Parthanatos Involves in the Damage of Hippocampal Neurons Following Epileptic Seizure: in Vivo and Vitro Study

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Research Article

Keywords: Epilepsy, Glutamate, PARP-1, Parthanatos, ROS

Posted Date: October 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1010695/v1>

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Abstract

Epileptic seizures, or status epilepticus can cause hippocampal neurons death, which have several detrimental effects including promoting the progression of epilepsy, the formation of hippocampal sclerosis and cognitive impairment. Exploring the underlying molecular mechanisms of seizure-induced neuronal death may ameliorate the serious impact of the brain caused by seizures and restrain the further progression of epilepsy. Parthanatos is a new form of programmed cell death characterized by hyper-activation of Poly (ADP-ribose) polymerase-1 (PARP-1), excessive synthesis of poly ADP-ribose (PAR) polymer, mitochondrial depolarization and translocation of apoptosis-inducing factor (AIF) to nucleus which was found in various neurodegeneration disorders but rarely reported in the field of epilepsy. This study aims to investigate whether parthanatos was involved in the mechanism of seizure-induced neuronal death in vivo and vitro study, and to provide new targets for neuroprotective treatment of epilepsy. In vivo study, our results showed that PARP-1 inhibitor PJ34 has protective effect on the damage of hippocampal neurons in rats after epileptic seizure and reduced the expression level of PARP-1 and the synthesis of PAR polymer as well as the translocation of apoptosis-inducing factor (AIF) from mitochondrial to the nucleus. In vitro study, we used glutamate-induced HT22 cell injury to establish cell damage model. Our results showed that glutamate induces HT22 cells death in a time dependent manner, accompanied with parthanatos-related biochemical events. Pretreatment with PJ34 or knockdown of PARP-1 with small interfering RNA effectively protected HT22 cells against the toxic effect of glutamate, and attenuated parthanatos-related biochemical events. Further, application of NAC was found to rescue HT22 cells death and reverse the above-mentioned parthanatos-related biochemical events via alleviating the overproduction of reactive oxygen species (ROS). In conclusion, our study confirmed that Parthanatos was involved in the damage of hippocampal neurons following epileptic seizure, and ROS may be the initial factor of Parthanatos.

1. Introduction

Epilepsy is a common chronic neurological disease affecting more than 68 million individuals worldwide [1]. The median prevalence of lifetime epilepsy is 5.8 per 1000 in developed countries and 10.3 per 1000 in developing countries [2]. The condition of prolonged epileptic seizures, or status epilepticus can cause different degrees of brain damage, especially in the hippocampus. Hippocampal neurons are sensitive to epileptic discharge and are prone to death, which may have several detrimental effects. Seizure-induced neuron death will further promote the progression of epilepsy and lead to the formation of hippocampal sclerosis (HS) [3]. In addition, studies have found that the damage of hippocampal neurons is closely related to cognitive impairment following epileptic seizure [4]. Therefore, it is particularly important to study the underlying molecular mechanisms of seizure-induced neuronal death which may ameliorate the serious impact of the brain caused by seizures and restrain the further progression of epilepsy.

Poly (ADP-ribose) polymerase-1 (PARP-1) dependent cell death, known as parthanatos, is a special pathway of cell death characterized by biochemical events including hyper-activation of PARP-1,

excessive synthesis of poly ADP-ribose (PAR) polymer, mitochondrial depolarization and translocation of apoptosis-inducing factor (AIF) to nucleus[5]. PARP-1 is a ribozyme maintaining the stability of the intracellular environment and responsible for DNA damage repair, transcriptional regulation, inflammation, metabolism, oncogene related signal transduction and cell differentiation and death [6]. PARP-1 can be activated by DNA damage via reactive oxygen species (ROS), alkylating agents light and ultraviolet (UV) [7-9]. Over activation of PARP-1 will rapidly deplete NAD⁺ and ATP to promote excessive synthesis and accumulation of PAR polymer which will further induce AIF translocation from mitochondrion to nucleus leading to bioenergetic collapse and cell death through inhibiting the glycolytic enzyme hexokinase [10, 11]. Currently, there are considerable evidences to support parthanatos in various neurological disorders. Pharmacological inhibition or genetic deletion of PARP-1 showed obvious neuroprotection in stroke, excitotoxic stress, Alzheimer disease, Parkinson's disease, and traumatic brain injury [12-17]. However, whether parthanatos involves in the mechanism of seizure-induced neuronal death is unknown.

