

The *Pael-R* gene does not mediate the changes in rotenone-induced Parkinson's disease model cells

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Abstract

In this study, we established cell models for Parkinson's disease using rotenone. An RNA interference vector targeting Parkin-associated endothelin receptor-like receptor (*Pael-R*) was transfected into the model cells. The results of reverse-transcription polymerase chain reaction and western blot analysis showed that *Pael-R* expression was decreased after RNA interference compared with the control group (no treatment) and the model group (rotenone treatment), while the rate of apoptosis and survival of dopaminergic cells did not differ significantly between groups, as detected by flow cytometry and an MTT assay. These experimental findings indicate that the *Pael-R* gene has no role in the changes in rotenone-induced Parkinson's disease model cells.

Key Words: nerve regeneration; neurodegeneration; Parkinson's disease; rotenone; *Pael-R*; RNA interference; apoptosis; mitochondria; neural regeneration

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Introduction

Parkinson's disease is a common neurodegenerative disease. It is mainly caused by the loss of dopaminergic innervation owing to degenerative necrosis of nigral dopaminergic neurons^[1-2]. Currently, the pathogenesis of Parkinson's disease is not entirely clear, but it has been generally considered to be the result of interactions among various genetic and environmental factors^[3-6].

Rotenone is a suppressant of mitochondrial compound I, and is widely used in pesticides^[7-8]. It was previously found that dopaminergic neurons become apoptotic if they are exposed to rotenone^[9-11]. Sherer et al.^[12-14] found that rotenone-treated rats showed behavioral and pathological features of Parkinson's disease; thus, such rats are considered to be an ideal Parkinson's disease model and have been widely used in research investigating the pathogenesis of Parkinson's disease. Presently, the pathways and molecular mechanisms by which rotenone induces apoptosis of dopaminergic neurons remain unclear.

Accumulating evidence has shown that *Parkin* is a common Parkinson's disease-causing gene and its mutation correlates with mitochondrial function through reducing mitochondrial complex activity^[15-18]. Moreover, *Parkin* mutation causes Parkin-associated endothelin receptor-like receptor (*Pael-R*) protein deposition, and the associated cytotoxicity leads to dopaminergic neuronal apoptosis^[19-21]. In this study, we speculated that the *Pael-R* gene is possibly involved in the action of rotenone on cells; therefore, this study aimed to investigate the role of the *Pael-R* gene in rotenone-induced Parkinson's disease model cells using RNA interference.

Results

Pael-R gene expression was downregulated by RNA interference

Total RNA was extracted from cultured PC12 cells representing the control group (no treatment at all), model group (rotenone treatment) and RNA interference group (rotenone treatment plus RNA interference vector). *Pael-R* gene primers were used for reverse-transcription polymerase chain reaction (RT-PCR) amplification, and 599 bp of the *Pael-R* gene interior fragment was obtained (Figure 1A).

The gray scale values of RT-PCR bands were analyzed and the absorbance ratios between the *Pael-R* gene and GAPDH gene in each group were calculated and used for further comparison. There was no significant difference between the control group without rotenone treatment and the model group in terms of the level of *Pael-R* gene expression ($P > 0.05$). This indicates that rotenone has no significant impact on the transcription of the *Pael-R* gene in induced dopaminergic neuron-like cells. The absorbance ratio from the RT-PCR results in the RNA interference group was 14% less than that in the model group ($P < 0.05$; Figure 1A). This evidence confirmed that the pRNA-U6/*Pael-R* interference vector suppresses the expression of the *Pael-R* gene.

The *Pael-R* protein level in all three cell groups was determined by western blot analysis. A *Pael-R* band was obtained at 67 kDa (Figure 1B). The gray scale analysis of RT-PCR results revealed similar levels of *Pael-R* protein in model and control cells ($P > 0.05$), suggesting that rotenone also has no significant influence on *Pael-R* protein levels. In the RNA interference group, the *Pael-R* protein level was lower than those in the control and model groups ($P < 0.05$; Figure

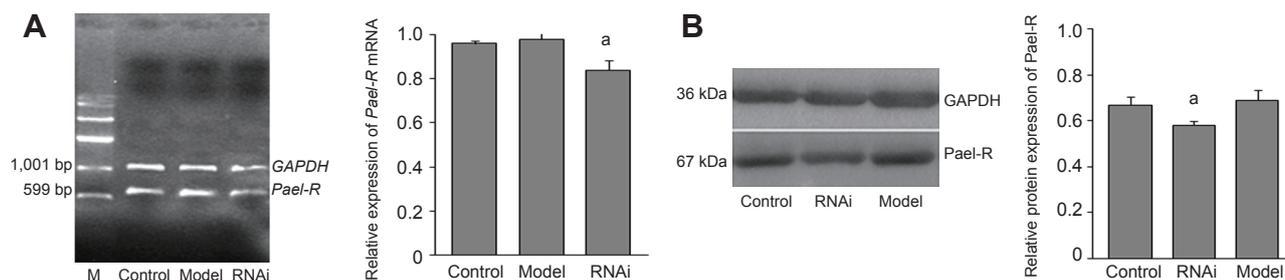


Figure 1 Effect of RNA interference (RNAi) on *Pael-R* gene and protein expression in Parkinson's disease model cells.

