Increased PINK1 Confers a Neuroprotective Role After Glutamate Excitotoxicity in Neuronal Cells

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Research

Keywords: Ischemic stroke, Glutamate excitotoxicity, PINK1, Primary cortical neurons, HT22

Posted Date: July 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-736719/v1>

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Abstract

Background: Ischemic insults often leads to mitochondrial dysfunction and neuronal injury. The neuronal damage induced by ischemia can be partly attributed to glutamate excitotoxicity. Previous studies have indicated that PINK1 plays a neuroprotective role against ischemic brain injury via regulating mitochondrial integrity and function. However, there are few reports elucidating the expression changes and effect of PINK1 on neuronal survival in glutamate excitotoxicity model.

Methods: We utilized HT22 cells and primary cortical neurons to establish glutamate excitotoxicity neuronal model. Cell Counting Kit-8 was used to investigate the effects of different concentrations of glutamate on neuronal activity; RT-PCR assay was used to investigate PINK1 transcription level following glutamate excitotoxicity; Western-blot and immunofluorescence assays were used to evaluate PINK1 translation level after glutamate injury. Moreover, we established HT22 cell stable transformants by lentiviral transfection to determine the role of PINK1 on neuronal damages in glutamate excitotoxicity.

Results: Different concentrations of glutamate inhibited the cell viability of HT22 line and primary cortical neurons. Following glutamate treatment at different times, the mRNA level, protein level and cellular fluorescence intensity of PINK1 firstly increased and then decreased. In addition, cells with low PINK1 expression could reinforce the inhibitory effect of glutamate on neuronal activity compared with normal cells, while high PINK1 expression showed a protective effect on neurons.

Conclusion: PINK1 may play a neuroprotective role in glutamate induced neuronal excitotoxicity.

Background

Ischemic insult always leads to neuronal damage or cell death which is the basis of pathophysiological mechanisms in various ischemic diseases such as cerebral stroke and so on[1]. These diseases affect millions of people each year, placing a heavy social and economic burden on low- and middle-income countries in particular[2, 3]. The mechanisms of neuronal damage or cell death induced by ischemic insult include many cellular catastrophic cascades such as glutamate excitotoxicity, mitochondrial dysfunction, neuroinflammation, oxidative stress, intracellular Ca^{2+} accumulation, voltage-gated ion channel opening, neuronal apoptosis and so on[4–8]. Glutamate excitotoxicity has been reported as a fundamental hallmark of ischemia-induced neurodegeneration.[9] Decreased cerebral blood flow to 20% will results in severe damage to brain tissue and minutes of hypoxia leads to ATP depletion, Na^+/K^+ pump failure, neuronal depolarization and excessive glutamate release. Because suddenly increasing glutamate cannot be taken up by neurons or astrocytes, the extracellular glutamate makes Ca^{2+} enter the cells; then the Ca^{2+} overload triggers the activation of intracellular proteases, esterases and endonucleases, which eventually leads to the cell death of neurons[5, 10, 11].

Mitochondrial dysfunction has been reported to be associated with a variety of diseases, including neurodegenerative diseases and cardiovascular diseases[12–19]. PINK1 is a protein kinase containing an

N-terminal mitochondrial targeting sequence (MTS), a transmembrane segment (TM) and a serine/threonine kinase domain[20–23]. As a serine/threonine kinase, PINK1 often acts in concert with the E3 ubiquitin ligase Parkin to sense mitochondrial functional status, which in turn marks damaged mitochondria for degradation through the autophagic pathway[24, 25]. PINK1 also regulates the quality and morphology of mitochondria[21]. In addition, PINK1 is also involved in apoptosis, cancer development[26], metabolic regulation, cardiac function and inflammation.

Many studies have used glutamate excitotoxicity model to indirectly simulate ischemic stroke in vitro. It is well-known that mitochondria are important organelles for ATP synthesis. Impaired mitochondria due to ischemia will directly affect the energy supply to tissues and this energy failure leads to rapid loss of ATP and uncontrolled leakage of ions through the cell membrane, which ultimately also leads to enormous Ca^{2+} influx. Previous Studies showed that PINK1 plays a neuroprotective role via regulating the integrity and function of mitochondria[27].