Glutamate is a primary mediator in excessive excitatory neurotransmission induced by neuronal excitotoxicity. A high concentration of extracellular glutamate results in activation of N-methyl-D-aspartate receptor (NMDAR) which allows Ca²⁺ to entry into the neuronal cells [18]. The increase of intracellular Ca²⁺ elicit ROS production, and the sequent accumulation of ROS can oxidative lipids, DNA, and proteins leading to cellular damage [19]. Considering activation of PARP-1 is directly mediated by DNA damage, we speculate that parthanatos might be involved in the mechanism of seizure-induced neuronal death, and ROS might be an important initiating factor in the process of parthanatos. Therefore, we examined the role of PARP-1 in the damage of hippocampal neurons following epileptic seizure in vivo and vitro study and investigated the underlying mechanism.

2. Materials And Methods

2.1 Animals

Male Wistar rats (280–300 g) were used in this study from the animal center at Jilin University in China and provided with water and normal rat chow ad libitum under standard laboratory conditions with a 12-h light/dark cycle. In the experiment, we tried to reduce animal suffering and the number of animals used.

2.2 Epileptic models and drug administration

Epileptic models were induced by kainic acid (KA, Abcam, Cambridge, UK) based on a standard method previously published[20]. 36 healthy rats were randomly divided into three experimental groups as the following:

sham group (n=6): rats were injected in the amygdala with a volume of PBS.

KA+ vehicle group (n=6): epileptic rats were treated with vehicle.

KA+ PJ34 group (n=6): epileptic rats were treated with PJ34 (15mg/kg/day, 3 days before seizure induction and 3 days after seizure induction).

2.3 Pathological assessments

Six rats were randomly selected from each group for pathological evaluation on day 3. The brain tissues including entire hippocampus of animals were conducted and used for Fluoro-Jade B (FJB) staining (Millipore, Burlington Massachusetts, US) according the method previously published [20]. The numbers of FJB-positive cells were counted and visualized under fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.4 Cell lines and culture

HT22 cell line was thawed and then cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were incubated at 5% CO₂ under 37 °C and used at mid-log phase in the experiments.

2.5 CCK8 assay

HT22 cells (5×10³ cells/ well) were seeded and incubated onto 96-well microplates for 24 h. In order to establish glutamate-induced excitotoxicity cell model to simulate neuronal damage following seizures, the cellular viability in different dose of glutamate (0, 5, 10, 20 mM) at different time points (6, 12, 24 h) were assessed by CCK8 assay (Dojindo, Japan), and were expressed as a ratio to the absorbance value of the control cells at 450 nm.

2.6 Cell treatment and transfection of small interfering RNA (siRNA)

HT22 cells were incubated in DMEM containing 10% FBS and 1% penicillin-streptomycin supplemented with 10 Mm glutamate for different time points (6, 12, 24 h) to induce cell injury. For achieving parthanatos inhibition, PARP-1 inhibitor PJ34 (Selleck, Texas, USA) was pretreated 1 h with 20 µmol/L followed by glutamate incubation in TH22cells. For achieving ROS inhibition, antioxidant NAC (Sigma-Aldrich, USA) was pretreated 1 h with 3 mmol/L followed by glutamate incubation in TH22cells.

HT22 cells (5×10³ cells/ well) were seeded onto a 10cm-culture dish. Transfection of siRNA was performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After the cells were transfected by siRNA overnight, glutamate was used for incubation in the cells at indicated dose for the subsequent experiments.

