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Possible involvement of Ca^{2+} signaling in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells

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Abstract

Rotenone, an inhibitor of mitochondrial respiratory chain complex I, is a useful tool to elicit animal model of Parkinson's disease. Rotenone-induced neuronal apoptosis may contribute to the etiology of Parkinson's disease. However, the mechanism of rotenone-induced apoptosis is not fully understood. In the present study, we show that Ca^{2+} signaling is essential for rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. By using Fluo-3/AM and Fura-2/AM, the fluorescent calcium indicator, rotenone was found to cause a rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). The intracellular Ca^{2+} chelator BAPTA attenuated rotenone-induced apoptosis. Notably, Ca^{2+} suppression also prevented rotenone-induced apoptotic related events including reactive oxygen species production, G2/M cell cycle arrest and caspase activation, suggesting that Ca^{2+} signaling is upstream to these events. In the absence of extracellular Ca^{2+}, the rotenone-induced $[Ca^{2+}]_i$ elevation was inhibited. Further, the voltage-dependent Ca^{2+} channel blocker nifedipine suppressed most of the elevation of $[Ca^{2+}]_i$ induced by rotenone. These results demonstrate that rotenone leads to an elevation in $[Ca^{2+}]_i$ through Ca^{2+} influx by the opening of voltage-gated Ca^{2+} channel. This study of rotenone may help to elucidate the neurodegenerative mechanisms in Parkinson's disease.

Keywords: Rotenone; Apoptosis; Ca^{2+} influx; G2/M cell cycle arrest; SH-SY5Y cells; BAPTA

Many studies have shown that oxidative stress and mitochondrial dysfunction are involved in the pathogenesis of Parkinson's disease. Mitochondrial dysfunction is suggested by a partial inhibition (20–40%) of respiratory chain complex I activity [16]. Rotenone, a plant-derived insecticide, is a specific inhibitor of mitochondrial complex I [19]. In rats rotenone has been shown to produce effects that closely resemble Parkinson's disease [6]. It is noteworthy that rotenone induces apoptosis in neural cells, which may contribute to the etiology of Parkinson's disease. Nakamura et al. showed the activation of both mitochondria- and endoplasmic reticulum-dependent caspase pathways in rotenone-induced apoptosis [11]. However, the mechanism underlying the induction of apoptosis by rotenone has not been well clarified. The studies of rotenone-induced apoptosis may also help to elucidate the neurodegenerative mechanisms in Parkinson's disease.

Apoptosis is a highly regulated cell death program that is induced in cells as a suicide response to unfavorable growth conditions or to exposure to pro-apoptotic external stimuli. The process of apoptosis is regulated by cell surface signals and expression of specific intracellular proteins. Ca^{2+} has been strongly implicated in induction of apoptosis and regulation of the apoptotic signaling pathways. Negre-Salvayre and Salvayre demonstrated the Ca^{2+} dependence of apoptosis as well as the protective effect of Ca^{2+} channel blockers and Ca^{2+} chelators [12]. During apoptosis, a variety of toxic insults, ultimately lead to cell death, are associated with increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [12,13]. Intracellular Ca^{2+} seems to be an important component of the mechanism of apoptosis. Ca^{2+} signaling is upstream to certain pathways that lead to apoptosis. Elevated $[Ca^{2+}]_i$ activates Ca^{2+}-dependent protein kinases and phosphatases during apoptosis [4]. Ca^{2+} has been also found to act on cell cycle at multiple points, including the reentry of quiescent cells into proliferation and the transition through G1/S, G2/M and the metaphase/anaphase boundaries [18].

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Given that excessive elevation in intracellular Ca^{2+} levels is a major factor leading to apoptosis in many cell types, we performed the present experiments to test the role of Ca^{2+} signaling in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells.

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) penicillin, 10% (v/v) fetal bovine serum, 5% CO_2 at 37 °C. Apoptosis was evaluated by using an Annexin V-FITC apoptosis detection kit (BD Biosciences Pharmingen). Briefly, cells were stained with annexin V-FITC and propidium iodide (PI). After incubation at room temperature in the dark, samples were analyzed on a flow cytometer (Becton Dickinson FACS Vantage SE, USA). This assay discriminates between intact (annexin V^-/PI^-), early apoptotic (annexin V^+/PI^-), and late apoptotic and necrotic cells (annexin V^+/PI^+).

