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# ISRADIPINE PREVENTS ROTENONE-INDUCED INTRACELLULAR CALCIUM RISE THAT ACCELERATES SENESCENCE IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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**Abstract—Previous research demonstrated that rotenone (RT) induces neuronal injury partially by increasing intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), and inducing oxidative stress, leading to a neurodegenerative disorder. However, the mechanism of RT-induced injury remains elusive. Recent work revealed that $Ca^{2+}$ signaling is important for RT-induced senescence in human neuroblastoma SH-SY5Y cells. In the present study, we found that in SH-SY5Y cells, RT increased $[Ca^{2+}]_i$, senescence associated β-galactosidase activity, aggregation of lipofuscin, production of reactive oxygen species, G1/G0 cell cycle arrest, and activation of p53/p21signaling proteins. In addition, RT decreased the expression of the signaling proteins for cell proliferation and survival, Cyclin-dependent kinase 2 (CDK2), cyclin D1, and Akt. Pretreatment of SH-SY5Y cells with isradipine, an L-type $Ca^{2+}$ channel blocker, or EGTA antagonized these effects of RT. These results suggested that application of isradipine might be a novel approach to prevent RT-induced neurodegenerative disorder such as Parkinson's disease.**


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*Abbreviations:* $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentrations; CDK2, Cyclin-dependent kinase 2; DHP, dihydropyridine; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Lipo, lipofuscin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethylthiahiazol-2-thiazolyl)-2,5-diphenyl-2H-tetraxolium bromide; NC, nitrocellulose filter; OCR, oxygen consumption rate; PD, Parkinson's disease; PBS, phosphate-buffered saline; PBST, PBS with Tween 20; PI, propidiun iodide; PI3-kinase–Akt, phosphatidylinositol 3′-kinase/Akt; ROS, reactive oxygen species; RT, rotenone; SA-β-gal, senescence association β-galactosidase; SDS, sodium dodecyl sulfate; SNc DA, substantial nigra dopamine; VDCCs, voltage-dependent $Ca^{2+}$ channels.

**Key words: rotenone (RT), Parkinson's disease, cell senescence, $Ca^{2+}$ signaling, SH-SY5Y cell.**

## INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder, which is strongly related to aging, especially in the population above the age of 65 (De Rijk et al., 1997; De Lau et al., 2004). To date, there are no effective drugs to cure or delay the progression of this disease. Chan et al. (2010) speculates a potential clue of the selective damage of substantial nigra dopamine (SNc DA) neurons in advancing age. These neurons rely on L-type $Ca^{2+}$ channels to maintain autonomous activity; hence, dysregulation of these $Ca^{2+}$ channels could pose a sustained stress on mitochondrial ATP-generating oxidative phosphorylation, cellular senescence acceleration and death. Therefore, L-type $Ca^{2+}$ channel blockers may be useful to reduce $Ca^{2+}$ influx in order to attenuate the senescence process of SNc DA neurons as well as PD progression.

Isradipine (Fig. 1A), a dihydropyridine (DHP) $Ca^{2+}$ channel antagonist, has much higher (>40-fold) affinity for CaV1.3 L-type channel than other known DHPs, such as nimodipine (Surmeier et al., 2010), and is readily available to the brain (Ilijic et al., 2011) with relative minor side effects (Fitton and Benfield, 1990). Isradipine has neuroprotective effects in the animal models of PD (Chan et al., 2007). Reduction of the $Ca^{2+}$ influx mediated by isradipine markedly reduces the neurotoxin sensitivity of adult SNc DA neurons, and also reduces the sensitivity of $Ca^{2+}$-binding protein to neurotoxin such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in *in vivo* study (Yamada et al., 1990; German et al., 1992).

Rotenone (RT) is a plant-derived pesticide, which inhibits activity of mitochondrial complex I and induces cellular oxidative stress (Turrens and Boveris, 1980) and inhibits mitochondrial basal oxygen consumption rate (OCR), decreased ATP-linked OCR, and stimulates glycolysis (Giordano et al., 2012). Numerous studies have used RT to produce an experimental animal model for PD by generating PD-like symptoms, such as bradykinesia, rigidity, resting tremor and cognitive decline (Ilijic et al., 2011). RT-induces neuronal injury partly by increasing intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and by inducing oxidative stress, which leads to neurodegeneration (Wang and Xu, 2005). Under