2.7 Lactate dehydrogenase cytotoxicity assay

HT22 cells (5×10³ cells/ well) were seeded and incubated in 96-well microplates for 24 h. Cells were then treated with target compounds at indicated concentrations for indicated periods. Cytotoxicity was evaluated at 490 nm with Lactate dehydrogenase cytotoxicity assay kit (Beyotime Biotech, Nanjing,

China) according to the manufacturer's protocol. Cell death ratio was calculated following: cell death ratio % = (A_{sample}-A_{control}/A_{max}-A_{control}) × 100%.

2.8 Immunofluorescence staining

HT22 cells (0.5×10⁴ cells/ well) were seeded and incubated in the 3cm-culture dishes for 24 h. The cells were treated with glutamate for 24 h, fixed in 4 % paraformaldehyde, washed with PBS, and incubated with 1% Triton X-100 for 10 min. Then, the cells were blocked with 5% bovine serum albumin (BSA) for 10 min, and incubated with primary antibody against PAR (1: 100, Millipore, Massachusetts, USA) and AIF (1: 100, Abcam, Cambridge, UK, USA) overnight at 4 °C. After that, the cells were incubated with fluoroconjugated secondary antibody against mouse (1: 200, Abcam, Cambridge, UK, USA) or rabbit (1: 200, Abcam, Cambridge, UK, USA) for 1 h at room temperature. After counterstaining nuclei with DAPI (Beyotime Biotech, Jiangsu, China) for 5 min, the cells were visualized under fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.9 Mitochondrial membrane potential (JC-1) assay

HT22 cells treated with glutamate at indicated doses for different time and pretreated 1 h with 20 μmol/L PJ34 or knocking down of PARP-1 with siRNA overnight followed by glutamate incubation, were collected and stained with JC-1 (Beyotime Biotech, Nanjing, China) followed the methods described previously by Ma et al [21]. After that, the cells were analyzed by flow cytometry (CytoFLEX S, Beckman Coulter Inc, California, USA). The excitation wavelength of JC-1 is 488 nm, and the approximate emission wavelength of the monomeric and J-aggregate forms is 529 and 590 nm, respectively.

2.10 Measurement of ROS production

The level of intracellular ROS in HT22 cells incubated with glutamate alone or combined with indicated compounds was evaluated according to the instruction of the redox-sensitive dye DCFH-DA (Beyotime Biotech, Nanjing, China). The cells were washed twice with PBS and stained with DCFH-DA (20 μmol/L) for 30 min in the dark and then measured by using Flow cytometry (CytoFLEX S, Beckman Coulter Inc, California, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The cells were also observed by fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.11 Measurement of 8-OHdG level

8-hydroxy-2 deoxyguanosine (8-OHdG) is a common biomarker of DNA damage, which can reflect the level of DNA oxidative damage in cells. 8-OHdG in cells was examined following the instruction of the 8-OHdG detection elisa kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). HT22 cells in different groups were collected by centrifugation at 800 × g for 5 min, and washed twice with PBS. Cells were crushed using PBS as the medium to form homogenate, and then centrifuged at 10000 × g for 10 min at 4 °C to get the supernatant for detection of 8-OHdG. Standard pores and measuring pores were set

in the experiment, and then each pore was operated by relevant reactions. OD value of each pore were determined at 450 nm wavelength by enzyme labeling.

2.12 Measurement of glutathione (GSH) level

The level of intracellular total GSH was detected following the instruction of the DTNB-GSSH reductase recycling assay kit (Beyotime Biotechnology, Nanjing, China). HT22 cells in different groups were collected by centrifugation at $800 \times g$ for 5 min, washed twice with PBS, and crushed using PBS as the medium to form homogenate, and then centrifuged at $10000 \times g$ for 10 min at 4 °C to get the supernatant for detection of intracellular total GSH. Blank pores, standard pores and measuring pores were set in the experiment, and the OD value of each pore were determined at 405 nm wavelength by enzyme labeling. GSH content was calculated according to formula ($\mu\text{mol/g}$ protein).

