

Original Article

Dopamine Release Suppression Dependent on an Increase of Intracellular Ca^{2+} Contributed to Rotenone-induced Neurotoxicity in PC12 Cells

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Abstract: Rotenone is an inhibitor of mitochondrial complex I that produces a model of Parkinson's disease (PD), in which neurons undergo dopamine release dysfunction and other features. In neurons, exocytosis is one of the processes associated with dopamine release and is dependent on Ca^{2+} dynamic changes of the cell. In the present study, we have investigated the exocytosis of dopamine and the involvement of Ca^{2+} in dopamine release in PC12 cells administrated with rotenone. Results demonstrated that rotenone led to an elevation of intracellular Ca^{2+} through Ca^{2+} influx by opening of the voltage-gated Ca^{2+} channel and influenced the soluble N-ethylmaleimide attachment protein receptor (SNARE) proteins expression (including syntaxin, vesicle-associated membrane protein 2 (VAMP₂) and synaptosome-associated protein 25 (SNAP-25)); pretreatment with a blocker of L-type voltage-activated Ca^{2+} channels (nifedipine) decreased the intracellular dopamine levels and ROS formation, increased the cell viability and enhanced the neurite outgrowth and exocytosis of synaptic vesicles. These results indicated that the involvement of intracellular Ca^{2+} was one of the factors resulting in suppression of dopamine release suppression in PC12 cells intoxicated with rotenone, which was associated with the rotenone-induced dopamine neurotoxicity. (DOI: 10.1293/tox.26.149; J Toxicol Pathol 2013; 26: 149–157)

Key words : rotenone, PC12 cells, Ca^{2+} , dopamine release, neurotoxicity

Introduction

Rotenone, a pesticide derived from the roots of plants of the Leguminosae family, is a specific inhibitor of mitochondrial complex I¹. It is a popular botanical pesticide that is harmless to plants, but toxic to insects, fish, and mammals^{2, 3}. In a rat model, rotenone has been shown to produce effects that closely resemble Parkinson's disease (PD)¹. Many epidemiological surveys have also revealed that rotenone is relevant to the pathogenesis of PD⁴. These observations have led to the development of a rodent model of PD based on chronic exposure to the mitochondrial complex I inhibitor, rotenone¹.

It has been shown that *in vitro* and *in vivo* treatment with rotenone decreased dopamine levels in the striatum, and inhibited dopamine uptake, thus reproducing the neurochemical features of PD⁵. It has also been demonstrated that rotenone has a supra-additive impact on the release of

monoamine transmitters⁵. In previous studies, we also have reported that rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism⁶. These conclusions urge us to explore whether the alteration of dopamine metabolism in neurons is the result of dopamine release dysfunction induced by rotenone. It is well known that exocytosis and endocytosis play important roles in dopamine release in neurons, which is associated closely with Ca^{2+} ion participation⁷. Therefore, in the present study, we investigated whether the dopamine release in neurons was influenced by rotenone, its association with intracellular free Ca^{2+} [Ca^{2+}]_i and its contribution to the neurotoxicity of PC12 cells. It is also noteworthy that the studies of the action of rotenone on [Ca^{2+}]_i and neurotransmitter release of PC12 cells may also contribute to understanding the etiology of the neurodegenerative mechanisms in PD.

Material and Methods

Materials

PC12 cells were obtained from Shanghai Cell Bank, Chinese Academy of Sciences. Rotenone, Fura-2/AM, FM1-43, nifedipine, and 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies of syntaxin, vesicle-associated

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membrane protein 2 (VAMP₂) and synaptosomal-associated protein 25 (SNAP-25) were purchased from Abcam (Santa Cruz, CA, USA). A Cell counting kit was purchased from DOJINDO LABORATORIES (Japan). A BCA Protein Assay Kit was purchased from Beyotime(China). An ECL⁺ detection system was purchased from Beyotime (China). All other reagents used were of the highest grade available.

Cell culture and treatment

The PC12 cell line was cultured at 37°C under an atmosphere of 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% horse serum and 5% fetal bovine serum (complete media). The culture medium was replenished at 3 to 4-day intervals based on the doubling time of PC12 cells. Cells were treated with the indicated concentration of rotenone dissolved in DMSO for 24 h. The control group was administrated with equal amounts of DMSO. The nifedipine pretreatment group was administrated with nifedipine (0.5 μM) for 30 minutes prior to rotenone intoxication.