(A) *Pael-R* gene expression detected by reverse-transcription polymerase chain reaction (RT-PCR). (B) *Pael-R* protein expression detected by western blot analysis. Expression of *Pael-R* gene and protein was decreased in Parkinson's disease model cells by RNA interference ($^aP < 0.05$, vs. model group). Both expression of *Pael-R* gene and protein was almost the same in the control group and the model group, but was lower in the RNAi group. Data are expressed as absorbance ratio of target gene/protein to GAPDH (mean \pm SD) and were analyzed using analysis of variance. M: Marker.

1B), indicating that transfection of cells with the pRNA-U6/*Pael-R* vector could effectively down-regulate *Pael-R* protein expression.

RNA interference had no significant influence on apoptosis of Parkinson's disease model cells

Morphological changes to cells were observed under a fluorescence microscope with Hoechst 33258 staining. There were no apoptotic cells in the control group. In the model group, some cells showed nuclear condensation or nuclear division and the nuclei appeared light blue with an irregular shape. These findings indicated the presence of apoptosis. In the RNA interference group, some cells showed nuclear condensation and division, which also confirmed the presence of apoptotic cells (Figure 2A–C).

We also examined the rates of apoptosis in the different cell groups by flow cytometry. The results were similar to those obtained with Hoechst 33258 staining. Cell apoptosis rates in the model and RNA interference groups were not statistically significantly different ($P > 0.05$; Figure 2D). This suggested that there is no significant influence of down-regulation of *Pael-R* gene expression on rotenone-induced apoptosis.

RNA interference had no significant influence on the viability of Parkinson's disease model cells

According to the results of the MTT assay, the cell survival rates in the model group and RNA interference group were significantly lower than that in the control group ($P < 0.05$). There was no statistically significant difference between the model group and the RNA interference group ($P > 0.05$; Figure 2E). These findings indicated that the cell survival rates decrease after rotenone treatment, but that this decrease in survival could not be improved by down-regulating *Pael-R* expression in cells.

Discussion

Widespread attention has been paid to rotenone as a potential cause of Parkinsonism. Recent studies have shown that long-term exposure to rotenone may cause changes at the cellular level in the form of injury to central dopaminergic neurons, obvious degeneration and apoptosis of substantia nigra dopaminergic neurons and the appearance

of Lewy bodies in neurons, tremor, unsteady gait and other Parkinson's disease symptoms^[22-23]. Currently, the precise mechanisms underlying rotenone-induced apoptosis in dopaminergic neurons are unclear. In the cell injury induced by rotenone, the following processes are known to participate: increased levels of reactive oxygen species; lipid peroxidation; progressive glutathione deficiency; mitochondrial depolarization; release of cytochrome C; and activation of caspase-3^[24-27]. These factors collectively lead to apoptosis, which, particularly when affecting dopaminergic neurons, is one of the important insults leading to Parkinson's disease^[28-29]. In 2006, Feng^[30] reported that apoptosis of dopaminergic neurons induced by rotenone may be related to damage to the intracellular microtubule system, thus leading to release of dopamine in cells, which causes cell toxicity. In addition, Pan-Montojo et al.^[31] found that rotenone causes alpha-synuclein deposition in the nervous system and further induces apoptosis of neuronal cells.

It has been found that some similar molecular mechanisms in the pathogenesis of Parkinson's disease are mediated by environmental toxins and genetic flaws^[32-33]. *Pael-R* protein deposition is an important factor during the course of apoptosis of dopaminergic neurons owing to *Parkin* gene mutations^[34-36]. We discussed the relationship between down-regulation of *Pael-R* gene expression and apoptosis of dopaminergic neurons induced by rotenone in this study. Furthermore, because the function of *Pael-R* is not well understood, it may be a component of other signaling pathways; therefore, we performed transient transfection of cells with an RNA interference vector to partially down-regulate the expression of *Pael-R*, and maintained a lower level of *Pael-R* expression in cells to avoid influences from other pathways.