2.13 Western blot analysis

Hippocampal tissue and HT22 cells were collected according to the standard protocols. Total proteins were extracted by using RIPA lysis buffer (Beyotime Biotec, Jiangsu, China) containing 1% proteinase inhibitors (Beyotime Biotec, Jiangsu, China). Nuclear and cytoplasmic proteins were isolated by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotec, Jiangsu, China). Mitochondria proteins from HT22 cells and hippocampal tissue were isolated following the instruction of Cell Mitochondria Isolation Kit (Beyotime Biotec, Jiangsu, China) and Tissue Mitochondria Isolation Kit (Beyotime Biotec, Jiangsu, China) respectively.

BCA Protein Assay Kit (Beyotime Biotec, Jiangsu, China) was used to quantify protein concentration of the samples. Equal amounts of protein samples were electrophoresed to 10 % SDS gels and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocked with 5% BSA for 1 h at room temperature, the membranes were incubated with anti-PARP-1 (1:1000, Abcam, Cambridge, UK, USA), anti-PAR (1:200, Millipore, Massachusetts, USA), anti-AIF (1:1000, Abcam, Cambridge, UK, USA), anti- β -actin (1:1000, Abcam, Cambridge, UK, USA), anti-COX IV (1:1000, Abcam, Cambridge, UK, USA), and anti-Histone H3 (1:1000, Abcam, Cambridge, UK, USA) overnight at 4 °C. After being incubated with HRP-conjugated secondary antibodies against mouse (1: 3000, Abcam, Cambridge, UK, USA) or rabbit (1: 3000, Abcam, Cambridge, UK, USA) for 1 h at room temperature, the membranes were washed three times with PBS and then analyzed using Odyssey infrared imaging system (LiCor, USA). The protein bands were quantified by Quantity One software (Bio-Rad laboratories).

2.14 Statistical analysis

All experimental data was conducted at least 6 independent experiments and expressed as mean $\pm$ SD. Statistical analysis was used by GraphPad Prism 5.0. Student's t-test or one-way analysis of variance Bonferroni's test was used for post-hoc comparisons. The value of P less than 0.05 was taken to indicate significance and P less than 0.01 was regarded as statistically very significance.

3. Results

3.1 PARP-1 inhibitor PJ34 attenuated neuronal loss in epileptic rats induced by KA

To evaluate the beneficial effects of PARP-1 inhibitor PJ34 on neuron loss in KA-induced epileptic rats, we administered PJ34 (15mg/kg/day, 3 days before seizure induction and 3 days after seizure induction) to the rats. At 3 day after seizure induction, FJB staining showed significantly increased hippocampal neuron damage in the CA1 and CA3 regions in KA+vehicle group compared with the sham group (CA1, 59.1 ± 3.9 vs 1.2 ± 0.8 , $P < 0.01$; CA3, 72 ± 4.7 vs 1.5 ± 1.0 , $P < 0.01$; $n = 6$), whereas in the KA+PJ34 group, FJB-positive neuronal damage were significantly fewer than that in KA +vehicle group (CA1, 29.5 ± 2.7 vs 59.1 ± 3.9 , $P < 0.01$; CA3, 48.3 ± 4.9 vs 72 ± 4.7 , $P < 0.01$; $n = 6$) (see Fig. 1). These results suggested that PARP-1 inhibitor PJ34 protected against KA-induced epileptic neuronal loss