Intracellular Ca^{2+} levels were determined with the Ca^{2+}-sensitive fluorochrome Fluo-3/AM and Fura-2/AM (Molecular Probes). After exposure to rotenone at various concentrations for various times, cells were harvested by centrifugation, and then loaded with Fluo-3/AM (2 μM) or Fura-2/AM (2 μM) in serum-free DMEM at 37 °C for 30 min. Cells were analyzed by a Becton Dickinson FACS Calibur flow cytometer (for Fluo-3) or a HITACHI F4500 fluorescence spectrophotometer (for Fura-2). Calibration of the fluorescence versus $[Ca^{2+}]_i$ was performed as described [2,8]. At the end of each experiment, 2 μM ionomycin or 10 nM digitonin was added to permeabilize the cells, resulting in the maximal fluorescence of Fluo-3 and Fura-2, respectively. The minimal fluorescence was determined by the addition of 5 mM EGTA.

Kinetic study of rise in intracellular Ca^{2+} was conducted as follows. SH-SY5Y cells were loaded with 5 μM Fluo-3/AM dye at 37 °C for 30 min, and then washed free of extracellular Fluo-3/AM dye and resuspended in Krebs-Ringer-HEPES (KRH) buffer (131 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 6 mM glucose, 20 mM HEPES, pH 7.4). Fluorescence was determined using a HITACHI F4500 fluorescence spectrophotometer with excitation and emission wavelengths of 488 nm and 525 nm, respectively. Treatment with rotenone was initiated after 1 min, and the increase in fluorescence was recorded for 9 min. Where the Ca^{2+} channel blocker nifedipine was used, cells were pretreated with nifedipine for 10 min before rotenone exposure.

Intracellular reactive oxygen species (ROS) was monitored by using the fluorescent probe DCFDA (Sigma–Aldrich). After treatment, cells were incubated with 10 μM DCFDA in DMEM at 37 °C for 30 min, then washed twice with PBS, and finally the DCF fluorescence intensity was measured in a microplate-reader (Fluoroskan Ascent Thermo Labsystems) at excitation wavelength 485 nm and the emission wavelength 538 nm.

Cell cycle distribution was analyzed by flow cytometry. Cells were fixed with ice-cold 70% ethanol overnight. Fixed cells were washed with PBS, followed by treatment with RNase A (0.1 mg/ml) at 37 °C for 30 min. Cells were stained with PI (0.5 mg/ml PI, 1% Triton x-100, 0.9% NaCl) in the dark at room temperature for 30 min. PI-stained cells were analyzed by flow cytometry. The data were processed for cell cycle distribution analysis by Modfit V 3.0 software.

Caspase activity was determined by using acetyl-Asp-Glu-Val-Asp-aldehyde-AFC (Clontech USA), a pseudosubstrate used to measure caspase activity (mainly for caspase-3). In the presence of caspase, this substrate is cleaved to the fluorochrome 7-amino-4-trifluoromethyl coumarin (AFC), which is quantified by measuring fluorescence intensity with a F-4500 HITACHI fluorescence spectrophotometer (400 nm excitation and 505 nm emission).

All results were expressed as mean ± S.E.M. Significance testing was performed by means of Student's *t*-tests. Differences between groups were considered significant at a value of $P < 0.05$.

The effect of rotenone on SH-SY5Y cell apoptosis was examined by annexin V-FITC-based flow cytometry. The data showed that rotenone increased the percentage of cells undergoing apoptosis (annexin V^+/PI^-) and decreased the number of viable cells (annexin V^-/PI^-) in a time- and dose-dependent manner (Fig. 1A). Recent studies suggested that the mechanism by which cells commit to apoptosis is related with the cell cycle [10]. In this study, we examined the effect of rotenone on the progression of cell cycle. The results showed that incubation of SH-SY5Y cells with rotenone caused a rise in the number of cells in the G2/M phase of the cell cycle and a concomitant decrease in G1 phase cells (Fig. 1B). When SH-SY5Y cells were exposed to