Nevertheless, there are few reports elucidating the expression model of PINK1 and its effect on neuronal survival in glutamate excitotoxicity model. In this study, we aim to study the change and role of PINK1 in glutamate excitotoxicity model in HT22 cell line and primary cortical neurons to provide guidance for inhibiting neuronal damage caused by glutamate excitotoxicity.

Methods

Ethics statement

The experiments involving animals were approved by the Animal Care and Use Ethical Committee of Air Force Medical University and complied with the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health.

Animals and experimental design

15 days C57 pregnant mice were purchased from Experimental animal center of Air Force Medical University. The neurons of the cortex of fetal rats were extracted and cultured for subsequent experiments. In this experiment, the primary cortical neurons and cell lines (HT22) were used to carry out the experiment, and all the cells used in the experiment were in good condition. The cells were divided into different glutamate concentrations and different glutamate stimulation time until RT-PCR, Western blot and immunofluorescence were used to analyze the cells.

Cell culture

HT-22 cells were purchased from Shanghai ZhongqiaoXinzhou Biotechnology Co., Ltd. the cells were cultured in DMEM containing 10% FBS at 37 °C in 5% CO₂ incubator. When the cell density reached 80%-90%, the cells were passaged according to the ratio of 1:3.

Extraction and culture of primary cortical neurons in mice

The culture dish was coated with 0.2mg/mL poly-L-lysine (PLL) overnight at 37 °C, then washed with sterile water for three times, and put into incubator for use. The culture dish was added with DMEM containing 10% FBS and pre-cooled on the ice box. C57 mice about 15 days of gestation were killed and disinfected with 75% alcohol. The primary neurons were extracted under the stereomicroscope. According to the required density of the experiment, the complete medium was used for appropriate dilution, and the cell suspension was made into seed plate. After 4-6 h, DMEM was replaced by the special fresh culture medium for neurons (50mL Neurobasal + 125uL Glu + 500ul antibiotic + 1ml B-27 supplement); after 5-7 days, the mature neurons were treated according to the experimental design.

Cell Counting Kit-8 assay

HT22 cells in good growth condition were prepared into single cell suspension and counted. The cells were seeded into 96-well plates according to the density of 2000 cells/100uL per well. After 4-6h, the adherent cells were treated with glutamate at the corresponding concentration and time point. After the treatment, the cells were exposed to the medium containing 10% CCK solution. The absorbance was measured at 450nm wavelength after incubation at 37°C for 2h.

Western Blot

Cells were collected in 1.5mL EP tubes and RIPA buffer was added to lyse the cell samples. BCA Kit was used for quantitative detection. SDS-PAGE gel electrophoresis was carried out under the total protein content of 30ug in each lane. After electrophoresis, the protein was transferred to PVDF membrane and then the membrane was blocked by 5% BSA at room temperature. The PVDF membrane was incubated with primary antibody diluents overnight at 4 °C. Then, the membrane was washed with TBST for 3 times, 5 minutes each time and then incubated with the second antibody diluent at room temperature for 1 h; then the PVDF membrane was washed with TBST for 3 times, 5 minutes each time. The protein expression was analyzed after ECL exposure.

Immunofluorescence

The cells were placed in 24 well plates and seeded with a suitable density. When the cell density reached 50% -80%, the cells were treated according to the specific experimental treatment, and then washed with PBS for 3 times, 5 minutes each time. The cells were fixed with 4% paraformaldehyde for 20 minutes and rinsed with PBS 3 times, 5 minutes each time. 0.3% Triton X-100 / PBS was added to slides for 20 minutes at room temperature, and then washed with PBS three times for 3 times, 5 minutes each time. Donkey serum was dripped onto the cells and sealed at room temperature for 30 minutes. After removing the blocking solution, diluted primary antibody was dripped onto each climbing piece and then put the piece into a wet box and incubated at 4 °C overnight. Then the cells were rinsed with PBST for 3 times, 5 minutes each time. Next, diluted fluorescent antibody was added onto the cells in dark environment and the slides were incubated in wet box for 1h at room temperature, and then rinsed with PBST for 3 times, 5 minutes each time. Then the slides were incubated with DAPI in dark for 5 minutes to stain the nuclei.