3.2 PJ34 regulated the expression levels of parthanatos-related proteins in epileptic rats induced by KA

To evaluate the effects of PJ34 on the protein levels of PARP-1, PAR polymer, and AIF (mitochondrial and nucleus) in the epileptic rats, western blot analysis was performed at 3d after seizure induction. As shown in **Fig. 2**, the protein levels of PARP-1 and PAR polymer were significantly increased in KA+vehicle group rats compared with the sham group, whereas with PJ34 administration, the protein levels of PARP-1 and PAR polymer were significantly decreased. Furthermore, the expression level of AIF was decreased in mitochondrial but increased in nucleus in KA+vehicle group rats compared with the sham group, which can be ameliorated by administration of PJ34. These results revealed that Parthanatos might be involved in the damage of hippocampal neurons after epilepsy in rats, and PARP-1 inhibitor PJ34 played neuroprotective effect and inhibited Parthanatos in epileptic rats.

3.3 Effects of glutamate on HT22 cells

In order to investigate the toxic effects of glutamate on HT22 cells, cellular viabilities of HT22 cells treated with glutamate at various concentrations for different periods were detected by CCK8 assay. As shown in **Fig. 3-A**, the average viabilities of HT22 cells decreased significantly with the increased doses of glutamate and the extended time of incubation, indicating that glutamate inhibited the viabilities of HT22 cells in a dose and time dependent manner. Thus, we found that glutamate induced cell death by approximately 50% was 10 mM incubated for 12h ($p < 0.01$ **Fig. 3-A**).

10 mM glutamate was chosen to observe the cell death ratio for different incubation time by using LDH release assay. The results showed that the cell death ratio increased respectively to $25.2 \pm 4.8\%$ ($p < 0.01$, $n=6$), $45.8 \pm 3.9\%$ ($p < 0.01$, $n=6$), $69.5 \pm 5.6\%$ ($p < 0.01$, $n=6$) after the HT22 cells incubated with glutamate for 6h, 12h and 24h (**Fig. 3-B**).

Furthermore, the effects of glutamate on mitochondrial membrane potentials in HT22 cells were detected using JC-1 staining. Flow cytometry analysis showed that the decline percentage of mitochondrial membrane potentials in HT22 cells were $7.08 \pm 1.74\%$ ($p < 0.01$, $n=6$) $\rightarrow 44.50 \pm 2.88\%$ ($p < 0.01$, $n=6$) $\rightarrow 55.33$

$\pm 4.46\%$ ($p < 0.01$, $n=6$) after incubating with glutamate for 6h, 12h and 24h compared with control group ($3.03 \pm 0.71\%$), (**Fig. 3-C**).

3.4 Glutamate induced the expression of parthanatos-related proteins in HT22 cells

In order to clarify whether glutamate initiated parthanatos in HT22 cells, we measured parthanatos-related proteins by western blot. Our results showed that the expression level of PARP-1 and PAR polymer were significantly increased in a time-dependent manner, and the translocation of AIF from mitochondrial to nucleus was progressively increased with the prolonged incubation time (6h, 12h, 24h) of glutamate in HT22 cells (**Fig. 4-A**). Meanwhile, immunofluorescence further confirmed the excessive synthesis of PAR polymer and translocation of AIF from mitochondrial to nucleus in TH22 cells treated with glutamate compared with the control (**Fig. 4-B**). All these results suggested that parthanatos might be involved in the mechanism of glutamate-induced damage in TH22 cells.

3.5 Inhibition of PARP-1 alleviated glutamate-induced parthanatos in HT22 cells

PJ34, a specific inhibitor of PARP-1, was used to clarify the role of PARP-1 in glutamate induced HT22 cell death. As shown in **Fig. 5-A**, LDH release assay showed that pretreatment with PJ34 decreased the death ratio of HT22 cells from $66.7 \pm 5.2\%$ to $50.0 \pm 4.3\%$ ($p < 0.01$). Similarly, JC-1 staining showed that pretreatment with PJ34 inhibit the decline percentage of mitochondrial membrane potentials in HT22 cells from $55.3 \pm 3.6\%$ to $39.7 \pm 3.1\%$ ($p < 0.01$, **Fig. 5-B**). At protein level, glutamate-induced the increased expression of PARP-1, accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in HT22 cells were all significantly inhibited by pretreatment with PJ34 (**Fig. 5-C**). Therefore, these results showed that inhibition of PARP-1 by using PJ34 effectively protected HT22 cells against the lethal effect of glutamate, and attenuated the increased expression of PARP-1, accumulation of PAR polymer, decline percentage of mitochondrial membrane potentials and the translocation of AIF into nucleus.