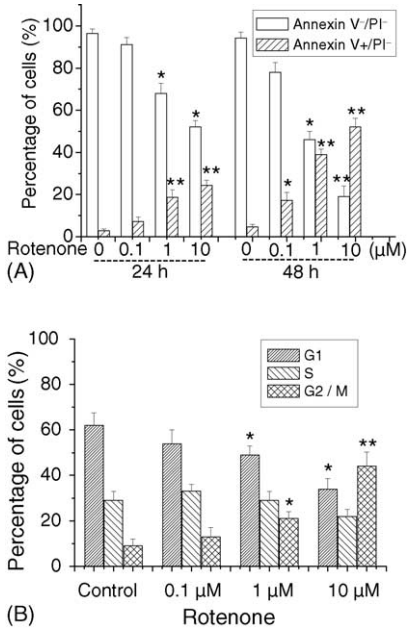

Fig. 1. Rotenone-induced apoptosis and cell cycle arrest by flow cytometry. Rotenone induced (A) apoptotic death in SH-SY5Y cells and (B) G2/M arrest of SH-SY5Y cells. Cells were exposed to different concentrations of rotenone for 24 h. Data are mean ± S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. control.

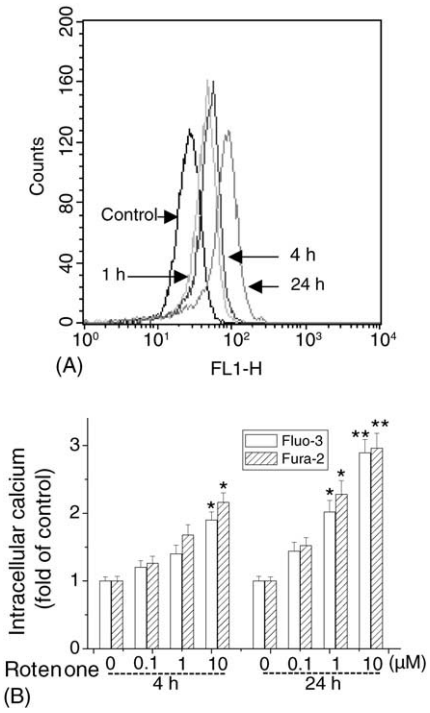

Fig. 2. Rotenone-mediated increase in cytosolic Ca^{2+} in SH-SY5Y cells. Intracellular Ca^{2+} levels were determined using flow cytometry with Fluo-3/AM fluorescent dye and spectrofluorometry with Fura-2/AM, respectively. (A) Effect of 10 μM rotenone on Fluo-3 fluorescence for up to 24 h and (B) statistic data of intracellular calcium in response to rotenone treatment normalized to control. Results are mean ± S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. control.

various concentrations of rotenone for 24 h, cells were progressively blocked in the G2/M phase of the cell cycle in a dose-dependent manner.

Increases in intracellular Ca^{2+} levels have been reported in many experimental models of apoptosis [12,13]. Therefore, we used the Ca^{2+}-sensitive fluorescence probe Fluo-3/AM and Fura-2/AM to monitor alterations in the intracellular Ca^{2+} by flow cytometry and spectrofluorometry, respectively. When SH-SY5Y cells were exposed to 10 μM rotenone for up to 24 h, the histogram of Fluo-3 fluorescence shifted to a higher intensity (Fig. 2A), indicating an increase in $[Ca^{2+}]_i$ over time. To determine the dose–response relationship, the effect of 0.1–10 μM rotenone was tested for 4 h and 24 h. As shown in Fig. 2B, $[Ca^{2+}]_i$ increased in a dose-dependent manner at both treatment times. These results suggest that rotenone elevates $[Ca^{2+}]_i$ in a time- and dose-dependent manner.

To determine the basis of the rise in $[Ca^{2+}]_i$ mediated by rotenone, we studied the increase in fluorescence using a spectrofluorometer. SH-SY5Y cells were preloaded with 5 μM Fluo-3/AM fluorescent dye before rotenone treatment. The increase in fluorescence was detected after rotenone was added to cells suspended in KRH buffer as shown in Fig. 3A (top trace). Experiments were also carried out in $CaCl_2$-free KRH buffer. Little increase in fluorescence was observed when rotenone was added to cells suspended in $CaCl_2$-free KRH buffer (Fig. 3B). However, a rapid increase and decrease in fluorescence were detected, respectively, when $CaCl_2$ (1 mM) and the calcium chelator EGTA (2 mM) were added to the cell suspension. Our data suggest that the rise in $[Ca^{2+}]_i$ is due to the Ca^{2+} influx from the medium. From these results, we hypothesize that pretreatment with rotenone might cause the opening of Ca^{2+} channels, allowing rapid influx of extracellular Ca^{2+}.