After rinsing with PBST for 3 times, 5 minutes each time, the Cells were observed under fluorescence microscope.

RT-PCR

The total RNA was extracted by Trizol method after the related treatments. Reverse transcription was performed according to the protocol in the 5X Prime Script RT Master Mix kit and RT-PCR was performed using the TB Green Premix Ex TaqII (TliRNaseH Plus) kit. The primer sequences for GAPDH were (forward) 5'-GGTGAAGGTCGGTGTGAACG-3' and (reverse) 5'-CTCGCTCCTGGAAGATGGTG-3'. The primer sequences for PINK1 are (forward) 5'-GATGACCTTGCAGTTGCTGGAG-3' and (reverse) 5'-CAGCAGCCAAAGTCTGAGATCACTA-3'. The expression of the relevant RNA was calculated by the $2^{-\Delta\Delta Ct}$ method and GAPDH was used as a control.

Statistical analysis

SPSS (17.0) software was used for statistical analysis of the data. T-test was used for the measurement data in accordance with the Pacific distribution between the two groups, and One-way ANOVA was used for the comparison between multiple groups. The data were expressed as mean $\pm$ standard deviation. $P < 0.05$ means the difference is statistically significant.

Result

Inhibitory effects of different concentrations of glutamate on neuronal activity

We used CCK8 assay to detect the effect of different concentrations of glutamate on HT22 cell line and primary cell activity. In HT22 cell line, the results showed that HT22 cell activity decreased significantly when treated with 2 mM and 8 mM glutamate for 24 h. Compared with the control group, there was statistical significance ($P < 0.05$). The inhibitory effect of 4 mM glutamate treatment for 24 h was weaker than that of 2 mM and 8 mM treatment groups, and there was no significant difference compared with the control group. When treated with high concentration glutamate (16, 32 mM), we did not observe the inhibitory effect of glutamate on the cells, and there was no significant difference compared with the control group. (Fig. 1a). Moreover, in primary cells, the results showed that cell activity decreased significantly after 24 h of glutamate treatment. Compared with the control group, the difference was statistically significant ($P < 0.05$). The inhibitory effect of 0.025 mM glutamate for 24 h was 50% compared with the control group. (Fig. 1b).

Glutamate treatment presented a time-dependent increase in mRNA levels of PINK1 in neurons

RT-PCR was used to detect mRNA level changes of PINK1 gene in HT22 cells treated with 8 mM glutamate for different periods of time. The results showed that the mRNA level of PINK1 presented a time-dependent increase, and the expression of PINK1 increased significantly at 6h of treatment. (Fig. 2a); Similarly, the mRNA level of PINK1 gene in primary cells treated with 0.025 mm glutamate for different

time was detected by RT qPCR. The results showed that the mRNA level of PINK1 increased in a time-dependent manner, which was consistent with the results of HT22. The expression of PINK1 increased significantly after 6 h of treatment, which was statistically significant compared with the control group. After 12 h and 24 h treatment, PINK1 mRNA level increased, but the increase was slower than that of 6 h treatment. (Fig. 2b).

The effect of glutamate treatment on PINK1 translation in neurons at different times showed a trend of first increase and then decrease