Measurement of intracellular Ca²⁺ levels with fura-2/AM fluorescence

Fura-2/AM is a specific indicator of intracellular calcium fluorescence. It can be hydrolyzed into Fura-2 by intracellular esterase. Fura-2 can be combined with cytosolic free Ca²⁺ reversibly with fluorescence emission. The binding ratio is 1:1. The maximum excitation wavelengths shift from 380 nm, before its combination with Ca²⁺, to 340 nm. There is a quantitative relationship between the emission fluorescence intensity and binding Ca²⁺ concentration. Therefore, the concentration of calcium ions is indicated by the fluorescence ratio excitation at 340 nm and 380 nm. In this experiment, we investigated how the intracellular Ca²⁺ levels were affected in PC12 cells treated with rotenone. Intracellular Ca²⁺ contents of PC12 cells were assayed with Fura-2/AM fluorescent dye. The levels of intracellular Ca²⁺ were reflected by the intensity of the Fura-2/AM fluorescent probe under a fluorescence microscope. Digital calcium imaging was performed as follows. A kinetic study of rise in intracellular Ca²⁺ was conducted in which PC12 cells were loaded with 2 μg/ml Fura-2/AM dye in the presence of pluronic acid at 37°C for 40 min, then washed free of extracellular Fura-2/AM dye and resuspended in Krebs-Ringer-HEPES (KRH) buffer (131 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 6 mM glucose, 20 mM HEPES, pH 7.4). The loading was terminated by washing with Hanks' Balanced Salt Solution for 25 min before starting the experiment. Treatment with rotenone was initiated 1 min later, and the changes in fluorescence were recorded for 18 min. PC12 cells were intoxicated with rotenone for 24 h with or without nifedipine, and then the cells were harvested. PC12 cells were loaded with Fura-2/AM dye and treated as above, and the intracellular Ca²⁺ was observed.

CCK-8 assay to evaluate PC12 cell viability

Cell Counting Kit-8 (CCK-8) was used to assess PC12 cell viability in accordance with the manufacturer's instructions. PC12 cells were maintained at 30%–70% confluence (exponential growth phase) and then were inoculated into 96-well plates at 1000 cells/well. PC12 cells were subjected to control group, rotenone (1.0 μM) treatment group and nifedipine pretreatment group respectively. They were then incubated in a carbon dioxide cell incubator (37°C, 5% CO₂) for 24 h. At the indicated times, CCK-8 solutions (10 μl) were added to each well, and incubation was performed for 2.5 h; optical density was then assayed with microplate reader at 450 nm. All results were normalized to optical density values measured from an identically conditioned well without medium.

Uptake of FM1-43 into PC12 cells

To examine the recycling of synaptic vesicles in PC12 cells treated with rotenone, FM1-43, a fluorescent dye, was introduced into the extracellular fluid. FM1-43, a lipophilic dye that has been shown to reflect neurosecretory changes through plasma membrane thickness due to vesicular exocytosis and endocytosis⁸, was used as a probe to understand the mechanism of neurotransmitter release in PC12 cells. PC12 cells were treated with 1 mM rotenone and solvent respectively for 24 h, and then were loaded with FM1-43 (10 mM) in a high K⁺ solution (50 mM) for 3 min. FM1-43 is "captured" in newly formed synaptic vesicles during the process of endocytosis, and the intensity of FM1-43 labeling is proportional to the amount of endocytosis and inversely proportional to the amount of exocytosis. After washing with phosphate buffered saline (PBS) for 30 min, the cells were examined. A band-pass filter (450–490 nm) was placed in the excitation path, and a dichroic mirror (510 nm) and a cut-off filter (520 nm) were placed in the emission path. Images were acquired and analyzed using a confocal scanning laser microscope, and its software.

Western immunoblotting analysis

After rotenone treatments, PC12 cells were harvested with trypsin, washed twice with cold phosphate-buffered saline (PBS) and resuspended in ice-cold RIPA lysis buffer. After 15 min on ice, the samples were centrifuged at 4°C for 15 min at 12,000 g. The supernatants were collected and stored at –80°C until analysis. Each sample containing 20 μg proteins was resolved in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and electroblotted for 40 minutes at 15 V onto a PVDF membrane. Membranes were blocked for 1 h at room temperature using 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and incubated for 24 h at 4°C with a monoclonal mouse anti-SNAP-25 antibody (1:750), vesicle-associated membrane protein 2 (VAMP₂) antibody (1:1000) and syntaxin antibody (1:800), respectively, in blocking solution. The membranes were rinsed for 5 min at room temperature in TBST 3 times, incubated for 1 h with a peroxidase-conjugated goat anti-mouse IgG 1:1000 in blocking solution, and again rinsed

with TBST. After a brief wash in TBST, bound antibodies were revealed by chemiluminescence with an ECL⁺ detection system. The membranes were further stripped of bound antibodies as recommended in the manufacturer's instructions, reblocked and probed with a monoclonal mouse anti-GAPDH 1:1000 as a protein loading control. The electrochemiluminescence stainings were quantified by densitometry with a VisioLab 2000 image analyzer. Values obtained for protein were normalized with their corresponding GAPDH values.

HPLC determination of dopamine contents in PC12 cells

PC12 cells cultured for 3–4 days and maintained at 30%–70% confluence (exponential growth phase), were divided into groups and were exposed to different treatment factors. At the indicated time, the cells in the flask were washed with PBS, harvested and mixed with 120 L ice-chilled perchloric acid (0.2 M) containing 0.5 mM EDTA and 0.05 mg/ml dihydroxybenzylamine (DHBA). Then the solution was ultrasonicated and centrifuged at 7000 g for 20 min at 4°C. The samples were collected and stored immediately at 80°C until analyzed.

Levels of dopamine were measured by HPLC coupled with a fluorescence detection system. Samples were eluted through a 150x4.6-mm ODS C18 column at a rate of 1.0 ml/min with a mobile phase consisting of 0.1 M Na₂HPO₄, 0.8 mM octyl sulfonic acid (OSA), 0.5 mM EDTA and 10% (v/v) methanol and adjusted to pH 3.63 with phosphoric acid. The ODS C18 column was kept at room temperature. The fluorescence detector was set with excitation at 254 nm and emission at 338 nm. The working standard solution was prepared in 0.2 M perchloric acid containing 0.5 mM EDTA and 0.05 mg/ml DHBA. The dopamine concentrations were determined with an external standard calibration curve. The concentrations of dopamine were expressed as micrograms in per gram of protein. The BCA Protein Assay kit was used to determine the protein concentration of the samples.