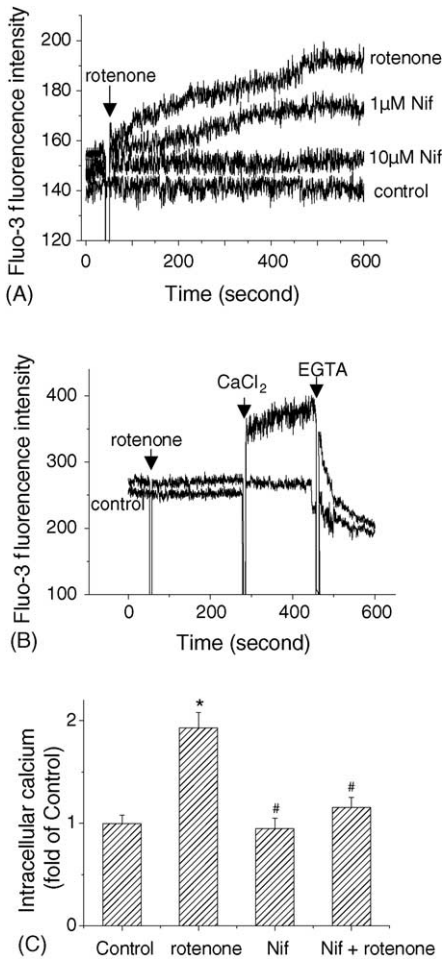

Fig. 3. Rotenone mediated rise in $[Ca^{2+}]_i$ through a voltage-dependent Ca^{2+} channel. SH-SY5Y cells were preloaded with Fluo-3/AM fluorescent dye before exposure to rotenone. (A) Kinetic study of Ca^{2+} mobilization using a F-4500 spectrofluorometer. Nifedipine (Nif), a voltage-dependent Ca^{2+} channel blocker, was applied at 1 and 10 μM to test its inhibitory effect on the rise of cytosolic Ca^{2+}. (B) Kinetic study on Ca^{2+} mobilization in SH-SY5Y cells suspended in KRH buffer (Ca^{2+} free). Rotenone was added, followed by $CaCl_2$ (1 mM), and subsequently, the Ca^{2+} chelator EGTA (2 mM). (C) Effect of nifedipine (Nif) on rotenone-induced $[Ca^{2+}]_i$ elevation by using a flow cytometer. SH-SY5Y cells were pretreated with Nif (2 μM) for 30 min before rotenone exposure for 4 h at 37 °C. * $P < 0.05$ vs. control; # $P < 0.05$ vs. rotenone alone.

To test this hypothesis, the classic voltage-gated Ca^{2+} channels, which might be involved in rotenone-induced Ca^{2+} influx, were investigated. When SH-SY5Y cells were treated with the voltage-dependent Ca^{2+} channel blocker nifedipine (Nif) 10 min prior to rotenone treatment, as shown in Fig. 3A, the rotenone-induce Ca^{2+} influx was found to be