3.6 Knockdown of PARP-1 alleviated glutamate-induced parthanatos in HT22 cells

To further investigate the role of PARP-1 in glutamate-induced HT22 cell death, we used transfection of siRNA to knock down PARP-1. LDH release assay showed that compared with the scramble siRNA group, knockdown of PARP-1 with siRNA in glutamate-induced HT22 decreased cell death ratio ($32.7 \pm 3.0\%$ vs $46.5 \pm 3.4\%$, $p < 0.01$, **Fig. 6-A**). Similarly, JC-1 staining showed that compared with the scramble siRNA group, knockdown of PARP-1 with siRNA in glutamate-induced HT22 inhibited the decline percentage of mitochondrial membrane potentials ($31.7 \pm 2.2\%$ vs $45.2 \pm 3.3\%$, $p < 0.01$, **Fig. 6-B**). Additionally, the increased expression of PARP-1, synthesis and accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in glutamate-induced HT22 cells were all significantly inhibited by knockdown of PARP-1 with siRNA (**Fig. 6-C**). Thus, these results confirmed that PARP-1 played an important role in glutamate-induce HT22 cell death.

3.7 ROS contributed to glutamate-induced parthanatos in HT22 cells

We investigated the role of ROS in the glutamate induced HT22 cell death and the relationship between ROS and parthanatos. As shown in **Fig. 7-A**, Flow cytometry showed that compared with the control group, the level of ROS increased respectively to $10.15 \pm 1.04\%$ ($p < 0.01$, $n=6$), $15.18 \pm 1.54\%$ ($p < 0.01$, $n=6$), $22.37 \pm 1.68\%$ ($p < 0.01$, $n=6$) after HT22 cells incubated with glutamate for 6h, 12h and 24h. The overproduced ROS at 24 h in HT22 cells treated with glutamate was attenuated by treatment with antioxidant NAC ($7.03 \pm 1.19\%$ vs $22.37 \pm 1.68\%$, $p < 0.01$, $n=6$, **Fig. 7-B**). DCFH-DA staining observed by fluorescence microscopy showed that the green fluorescence in HT22 cells treated with glutamate with 24 h was much more than the control group, and the green fluorescence can be decreased by pretreatment with antioxidant NAC (**Fig. 7-C**).

We further investigated the level of 8-OHdG and GSH in HT22 cells following incubated with glutamate and the effect of pretreatment with antioxidant NAC. Our results showed that the level of 8-OHdG increased respectively to 37.7 ± 4.0 ng/mg protein ($p < 0.01$, $n=6$), 42.5 ± 3.6 ng/mg protein ($p < 0.01$, $n=6$), 50.3 ± 4.0 ng/mg protein ($p < 0.01$, $n=6$) after HT22 cells incubated with glutamate for 6h, 12h and 24h, compared with the control group (29.2 ± 3.2 ng/mg protein, **Fig. 8-A**). Pretreatment with antioxidant NAC effectively inhibit the increased level of 8-OHdG in HT22 cells induced by glutamate (35.8 ± 3.5 vs 50.3 ± 4.0 ng/mg protein, $p < 0.01$, $n=6$, **Fig. 8-A**). Meanwhile, the level of GSH decreased respectively to 16.0 ± 1.4 nmol/mg protein ($p < 0.05$, $n=6$), 12.7 ± 2.2 nmol/mg protein ($p < 0.01$, $n=6$), 8.3 ± 1.6 nmol/mg protein ($p < 0.01$, $n=6$) after HT22 cells incubated with glutamate for 6h, 12h and 24h, compared with the control group (19.1 ± 1.7 nmol/mg protein, **Fig. 8-B**). Pretreatment with antioxidant NAC effectively increased the level of GSH in HT22 cells induced by glutamate (17.1 ± 1.9 vs 8.3 ± 1.6 nmol/mg protein, $p < 0.01$, $n=6$, **Fig. 8-B**).