Intracellular ROS measurement

Intracellular reactive oxygen species (ROS) were analyzed by using dichlorofluorescein diacetate (DCF-DA) as a probe. DCF-DA, a nonfluorescent compound, is deacetylated by viable cells to 2,7-dichlorofluorescein (DCF) by hydrogen peroxide. First, PC12 cells were treated with the designated experimental conditions. Then PC12 cells were collected and incubated with 100 mM DCF-DA (dissolved in DMSO) for 30 min at 37°C. Cells were then washed three times with PBS (pH 7.4) and the intracellular accumulation of fluorescent DCF-DA was measured by a Cyto-Fluor Multi-Well Plate Reader with excitation at 485 nm and emission at 530 nm, respectively. The results were expressed as the intensity of DCF fluorescence.

Observation of neurite outgrowth of PC12 cells

Cells were cultured on cover slips. Following treatment, the cells were washed in ice-cold Krebs-Ringer buf-

fer (KRB) and fixed with 10% paraformaldehyde. The cover slips were mounted with Mowiol and allowed to dry before viewing under phase-contrast conditions with a Zeiss Axiovert S100 microscope. From each slide, at least 100 cells from five randomly selected fields were counted. Measurements were carried out using the NIH/Scion image software.

Statistical analysis

All values in the experiments were expressed as $\bar{x} \pm s$. Statistical analyses were carried out using the SPSS 10.0 software. The data were evaluated by one-way analysis of the variance (ANOVA). $P < 0.05$ and $P < 0.01$ were taken as levels of statistical significance.

Results

The intracellular Ca²⁺ in PC12 cells

A concentration-dependent increase in the intracellular Ca²⁺ levels was displayed in PC12 cells exposed to different concentrations of rotenone under digital calcium imaging. Experiments revealed that the increase in intracellular Ca²⁺ developed faster at higher concentrations of rotenone: 1.0 μM rotenone caused an elevation of [Ca²⁺]_i within ~3 min in the majority of exposed cells. The averaged time course of changes in intracellular Ca²⁺ in all cells within the field of view indicated that the rotenone-inducing increase in intracellular Ca²⁺ occurred with an ~3 min delay and resulted in a substantial elevation of intracellular Ca²⁺ by the end of the recording period, 18 min (Fig. 1B, C, D). However, the intracellular Ca²⁺ was stable throughout the recording period in PC12 cells of the control group (Fig. 1A). In addition, intracellular Ca²⁺ was also increased in PC12 cells after rotenone treatment for 24 h, maintaining a relative stable level (Fig. 1G). However, intracellular Ca²⁺ was reduced in PC12 cells exposed to rotenone with nifedipine pretreatment (Fig. 1H).

Factors affecting rotenone-inducing Ca²⁺ influx in PC12 cells

Pretreatment with the blocker of L-type voltage-activated Ca²⁺ channels (nifedipine) resulted in suppression of the rotenone-inducing intracellular Ca²⁺ increase in the PC12 cells to a certain extent (Fig. 1E). Another experiment showed that in the absence of extracellular Ca²⁺, there was no increase in intracellular Ca²⁺ during exposure of PC12 cells to rotenone. Reintroduction of extracellular Ca²⁺ with CaCl₂ immediately after cell exposure to Ca²⁺-free solution, resulted in an equally fast recovery of increase in intracellular Ca²⁺. Moreover, when extracellular Ca²⁺ was abolished using the chelator (EGTA) of Ca²⁺ during the rising phase of the rotenone-inducing intracellular Ca²⁺ increase, the intracellular Ca²⁺ declined to almost its initial level within the next few minutes (Fig. 1F).