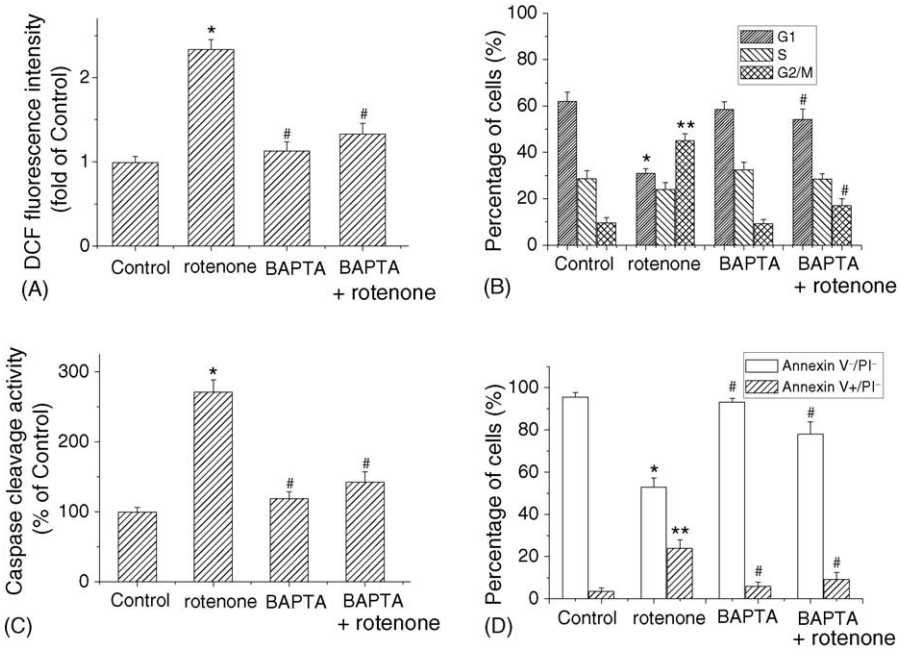

Fig. 4. The effect of intracellular Ca^{2+} chelator BAPTA on intracellular events associated with rotenone-induced apoptosis in SH-SY5Y cells. BAPTA (2 μM) was applied 30 min before the addition of 10 μM rotenone for 24 h incubation. (A) Effect of BAPTA on rotenone-induced ROS generation evaluated by the fluorescence intensity of DCF. (B) BAPTA alleviates G2/M cell cycle arrest induced by rotenone. (C) BAPTA inhibited rotenone-induced increase in caspase activity measured by fluorometric assay. (D) BAPTA ameliorated the percentage of rotenone-induced apoptotic cells detected by flow cytometry. Data are expressed as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$ vs. rotenone alone treatment.

attenuated in a concentration-dependent manner. This suggests that rotenone activates the opening of voltage-gated Ca^{2+} channels in SH-SY5Y cells, leading to Ca^{2+} influx. Although nifedipine binds with high affinity to calcium channels, it can also interact with some other cellular structures at the concentrations used [20], which may explain why the effect of nifedipine on $[Ca^{2+}]_i$ did not correspond very well with the expected sensitivity of calcium channels in Fig. 3A. The effect of nifedipine was also investigated by flow cytometry, 2 μM nifedipine was needed to block Ca^{2+} influx ($P < 0.05$) mediated by rotenone (Fig. 3C). This result implicated the involvement of voltage-gated Ca^{2+} channel in rotenone-induced Ca^{2+} influx as well.

In several reports addressing the rotenone-induced apoptosis, the activation of apoptotic cascades was attributed to signaling events other than Ca^{2+} elevation, such as the increase of ROS [14]. In order to determine the relationship between Ca^{2+} and the major apoptotic pathways, SH-SY5Y cells were pretreated with the intracellular Ca^{2+} chelator BAPTA/AM (2 μM) for 30 min before exposure to 10 μM rotenone for 24 h. As oxidative stress and changes in $[Ca^{2+}]_i$ are intimately related, the relationships between ROS generation and the increase in $[Ca^{2+}]_i$ was investigated following rotenone treatment. As shown in Fig. 4A, BAPTA treatment significantly reduced rotenone-induced ROS production. At the concentration tested, BAPTA reduced 61% of rotenone-induced ROS. On the other hand, although catalase reduced rotenone-induced ROS markedly, it does not decrease $[Ca^{2+}]_i$ elevation (data not shown). These results imply that Ca^{2+} is involved in rotenone-induced ROS generation in SH-SY5Y cells. Ca^{2+} plays an important role in regulating cell cycle [18]. As shown in Fig. 4B, pretreatment of BAPTA/AM (2 μM) suppressed rotenone-induce G2/M cell cycle arrest.

As activation of caspases is the key steps of the apoptosis process, its activity was examined by fluorometric assay in this study. The effect of BAPTA on MPP^+-induced caspases activation is shown in Fig. 4C. BAPTA attenuated rotenone-induced activity of caspase from 271% to 142%. As shown in Fig. 4D, BAPTA greatly ameliorated rotenone-induced apoptosis detected by flow cytometry. The number of apoptotic cells decreased from 24.2 ± 2.1% to 9.2 ± 1.3%. Since BAPTA alone did not interfere with apoptosis, the data indicate that intracellular Ca^{2+} is clearly involved in the rotenone-induced apoptotic processes in SH-SY5Y cells.

In the present study, we demonstrate the important role of Ca^{2+} during the rotenone-induced apoptosis. Our findings are as follows: (1) rotenone-induced apoptosis is largely Ca^{2+}-dependent; (2) the rise of $[Ca^{2+}]_i$ is mainly due to Ca^{2+} influx via the voltage-gated Ca^{2+} channel; and (3) the increased intracellular Ca^{2+} is involved in rotenone-induced apoptotic events.