Our results demonstrate that dopaminergic neuron-like cells show obvious apoptosis after the treatment with rotenone. The results of gene expression analyses indicate that there were no significant differences in *Pael-R* transcript and protein levels before and after rotenone treatment. This finding suggests that rotenone has no obvious effect on *Pael-R* gene expression, and that *Pael-R* probably does not participate in the course of apoptosis of dopaminergic neuron-like cells induced by rotenone. Further investigations revealed that after down-regulating *Pael-R* gene expression by RNA

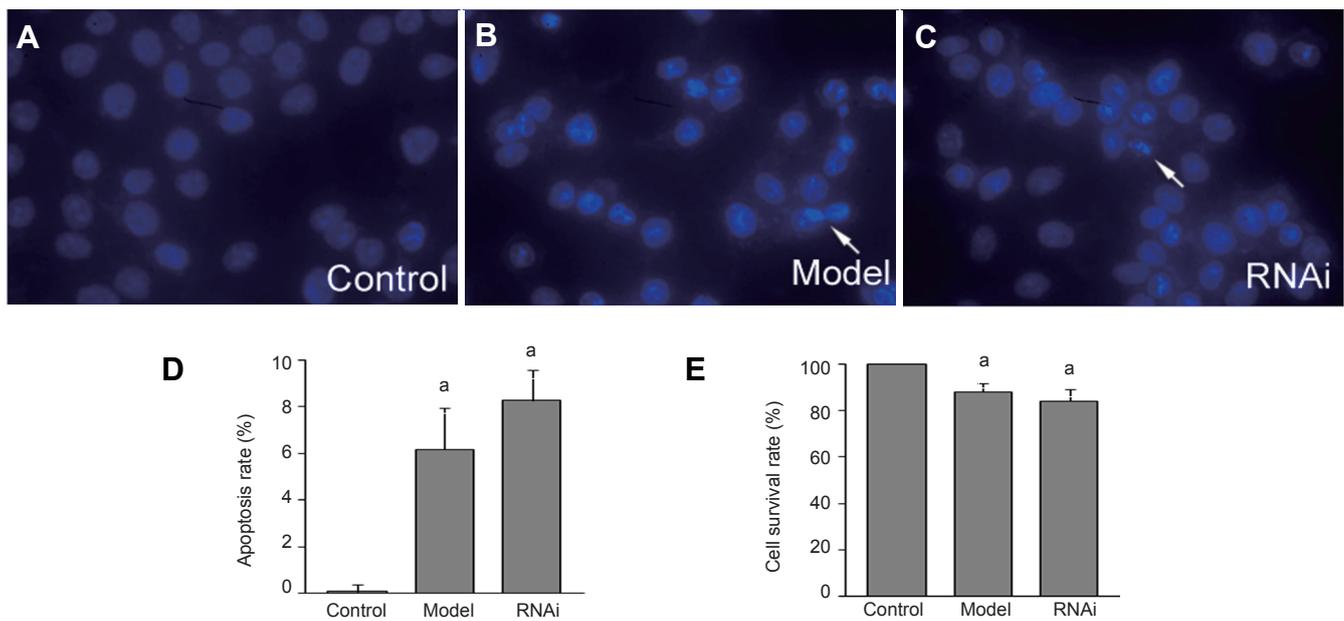


Figure 2 Effect of RNA interference (RNAi) on apoptosis and survival rate of Parkinson's disease model cells.

(A–C) Morphological changes to apoptotic cells that appeared in model and RNAi groups were detected by Hoechst 33258 staining (fluorescence inverted microscope, $\times 400$); apoptotic cells were stained as blue. (D) Apoptosis rate was detected by flow cytometry. (E) Cell survival rate was detected by MTT assay. The rate of apoptosis and survival rate in Parkinson's disease cells in the model group was not significantly different from that in the RNAi group. Data are expressed as mean \pm SD and were analyzed using analysis of variance. ^a $P < 0.05$, vs. control group.

interference in the rotenone-treated cells, there were no significant changes in cell viability and level of apoptosis. This result suggests that down-regulating *Pael-R* gene expression could not promote the survival of Parkinson's disease model cells, and that down-regulation of *Pael-R* gene has no protective action in rotenone-induced Parkinson's disease model cells.

Materials and Methods

Design

A controlled cytobiology study.

Time and setting

The experiments were performed at the Cancer Research Institute, Xiangya Medical School of Central South University, China from June 2007 to March 2008.

Materials

PC12 cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, China, and rotenone was purchased from Sigma, St. Louis, MO, USA.

Methods

Induction of dopaminergic neuron-like cells

PC12 cells were incubated in culture plates covered with poly-L-lysine, and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) including 10% fetal calf serum (Invitrogen) in an incubator with 5% CO₂ at 37°C. PC12 cells were plated at a density of 2.5–10 $\times 10^4$ cells/cm² during induction and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 50 ng/mL nerve growth factor (Invitrogen). The medium was changed every

2–3 days. Neurites could be observed 1 week after induction and culture; the cells then became dopaminergic neuron-like cells at 10 days^[37-38].