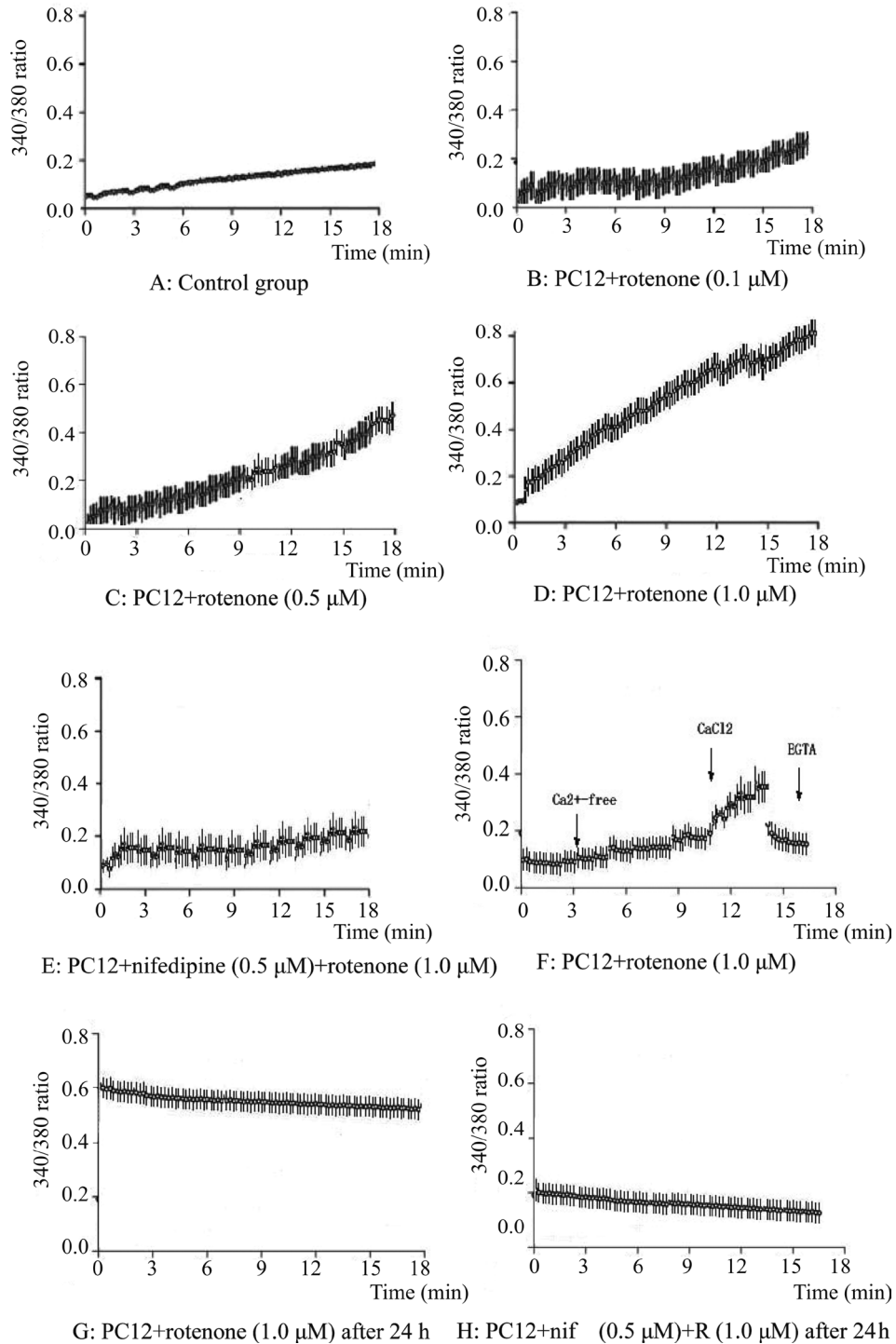


Fig. 1. Intracellular Ca²⁺ levels were determined with Fura-2/AM fluorescent dye. A: The intracellular Ca²⁺ of PC12 cells in the control group was stable during the observation time. B, C, D: Intracellular Ca²⁺ of PC12 cells was increased by treatment with rotenone (0.1 μM, 0.5 μM, 1.0 μM) in a concentration dependent manner. E: Effects of nifedipine on the increase in intracellular Ca²⁺ of PC12 cells intoxicated with rotenone (1.0 mM). PC12 cells were pretreated with nifedipine (0.5 μM) followed by rotenone administration. The enhancement of intracellular Ca²⁺ induced by rotenone was retarded in PC12 cells with nifedipine pretreatment. F: Effects of Ca²⁺ concentration in cell medium (Ca²⁺ free, CaCl₂ or EGTA) on the increase in intracellular Ca²⁺ in PC12 cells intoxicated with rotenone (1.0 μM). Rotenone was added first without Ca²⁺, followed with CaCl₂ (1 mM) added, and then the Ca²⁺ chelator EGTA (2 mM) was added to the medium. First, intracellular Ca²⁺ showed no obvious changes in PC12 cells treated with rotenone in the medium without intracellular Ca²⁺, this was followed by a swift rise in intracellular Ca²⁺ with Ca²⁺ addition, and then a quick drop in intracellular Ca²⁺ in the PC12 cells when EGTA was added. G: A stable increase in intracellular Ca²⁺ was induced in PC12 cells after rotenone treatment for 24 hours. H: With nifedipine pretreatment, a decrease in intracellular Ca²⁺ was observed in PC12 cells exposed to rotenone (1.0 μM) for 24 hours.

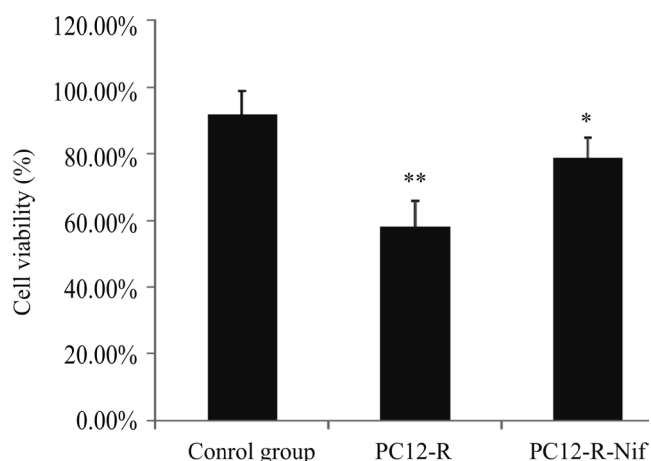


Fig. 2. PC12 cell viability was observed with CCK-8. Rotenone treatment (1.0 μM) decreased the PC12 cell viability obviously compared with that in the control group. However, nifedipine (0.5 μM) pretreatment significantly improved the viability of PC12 cells exposed to rotenone alone. Data are expressed as $\bar{x} \pm s$, * $P < 0.05$ vs PC12+rotenone group (1.0 μM); ** $P < 0.01$ vs control group.