Increases in intracellular Ca^{2+} levels have been reported in many experimental models of apoptosis [12,13]. Our current results show that rotenone treatment results in a concentration-dependent rise in intracellular Ca^{2+} in SH-SY5Y cells. Previous studies showed that the major pathway of intracellular Ca^{2+} increase are Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular calcium

store. These two pathways appeared to be involved in the source of intracellular Ca^{2+} increase associated with apoptosis. Our results with $CaCl_2$-free KRH buffer and EGTA demonstrated that rotenone induced the influx of extracellular Ca^{2+} in SH-SY5Y cells. Nifedipine, a blocker of voltage-dependent Ca^{2+} channel, was shown to inhibit the rotenone-induced extracellular Ca^{2+} influx, suggesting the involvement of voltage-gated Ca^{2+} channel in Ca^{2+} influx. Recently, Bonsi et al. reported that a significant $[Ca^{2+}]_i$ rise matched the cell membrane depolarizing phase in striatal cholinergic interneurons treated by rotenone [5]. Thus, we hypothesize that rotenone might cause a rapid Ca^{2+} influx through voltage-gated channels that are activated by depolarizing the cell membrane.

Several recent studies have reported the involvement of Ca^{2+} in apoptotic processes [12,13]. The elevations in intracellular Ca^{2+} cause proapoptotic protein expression [12]. The rise of $[Ca^{2+}]_i$ has been demonstrated to cause mitochondrial inner transmembrane potential collapse and to induce the release of cytochrome *c* [9]. Interestingly, previous studies showed that rotenone causes mitochondrial inner transmembrane potential collapse and cytochrome *c* release, and then activates of caspase-3 [14]. In the present study, we found that lowering $[Ca^{2+}]_i$ with an intracellular chelator (BAPTA) attenuated rotenone-induced caspase activation and apoptotic death in SH-SY5Y cells. BAPTA suppressed the activation of caspase induced by rotenone, suggesting that Ca^{2+} may act upstream to caspase. These data indicate that rotenone-induced apoptosis is mediated, at least in part, by elevation of $[Ca^{2+}]_i$, suggesting the involvement of Ca^{2+} signaling in the apoptotic process evoked by rotenone.

In this study, we show that rotenone gives rise to cell cycle arrest predominantly at the G2/M checkpoint. This result agrees with the results of Armstrong et al. on human B lymphoma PW cell lines [1]. We speculated that rotenone-induced apoptosis of SH-SY5Y cells might occur at G2/M phase or in the process of transition from S to G2/M phase. Rotenone-induced G2/M arrest was blocked by pretreatment of BAPTA, indicating the involvement of Ca^{2+} in cell cycle arrest.

The rotenone-induced ROS production was reduced in the presence of Ca^{2+} chelator BAPTA, indicating that Ca^{2+} is involved in the generation of ROS induced by rotenone. Sousa et al. reported that Ca^{2+} stimulated rotenone-induced increase of ROS production in isolated rat forebrain mitochondria [17]. Here, the question has been raised how rotenone induces generation of ROS. Rotenone blocks electron flow through respiratory chain complex I, followed by enhancement of $O_2^{-\bullet}$ formation [19]. It appears to be the primary mechanism of rotenone-induced formation of $O_2^{-\bullet}$, which can rapidly evolve to H_2O_2. The ability of Ca^{2+} to stimulate mitochondrial ROS generation may be attributed to an increased lipid packing and lipid domain formation that may contribute to electron leakage from the respiratory chain complex I [7]. Saeko et al. demonstrated that NADPH oxidase also served as a source of rotenone-induced ROS generation [14]. The activity of NADPH oxidase could be regulated by intracellular Ca^{2+} [15]. On the other hand, Bhattacharyya et al. showed that the rise of $[Ca^{2+}]_i$ accompanied the decrease in the activity of antioxidant enzymes [2], indicating an interesting possibility that the cells treated by rotenone may have a calcium-inactivated mechanism for removing ROS. Oxygen stress and Ca^{2+} influx to the cytosol, has been proposed to play an important role in the neuropathology of Parkinson's disease [3]. Under these conditions, Ca^{2+}-stimulated ROS release may be an important link between the partial mitochondrial complex I inhibition and the oxidative damage observed in Parkinson's disease.

The present study may provide insight into the mechanism of Parkinson's disease etiology. Further detailed studies in the mechanisms of rotenone-induced apoptosis are necessary before definite conclusions can be drawn.

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