The expression of PINK1 was detected by Western blot. In HT22 cells, the cells were treated with 8 mM glutamate for 3, 6, 12 and 24 h. The expression of PINK1 had a certain regularity at different time and reached the peak at 6 h. (Fig. 3a). After quantification, the expression of PINK1 reached the peak at 6 h, which was statistically significant compared with the control group ($p < 0.05$). In addition, after 3 h and 12 h treatment, the expression level of PINK1 also increased, which was statistically significant compared with the control group. However, the results showed that the expression of PINK1 had no statistical significance after glutamate treatment for 24 h. (Fig. 3c) Therefore, in the following experiment, the glutamate excitotoxicity model was established in HT22 cells under the condition of 8 mM / 6 h. In the primary neurons, the cells were treated with 0.025 mM glutamate for 3 h, 6 h, 12 h and 24 h. Western blot showed that the expression of PINK1 changed significantly at different time points. After 3 h, 6 h and 12 h, the expression of PINK1 increased significantly; (Fig. 3b). The quantitative results showed that the expression of PINK1 increased significantly after glutamate treatment for 3 h, 6 h, 12 h and 24 h, and reached the peak at 6 h, which was statistically significant compared with the control group. (Fig. 3d) Therefore, in the following experiment, the glutamate excitotoxicity model of primary neurons was established with 0.025 mM/6 h. Therefore, in the following experiment, we used 0.025 mM / 6 h to establish the glutamate excitotoxicity model of primary neurons.

The protein expression of PINK1 in neurons showed a trend of first increase and then decrease and localized in the cytosol

We further explored the expression and localization of PINK1 by immunofluorescence staining. The results showed that HT22 cells were treated with 8 mM glutamate for 3 h, 6 h, 12 h and 24 h, respectively. It was found that the fluorescence intensity was the highest at 6 h. The fluorescence intensity at 3 h was the second. After 12 h and 24 h treatment, the fluorescence intensity was the weakest. (Fig. 4a) The results of quantification confirmed our preliminary observation. The fluorescence intensity of cells after 6 h treatment was the strongest, which was statistically significant compared with the control group ($P < 0.05$). The fluorescence intensity after 3 h treatment was the second, and the results after 12 h and 24 h treatment were not statistically significant compared with the control group. (Fig. 4c) Similarly, the primary cells were treated with 0.025 mM glutamate for 3 h, 6 h, 12 h and 24 h, respectively. Preliminary observation showed that the fluorescence intensity was the highest at 6 h. The fluorescence intensity at 3 h and 12 h followed. After 24 h treatment, the fluorescence intensity was lower than that of the control group (Fig. 4b). The results of quantification confirmed our preliminary observation that the fluorescence

intensity of cells treated for 6 h was the strongest, which was statistically significant compared with the control group ($P < 0.05$), followed by that of cells treated for 12 h, and that of cells treated for 3 h was weaker, and that of cells treated for 24 h was lower than that of the control group (Fig. 4d). On the other hand, immunofluorescence results showed that PINK1 was in the cytoplasm.

PINK1 lentivirus was successfully transfected into HT22 cells

HT22 cells were transfected with LV-PINK1, CON-313, PINK1-RNAi (58770-2), PINK1-RNAi (58771-1), and PINK1-RNAi (58772-1) lentiviruses for 72 h (Fig. S a). Proteins and RNA were extracted, and PINK1 transfection efficiency was detected by Western Blot, RT-PCR, and immunofluorescence staining. The results showed that PINK1 was significantly reduced at both transcriptional (Fig. S b) and translational (Fig. S c) levels after PINK1-RNAi (58771-1) transfection into HT22 cells, and empty virus vector CON-313 had no significant effect on PINK1 expression in HT22 cells. The results of fluorescence assay showed that LV-PINK1 and PINK1-RNAi (58771-1) lentiviruses were successfully transfected into HT22 cells (Fig. S d).

PINK1 protects HT22 cells from glutamate induced excitotoxicity

This part of the experiment was divided into five groups: control group (without any intervention), L-Glu group (to establish glutamate excitotoxicity model group), vehicle/L-Glu group (using control empty virus intervention and glutamate treatment), PINK1-RNAi/L-Glu group (using PINK1 interference lentivirus intervention and glutamate treatment), and LV-PINK1/L-Glu group (using PINK1 overexpression lentivirus intervention and glutamate treatment). The results showed that compared with the control group, the cell viability of the L-Glu group was significantly decreased; there was no significant difference in the cell viability of the vehicle/L-Glu group; compared with the vehicle/L-Glu group, the cell viability of the PINK1-RNAi/L-Glu group was significantly decreased while the cell viability of the LV-PINK1/L-Glu group was significantly increased (Fig. 5).