The viability of PC12 cells

The viability of PC12 cells treated with rotenone was decreased significantly compared with that in the control group ($P < 0.01$). However, the viability of PC12 cells pretreated with nifedipine was increased and significantly different compared with that in the group treated with rotenone alone (Fig. 2).

Synaptic vesicle recycling in PC12 cells

FM1-43 labeling occurred in both the PC12 cells of the control group and the experimental groups following with high K^+ stimulation. PC12 cells of the control group were extensively labeled along the contours of the cell body, mainly with dot-like distribution (Fig. 3A). On the other hand, the PC12 cells treated with rotenone were labeled more intensely, mainly clustering around the nucleus, and the particle density was obviously increased (Fig. 3B). The intensity of the fluorescence of the spots was measured and calculated. The fluorescence intensity was greater in PC12 cells treated with rotenone (1.0 μM) compared with that in the control group (Fig. 3D). The fluorescence intensity represented the numbers of vesicles. Therefore, the number of vesicles in the cytoplasm labeled with FM1-43 was increased in PC12 cells induced by rotenone in comparison with PC12 cells of the control group (Fig. 3D).

Because the depolarization-inducing synaptic vesicle recycling is dependent on the influx of extracellular Ca^{2+} , the Ca^{2+} dependency of the suppression of synaptic vesicles release induced by rotenone was tested in the stimulation medium pretreated with nifedipine. As shown in Fig. 3D, the fluorescence intensity of FM1-43 labeling was weakened in PC12 cells pretreated with nifedipine compared with that of PC12 cells treated with rotenone alone, which indicated

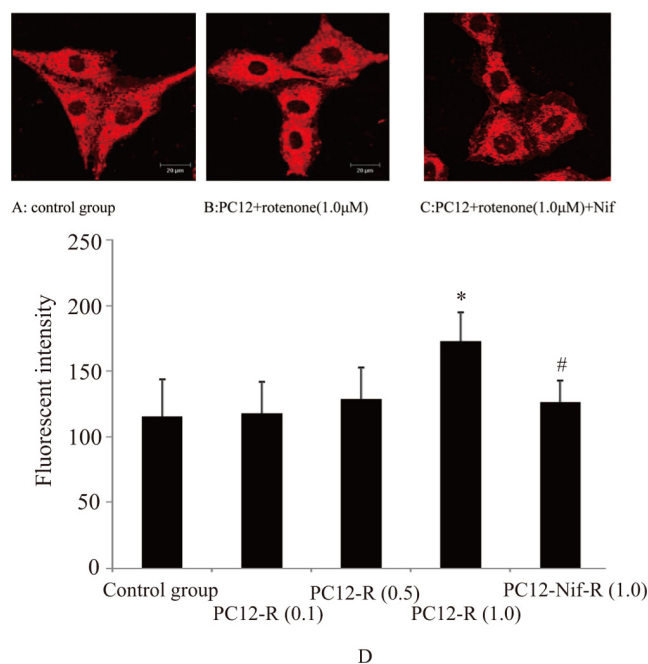


Fig. 3. Synaptic vesicle recycling observed with FM1-43 in PC12 cells. The results showed that the FM1-43 fluorescent dye was diffused along the PC12 cell body in the control group (A); however it preferentially concentrated around the nucleus of rotenone-treated PC12 cells (B). Nifedipine pretreatment retarded these changes as shown in C. The fluorescence intensity of synaptic vesicles labeled with FM1-43 was calculated. The results showed that the distribution of the fluorescence intensity was greater in PC12 cells treated with rotenone (1.0 μM) compared with that in the control group; however, the fluorescence intensity was lower in PC12 cells pretreated with nifedipine in comparison with that in PC12 cells treated with rotenone alone (1.0 μM) (D). Data are expressed as $\bar{x} \pm s$, * $P \leq 0.01$ vs control group; # $P < 0.01$ vs rotenone (1.0 μM) group.

that the numbers of synaptic vesicles in the cytoplasm decreased in PC12 cells treated with nifedipine. These synaptic vesicles were recycled in a Ca^{2+} -dependent manner, and nifedipine pretreatment could slow down the increase in synaptic vesicles in PC12 cells induced by rotenone.

Soluble N-ethylmaleimide attachment protein receptor complex (SNARE) protein expression in PC12 cells

The neuronal SNARE formed by synaptobrevin (also known as VAMP_2), syntaxin and SNAP-25 plays a central role in Ca^{2+} -triggered neurotransmitter release. The effect of rotenone on SNARE protein expression was studied by Western immunoblotting analysis in PC12 cells. The VAMP_2 protein expression level was increased significantly in PC12 cells treated with 1.0 μM rotenone compared with that of the control group (Fig. 4A-1, 2). SNAP-25 protein was detected in the control cells, and its expression level was decreased with rotenone treatment (Fig. 4B-1, 2). Under the same experimental conditions, syntaxin protein was also detected in PC12 cells and decreased markedly in the

