words, disruption in dopamine disposition and/or metabolism could underlie the progressive degeneration of dopaminergic neurons in PD.

As a specific inhibitor of mitochondrial complex I, the metabolic consequence of mitochondrial electron flow blockage induced by rotenone would be the incomplete oxidation of glucose and consequent lactic acid accumulation. Indeed, we showed that rotenone treatment also induced an increase in lactic-acid accumulation. The results support the notion that mitochondrial dysfunction is responsible for rotenone-induced cell death in PC12 cells. Mitochondria in PC12 cells can become more vulnerable to hydrogen peroxide-induced oxidative stress when complex I is inhibited.

Taken together, our results demonstrated that both oxidative stress and mitochondrial dysfunction were responsible for rotenone-induced apoptotic cell death in PC12 cells, and that rotenone-induced ROS accumulation was mainly DA auto-oxidation mediated.

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