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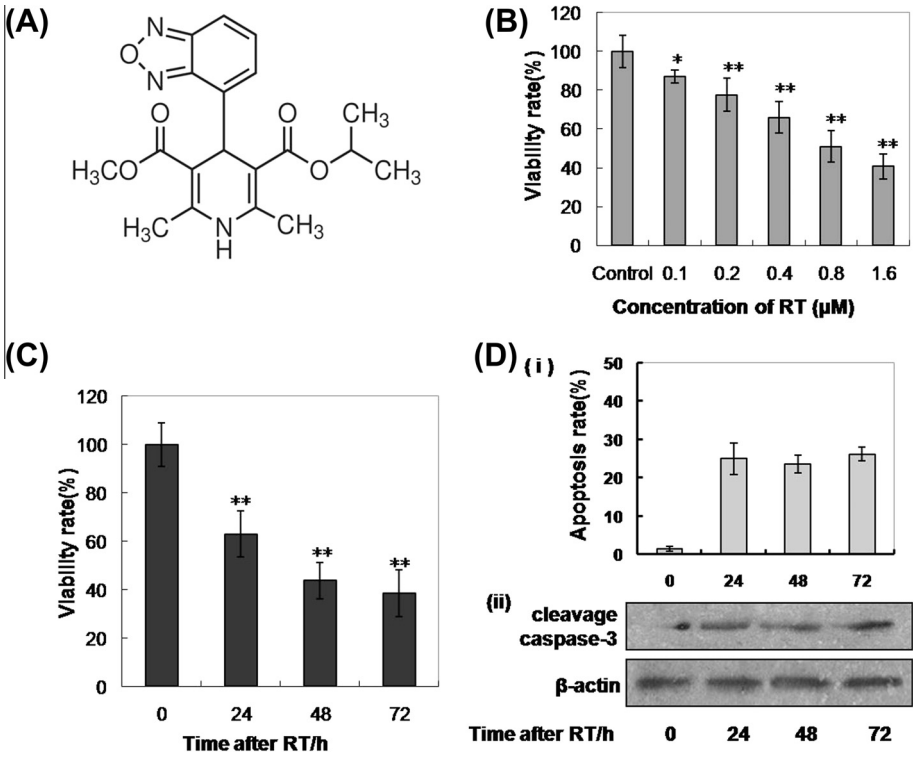


**Fig. 1.** Rotenone (RT)-induced SH-SY5Y cell death. (A) Chemical structure of Isradipine. (Molecular formula: $C_{19}H_{21}N_3O_5$; molecular weight: 371.39). SH-SY5Y cells were treated with RT (0–1.6 μM) for 24 h (B) or with 0.4 μM RT for 0–72 h (C). Data are shown as mean ± S.D. ($n$ = 6). (D) SH-SY5Y cells were treated with 0.4 μM RT for various times to detect apoptosis rate, Data are shown as mean ± S.D. ($n$ = 3). (E) Western blot detected cleaved caspase-3 after treatment with 0.4 μM RT for different times in SH-SY5Y cells. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with control.

conditions of $[Ca^{2+}]_i$-overload, mitochondria can buffer intracellular $Ca^{2+}$ by fast sequestration (Werth and Thayer, 1994). Exposure of neurons to excitotoxins (e.g., glutamate) is similar to the exposure of mitochondria to high $[Ca^{2+}]_i$, which leads to mitochondrial generation of hydroxyl and carbon-centered radicals (Dykens, 1994). Previous *in vitro* studies revealed that mitochondrial free radical generation linked directly to glutamate-induced increase in mitochondrial $Ca^{2+}$ concentrations (Dugan et al., 1994). However, the mechanism of RT-induced injury remains elusive. Several studies have reported that RT-induces cell apoptosis in SH-SY5Y cell through mitochondrial dysfunction (Kitamura et al., 2002; Newhouse et al., 2004; Bauereis et al., 2011) and regulation of $[Ca^{2+}]_i$ (Ilijic et al., 2011). $Ca^{2+}$ influx is an energy-required process supplied by ATP that is provided by mitochondria through oxidative phosphorylation or flux of ion gradient (Wilson and Callaway, 2000). Oxidative phosphorylation process produces superoxide and reactive oxygen species (ROS), and $Ca^{2+}$ entry, which further increases mitochondrial oxidative stress that might accelerate senescence of SNc DA neurons (Chan et al., 2010). There is increasing evidence that voltage-dependent $Ca^{2+}$ channels (VDCCs; such as L-type $Ca^{2+}$ channels) play an important role in senescence-related cognitive impairment, and L-type VDCC antagonists (e.g., nimodipine and isradipine) can improve cognition in old animals (Deyo et al., 1989; Disterhoft et al., 2004) and PD-like animals (Ilijic et al., 2011). Therefore, L-type $Ca^{2+}$ channel blockers may be useful to reduce $Ca^{2+}$ influx in order to attenuate the senescence process of SNcDA neurons as well as PD progression.

In a preliminary study, we found that RT induced senescence more significantly than apoptosis in SH-SY5Y cell. In the present study, we focused on RT-induced senescence and death of SH-SY5Y cells as an *in vitro* PD model to determine the effect of isradipine on this model. We hypothesized that isradipine prevented RT-induced $[Ca^{2+}]_i$ rise, which delayed senescence and death in SH-SY5Y cells, through inhibition of the p53/p21 pathway and activation of the phosphatidylinositol 3′-kinase–Akt (PI3K/Akt) pathway.

## EXPERIMENTAL PROCEDURES

### Chemicals

RT was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) followed by Dulbecco's modified Eagle medium (DMEM) to prepare the stock solutions (1.6 μM), and was stored at 4 °C before use, the concentration of DMSO was less than 0.3%. Fluo-3/AM was purchased from Dojindo Laboratories (Japan), and EGTA, DMSO, and 3-(4,5-dimethylthiahiazol-2-thiazolyl)-2,5-diphenyl-2H-tetraxolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Isradipine was supplied by Anhui Newstar Pharmaceutical Development Co.,