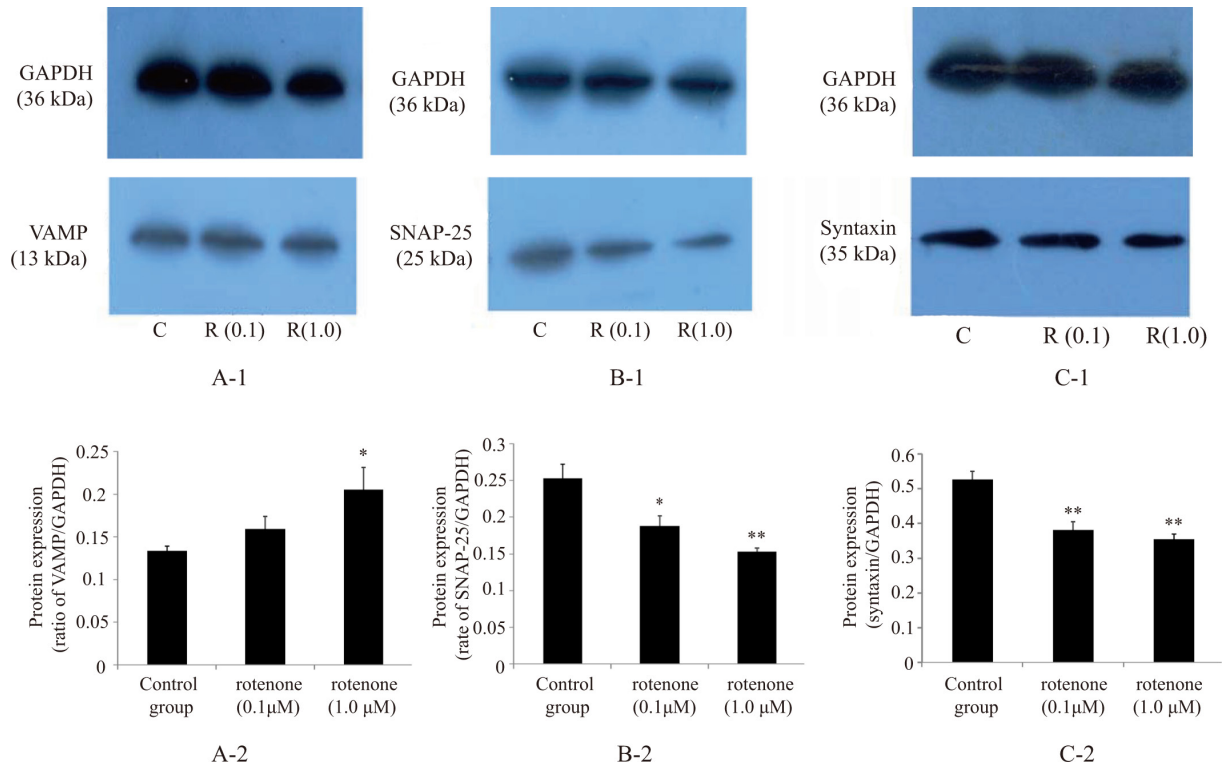


Fig. 4. Western immunoblotting analysis of SNARE expression in PC12 cells. The Western immunoblotting ratio was calculated from 3 batches of independent experiments and was as shown in the figure. VAMP₂ protein expression was increased significantly by 1.0 μM rotenone treatment; however protein expression of SNAP-25 and syntaxin was decreased markedly by rotenone treatment. Data are expressed as $\bar{x} \pm s$, ($n=3$), * $P < 0.05$; ** $P < 0.01$.

experimental group (Fig. 4C-1, 2). As the SNARE contains several potential Ca^{2+} -binding sites on the surface, the variations of the three proteins expression might be associated with the influx of Ca^{2+} in the PC12 cells induced by rotenone as mentioned above. Thus, SNARE may be involved directly in the neurotoxicity of PC12 cells induced by rotenone.

Capabilities of dopamine release of PC12 cells

To study the dependence of Ca^{2+} on dopamine release in rotenone-intoxicated PC12 cells pretreated with nifedipine, intracellular dopamine levels were measured. Intracellular dopamine levels were significantly increased after rotenone (1.0 μM) treatment in compared with those in the control group (Fig. 5). However, the cytoplasmic dopamine content was decreased markedly in PC12 cells exposed to rotenone with nifedipine pretreatment (Fig. 5).

Changes in ROS levels in PC12 cells

The contents of ROS in PC12 cells can be reflected by the intensity of DCF-DA. The intensity of DCF-DA was increased in PC12 cells intoxicated with rotenone compared with that in the control group, indicating that the levels of ROS formation in PC12 cells was stimulated by rotenone. Pretreatment with nifedipine decreased the ROS formation in PC12 cells exposed to rotenone (Fig. 6).

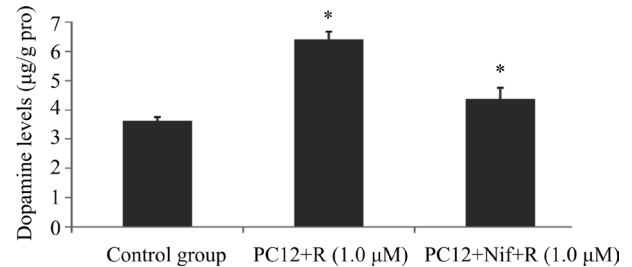


Fig. 5. Intracellular dopamine in PC12 cells was collected and analyzed. Intracellular dopamine levels were significantly increased following treatment with 1.0 μM rotenone alone compared with that in the control group. The level of intracellular dopamine of PC12 cells incubated with nifedipine pretreatment was lower obviously compared with that in PC12 cells treated with rotenone. Data are expressed as $\bar{x} \pm s$, ($n=6$), ** $P < 0.01$.