Discussion

In this study, we found that different concentrations of glutamate inhibited cell viability in both HT22 cells and mouse primary cortical neurons. The maximum inhibitory effect on the viability of HT22 cells and primary cells was achieved at 8 mM/24 h and 0.025 mM/6 h, respectively. After glutamate treatment for different periods of time, both the mRNA and protein levels of PINK1 showed a trend of first increase and then decrease. In addition, we found that knockdown of PINK1 intensified the reduction of cell viability by glutamate, while overexpression of PINK1 alleviated the reduction of cell viability by glutamate and played a cytoprotective role.

It is well-known that the increase of extracellular glutamate level can lead to excessive activation of glutamate excitability, resulting in calcium and magnesium ion exchange, intracellular calcium ion accumulation, and ultimately toxicity to cells [28]. When we study the damage effect of glutamate on cells, we should fully consider the effect of ions contained in the culture system on cells. Therefore, the culture system used in this study is Hank's balanced salt buffer without calcium and magnesium ions.

However, it was also reported that they used complete culture medium until detection, such as BME, DMEM neurobasal, etc.

It has been reported that glutamate excitotoxicity may be a common pathway for neuronal death such as neurological diseases and ischemic injury[29, 30]. Therefore, glutamate excitotoxicity model is an important experimental method to understand neuronal injury. Under normal physiological conditions, glutamate can control activity-dependent depolarization of neurons and contribute to neuronal differentiation and survival. However, when the extracellular glutamate level increases, it can lead to excessive activation of glutamate excitability and ultimately toxicity to cells[31]. HT22 cell line, namely mouse hippocampal neuron cell line, was used in this study. It is an excellent cell model for studying glutamate toxicity in vitro. It has been reported that HT22 cells have obvious morphological changes when exposed to 25 mM glutamate for 24 h, and the cell viability decreases by about 50%[32]. Some studies have also confirmed that Homer1a can protect HT-22 cells from glutamate induced oxidative damage, and the concentration / exposure time of glutamate used is 10 mM / 24 h[33]. Therefore, the concentration and time of glutamate causing cytotoxicity in vitro is not the same due to differences in experimental conditions, cell species status, culture process, drug solvent, and so on. The more appropriate concentration and time of glutamate induced excitotoxicity in cells usually reduced the cell viability to about 50%, while the glutamate treatment concentration/exposure time used in this experiment was 8 mM/6 h for HT22 cell line and 0.025 mM/6 h for primary cortical neurons, and the cell viability was half that of normal cells at this concentration and time. Interestingly, we found that the viability of the HT22 cell line instead rose in response to 4 mM/16 mM/32 mM glutamate concentrations, tending towards the control group. We speculate that this may be a self-protective effect of cells, originating from the compensatory effect of cells.

It has been found that the expression level of PINK1 increased significantly after mitochondrial dysfunction induced by ischemia[34]. It is puzzling that our research on PINK1 in glutamate excitotoxicity model found that the expression of PINK1 in mRNA and protein levels and fluorescence intensity showed a trend of first rising and then declining. We speculate that it may be because under the stimulation of glutamate, cells trigger their own "compensatory" mechanism to maintain normal physiological functions, resulting in the up-regulation of PINK1 expression.

PINK1 protein can be divided into several structural and functional regions: mitochondrial targeting sequence (MTS), outer membrane localization signal (OMS), transmembrane domain (TMD), and kinase domain (KD). It has been reported that PINK1 is constantly transferred to the inner membrane of mitochondria under normal physiological conditions. When the mitochondrial membrane potential is damaged, the pathway of PINK1 entering the inner membrane is blocked, and PINK1 accumulates in the outer membrane of mitochondria[35]. Our immunofluorescence results showed that PINK1 was in the cytoplasm, which was consistent with the reported results, suggesting that the function of PINK1 mainly functions in the cytoplasm.