Establishment of a rotenone-induced Parkinson's disease cell model

Induced dopaminergic neuron-like cells were plated in 6-well plates at a density of 5 $\times 10^5$ cells/well. Rotenone was added after 24 hours to a final concentration of 1 μ mol/L. The rotenone-induced Parkinson's disease cell model was established after 24 hours of treatment^[39-40].

Down-regulation of the *Pael-R* gene using RNA interference in rotenone-induced Parkinson's model cells

The pRNA-U6/*Pael-R* interference vector was designed for rat *Pael-R* gene and constructed as previously described^[41]. Parkinson's disease model cells treated with rotenone were plated in 6-well plates at a density of 5 $\times 10^5$ cells/well for 24 hours. Then, the pRNA-U6/*Pael-R* vector was transfected into cells together with Lipofectin 2000 (Invitrogen) according to the manufacturer's protocol. Specifically, 4 μ g of vector was mixed with 10 μ L of Lipofectin 2000 reagent, and cells were incubated with the mixture for 24 hours. Cells can be collected for tests 48 hours after transfection.

Detection of *Pael-R* gene expression by RT-PCR

Differences in the levels of transcription of the *Pael-R* gene were detected by RT-PCR after extracting total RNA from cells. *Pael-R* gene primer was designed using Primer3 software (Howard Hughes Medical Institute, Chevy Chase, MD, USA) to obtain 599 bp of the *Pael-R* gene interior fragment. The upstream primer was 5'-AAC CGA CGC GTG AGA

CTG AA-3', and the downstream primer was 5'-TGC GCC ATC ATA AGT GAG AGC-3'. The rat *GAPDH* gene served as an internal reference; the upstream primer was 5'-TGG TGA AGG TCG GTG TGA AC-3', the downstream primer was 5'-TTA CTC CTT GGA GGC CAT GT-3', and the product was 1,001 bp. The gray scale values for bands in the RT-PCR results were then analyzed by the Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) and results were calculated according to the formula: absorbance rate of *Pael-R* gene/*GAPDH* gene.

Detection of Pael-R protein by western blot analysis

Proteins from all three cell groups were prepared by cell lysis. Differences in the levels of Pael-R protein were assessed using goat-anti-rat Pael-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total protein (20 µg) was taken from each group for electrophoresis and subsequent transfer of proteins onto a polyvinylidene fluoride membrane. Membranes were blocked for 1 hour at room temperature, then incubated in primary antibody (1:1,000) overnight at 4°C, followed by incubation with rabbit anti-goat IgG (1:5,000; Sigma) for 1 hour at room temperature. Next, samples were treated for chemiluminescence with ECL fluid. Gray scale value analysis was then performed using the Gel Doc XR+ System (Bio-Rad) according to the formula: absorbance rate of *Pael-R* protein/*GAPDH* protein.

Cell morphological changes tested by Hoechst 33258 staining

Hoechst 33258 solution (Sigma) was added into the three groups of cultured cells to a final concentration of 10 µg/mL; then, cells were incubated for 5 minutes at room temperature. Cell morphology was observed after PBS elution under an inverted fluorescence microscope (Leica, Wetzlar, Germany).

Cell apoptosis test by flow cytometry

Cells in the three groups were collected and the level of cell apoptosis in each group was assessed by cell cytometry (BD, Franklin Lakes, NJ, USA) using annexin V-FITC/propidium iodide double staining^[24].

Cell viability tested by MTT assay

Cells from the three groups were plated into 96-well plates at a density of 1×10^4 cells/well. MTT solution (10 µL, 5 g/L, Sigma) was added into each well at 48 hours after RNA interference. Cells were incubated for 4 hours in 5% CO₂ at 37°C, then culture medium was removed and 150 µL of dimethyl sulfoxide (Sigma) was added into each well and cultured for 10 minutes until all particles were completely dissolved. Finally, the absorbance value at 490 nm was measured using a Microplate Reader (Bio-Rad) and the cell survival rate was calculated. Survival rate (%) = treated group absorbance/untreated group absorbance \times 100%.

Statistical analysis

Statistical analysis was performed using SPSS software (Version 14.0, SPSS, Chicago, IL, USA). Data are expressed as mean \pm SD and were analyzed using one-way analysis of variance^[35]. Statistical significance was defined as $P < 0.05$.

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Author contributions: Zou T and Hu ZP designed the study and contributed to manuscript development. Zou T, Tang XQ and Huang ZL conducted the experiments. Zou T and Xu NG participated in data integrity and analysis. All authors had approved the final version of the paper.

Conflicts of interest: None declared.

Peer review: Using RT-PCR, western blot analysis, MTT and flow cytometry, we detected the effects of RNA interference-mediated *Pael-R* gene down-regulation in rotenone-induced Parkinson's disease model cells. The experiment purpose could be achieved by the techniques.

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