The neurite outgrowth of PC12 cells

PC12 cells in the control group were polygonal and had long axons. However, after rotenone treatment for 24 h, the neurites of PC12 cells were dramatically shortened. Even some of the cells had almost no axons, becoming round, taking a spherical shape and forming a monolayer. However, PC12 cells pretreated with nifedipine (0.5 μM) and exposed

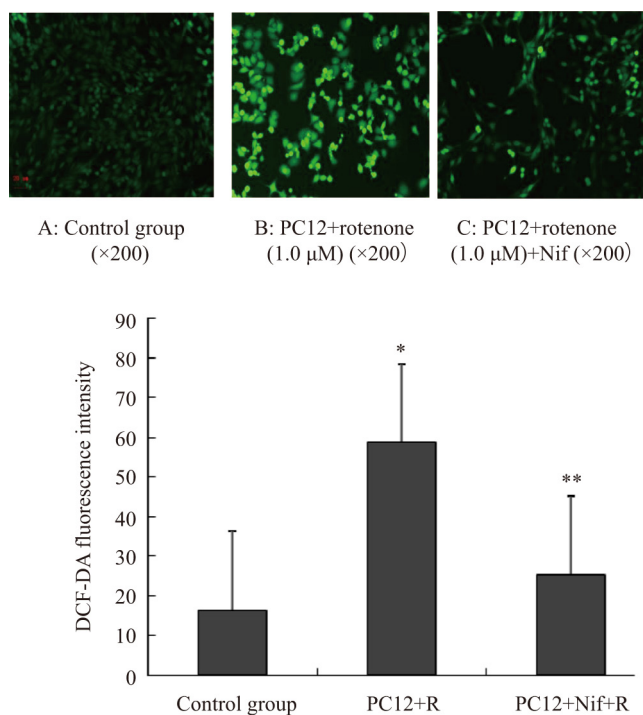


Fig. 6. ROS content in PC12 cells intoxicated with rotenone. The top panel shows microscopic images of DCF-DA fluorescence intensity, which reflected the ROS levels in PC12 cells. DCF-DA fluorescence intensity in PC12 cells treated with rotenone was more stronger than that in the control group, which indicated an increase in ROS levels induced by rotenone in PC12 cells. Nifedipine (0.5 μ M) pretreatment could decrease the ROS levels in PC12 cells induced by rotenone. Data are expressed as $\bar{x} \pm s$, * $P < 0.01$ vs control group; ** $P < 0.01$ vs PC12+rotenone group.

to rotenone also differed morphologically from the controls. Nifedipine pretreatment, to some extent, could relieve the shortening of the processes of PC12 cells induced by rotenone (Fig. 7).

Discussion

Rotenone is a lipophilic compound that freely crosses cell membranes and accesses cytoplasm and mitochondria and has been proved to have neurotoxicity and has abilities to produce many pathological and chemical features of PD in rats. Now, the neurotransmitter dopamine is believed to be involved in the neurodegeneration of PD⁹. Dopamine release from neurons takes place at specialized structures called synapses¹⁰. Dopamine release occurs through vesicular exocytosis and is action potential- and Ca^{2+} -dependent.¹¹

Intracellular Ca^{2+} has been strongly implicated in the regulation of the dopamine release of exocytosis signaling pathways¹². And furthermore, it has also been reported that a rotenone-induced increase in Ca^{2+} concentration was involved in its toxic mechanism, which was associated with oxidative stress. In the present study, we also found that

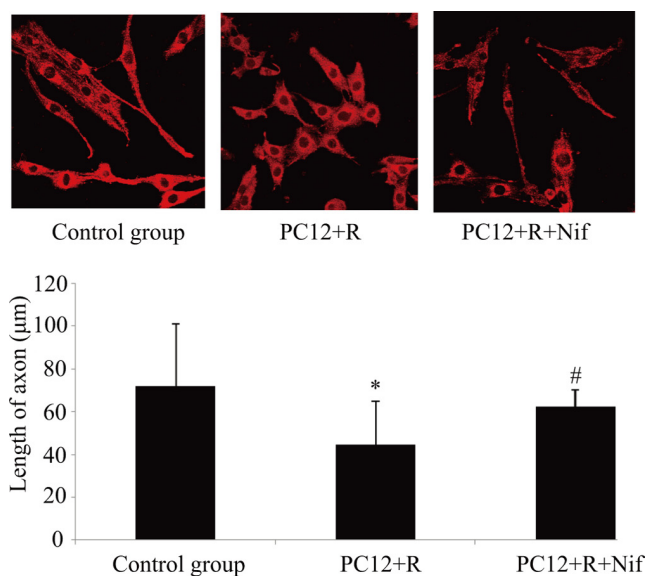


Fig. 7. Effects of nifedipine on the neurite outgrowth of PC12 cells processes treated with rotenone. Neurite length was reduced in PC12 cells intoxicated with rotenone and the difference was significant ($P < 0.05$). The processes of nifedipine pretreatment in PC12 cells exposed to rotenone were longer in contrast with those in PC12 cells intoxicated with rotenone alone. Data are expressed as $\bar{x} \pm s$, * $P < 0.05$ vs control group; # $P < 0.05$ vs PC12+rotenone.