In contrast to other studies, this assay is the first to use cell lines and primary cells to comprehensively investigate the effects of glutamate on cells and reveal the expression pattern of PINK1 in neurons. However, the shortcomings are: 1. There is a lack of validation of in vitro experiments; 2. This study focuses on ischemic stroke and should be further explored using clinical specimens; 3. In this study, the role of PINK1 was studied using lentiviruses, and agonists and inhibitors of PINK1 can also be selected for study.

Conclusion

Glutamate excitotoxicity can cause neuronal damage, and then lead to neuronal dysfunction, leading to a series of changes in related pathways. We found that the expression of PINK1 in neurons increased at first and then decreased after glutamate treatment. We found that PINK1 may play a protective role in neurons. However, the specific molecular mechanism has not yet been elucidated, which needs further study.

Declarations

Acknowledgements

The authors thank Dr. Xiu-Quan Wu and Dr. Xiao-Wei Fei for participating in the discussion of the manuscript. We especially thank the relevant researchers in the Neurosurgery Laboratory of the First Affiliated Hospital of Military Medical University, Chinese PLA Air Force for their participation and help.

Authors' Contributions

YND, and XQW conceived and designed the experiments; YND and XQW performed the experiments; YND and XWF contributed reagents/ materials; YND contributed data analyzed; YND wrote the manuscript and ZF revised it. All authors read and approved the final manuscript.

Funding

This research was funded by the National Natural Science Foundation of China, grant number 81771239.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All experiments involving the animals were approved by the Institutional Animal Ethics Committee of Air Force Medical University. All procedures performed in the study involving the animals were followed "The Guide for the Care and Use of Laboratory Animals" guidelines for animal welfare.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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Figures

Figure 1

Effects of different concentrations of glutamate on neuronal activity. a. HT22 cells were treated with different concentrations (2, 4, 8, 16, 32 mM) of glutamate for 24 h, incubated with CCK-8 and measured after 2 h by the CCK-8 assay. b. Primary cortical neurons were treated with different concentrations (0.01, 0.025, 0.05, 0.1, 0.25,0.5mM) of glutamate for 24 h, incubated with CCK-8 and measured after 2 h by the CCK-8 assay. Data are expressed as mean ± standard deviation of three independent experiments. *P < 0.05 vs. the control group.

Figure 2

The expression of PINK1 was detected by mRNA level. a. HT22 cells were treated with 8 mM glutamate for 3, 6, 12 and 24 h, respectively. The expression of PINK1 was detected by RT-PCR. *: P < 0.05 vs. the control group. b. Primary cortical neurons were treated with 0.025 mM glutamate for 3, 6, 12 and 24 h, respectively. The expression of PINK1 was detected by RT-PCR. *: P < 0.05 vs. the control group

Figure 3

Expression of PINK1 under glutamate treatment. a. HT22 cells were treated with 8 mM glutamate for 3, 6, 12 and 24 h respectively. The expression of PINK1 was detected by Western blot. b. The primary cortical neurons were treated with 0.025 mM glutamate for 3, 6, 12 and 24 h respectively. The expression of PINK1 was detected by Western blot. c. Quantification of PINK1 in HT22. d. Quantification of PINK1 in primary cortical neurons. *: $P < 0.05$ vs. the control group

Figure 4

The expression and localization of PINK1 were detected by immunofluorescence. a. HT22 cells were treated with 8 mM glutamate for 3, 6, 12 and 24 h respectively. The expression and localization of PINK1 were detected by immunofluorescence staining. Scale bar: 50 μ m b. Primary cortical neurons were treated with 0.025mM glutamate for 3, 6, 12 and 24 h, respectively. The expression and localization of PINK1

were detected by immunofluorescence staining. Scale bar: 100 μ m. c. Quantification of PINK1 in HT22. d. Quantification of PINK1 in Primary cortical neurons. *: $P < 0.05$ vs. the control group

Figure 5
CCK-8 assay for HT22 cells. Cell viability was measured by microplate reader at 450 nm, *: $P < 0.05$ vs. the control group; ns: no statistical difference

Supplementary Files

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