rotenone treatment resulted in a concentration-dependent rise in intracellular Ca^{2+} in PC12 cells, with the presence of extracellular Ca^{2+} as a precondition, which was connected with the dysfunction of the neurotransmitter release. The increase in intracellular Ca^{2+} partially contributed to the suppression of dopamine release in PC12 cells intoxicated with rotenone. It has been reported that an influx of extracellular Ca^{2+} through Ca^{2+} -permeable ion channels, a decrease in the outflow of intracellular Ca^{2+} and a release of Ca^{2+} from intracellular stores are the three main mechanisms, that contribute to the increase of intracellular Ca^{2+} in cells. Our current results showed that in the absence of extracellular Ca^{2+} or with the removal of extracellular Ca^{2+} by adding the Ca^{2+} chelator (EGTA) to the medium, there was no increase in intracellular Ca^{2+} during the exposure of PC12 cells to rotenone, suggesting that there was no Ca^{2+} released from intracellular stores. Pretreatment with nifedipine, a blockers of L-type voltage-activated Ca^{2+} channels, effectively suppressed the rotenone-induced intracellular Ca^{2+} increase in PC12 cells. Therefore, the influx of extracellular Ca^{2+} mainly via L-type voltage-activated Ca^{2+} channels might be involved in the mechanism of rotenone-induced neurotoxicity.

FM 1-43 was developed about 10 years ago. It not only serves as a very useful tool for simple synapse identification in a living preparation, but also provides quantitative information about recycling pools of vesicles⁸. During periods of PC12 cell activity, recycling synaptic vesicles are labeled as they internalize t FM 1-43 dye. After washing out

the surface-bound probe, the recycling synaptic vesicles are easily visualized. The present results showed that the FM1-43-labeled synaptic vesicles were extensively scattered in the contours of the cell body, with weak fluorescence intensity in PC12 cells of the control group. However, in PC12 cells treated with rotenone, FM1-43-labeled vesicles were mainly clustered around the nucleus with stronger fluorescence intensity. The number of synaptic vesicles increased in PC12 cells treated with rotenone, indicating there might be a suppression of vesicle exocytosis and an enhancement of endocytosis. The balance of endocytosis and exocytosis was destroyed by rotenone. It can be concluded that rotenone led to suppression of dopamine release and was involved in PC12 cells neurotoxicity.

Of course, exocytosis occurs in a series of steps and also is an intricate network involving many factors^{13,14}. The process includes docking of synaptic vesicles to the presynaptic plasma membrane, one or more priming reactions that leave the vesicles ready for release, and the actual release triggered by Ca^{2+} influx into presynaptic terminals^{14–17}. Thus, there are many factors that need to be considered. SNARE proteins play a key role in Ca^{2+} -triggered neurotransmitter release in neurons^{18,19}. However, the precise point of action of the SNARE in neurotransmitter release remains unclear. Syntaxin, SNAP-25 and VAMP₂ are essential for regulated exocytosis in diverse cell types²⁰. In PC12 cells intoxicated with rotenone, while the protein expression of VAMP₂ increased, the syntaxin and SNAP-25 protein expression all decreased compared with those in the control group. The SNARE contains several potential Ca^{2+} -binding sites on the surface²⁰, suggesting that it may be involved directly in Ca^{2+} -binding during neurotransmitter release. So the influx of Ca^{2+} induced by rotenone might have given rise to changes in protein expression of the SNARE or the binding action of SNARE, which further led to the dopamine release dysfunction in PC12 cells induced by rotenone. Therefore, the dopamine release dysfunction might participate in the mechanism of rotenone-induced neurotoxicity due to effects of rotenone on SNARE proteins.

Increasing evidence shows that this form of neurotransmitter discharge can be modulated by changes in intracellular Ca^{2+} concentration²¹. Nifedipine pretreatment markedly decreased the synaptic vesicle accumulation in PC12 cells intoxicated with rotenone, which suggested that nifedipine could alleviate the rotenone-induced exocytosis suppression of PC12 cells. The increase in intracellular Ca^{2+} in PC12 cells treated with rotenone may be the cause of exocytosis dysfunction, leading to the accumulation of synaptic vesicles and the intracellular dopamine increase. In fact, it has been reported many times that endocytosis and exocytosis of neurons are dependent on intracellular Ca^{2+} . As rotenone treatment evoked a rise in intracellular Ca^{2+} , dopamine release from synaptic vesicle exocytosis was partially suppressed in a Ca^{2+} -dependent manner.

Thus, it can be summed up that rotenone induced neurotoxicity was associated with the influx of intracellular Ca^{2+} via the voltage-gated Ca^{2+} channel from the extracel-

lular Ca^{2+} , and that suppression of dopamine release was partially dependent on the increased intracellular Ca^{2+} . The current study may further add to the current knowledge of the mechanism of rotenone neurotoxicity and also the PD etiology. Of course, further detailed studies on the mechanisms of rotenone-induced suppression of dopamine release are necessary before definite conclusions can be drawn.

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