Western blot analysis showed that glutamate-induced increased expression of PARP-1, synthesis and accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in HT22 cells were all significantly inhibited by pretreatment with NAC (**Fig. 8-C**). Therefore, the above results suggested that ROS might an initial factor contributing to the activation and upregulation of PARP-1 in glutamate-induce HT22 cell death.

4. Discussion

Recently, treatment of epilepsy is mainly relied on drugs of controlling epileptic symptoms. Although there are a variety of antiepileptic drugs (AEDs) available, 30- 40% of patients are resistant to drugs and the epileptic seizures could not be controlled, therefore becoming refractory epilepsy [22]. In fact, in the stage of clinical diagnosis of epilepsy, the corresponding molecular pathological changes have been established. At this stage, treatment of AEDs just controlling epileptic symptoms has been in a backward passive situation. Therefore, in-depth study of the pathogenesis of epilepsy can open a new way for the treatment of inhibition of epileptogenesis. As described previously, epileptic seizures or status epilepticus can cause hippocampal neurons death, which could promote the progression of epilepsy. In this study, we used KA injected into the right amygdala of the rats to establish epilepsy model, and the results showed that neurons injury were mostly located in CA1 and CA3 regions of hippocampus. Application of PARP-1

inhibitor PJ34 showed significantly neuroprotective effect on the damage of hippocampal neurons after epileptic seizures in rats. Meanwhile, it also reduced the expression of PARP-1 and the synthesis of PAR in hippocampus, and inhibited the translocation of AIF from mitochondrial to the nucleus.

Glutamate is a primary mediator in excessive excitatory neurotransmission induced by neuronal excitotoxicity following epileptic seizure. In vitro study, we used glutamate to induce HT22 cell damage to simulate the hippocampal neuron death following epilepsy, and investigated whether parthanatos was involved in glutamate-induced neuronal death. In this process, we explored the role of PARP-1 by using PARP-1 inhibitor PJ34 and knockdown of PARP-1 with siRNA for developing effective treatment strategies against neuron damage. Our results observed that glutamate induced HT22 cell death accompanied with increased expression of PARP-1, excessive accumulation of PAR polymer and translocation of AIF from mitochondrial to nucleus. Inhibition of PARP-1 by using PARP-1 inhibitor PJ34 or knockdown of PARP-1 with siRNA effectively protected HT22 cells against the toxic effect of glutamate, and attenuated the level of PARP-1 and PAR polymer, as well as alleviated AIF translocation into nucleus and mitochondrial depolarization. Antioxidant NAC alleviated the overproduction of ROS, rescued HT22 cell death induced by glutamate, and reversed the above-mentioned parthanatos biochemical events. Considering these results, we conducted that glutamate induced HT22 cell death may be in a ROS-mediated PARP-1 dependent manner.

Parthanatos is observed in the mechanisms of multiple diseases including cerebral hypoxia/ischemia, inflammation, diabetes, and trauma [17, 23-25]. Over-activation of PARP-1 is the initiating step in the process of Parthanatos. PARP-1 is regarded as “DNA damage sensor”, under normal physiological conditions, PARP-1 is activated by DNA strand breakage for repairing cellular DNA damage and maintaining cellular homeostasis [26]. Under pathological conditions, PARP-1 is over activated by massive DNA damage results in excessive synthesis and accumulation of PAR polymer, which translocate into cytoplasm to produce cytotoxic effects leading to parthanatos [27]. In our study, we observed that the level of PARP-1 and PAR polymer increased during the process of HT22 cells damage induced by glutamate in a time dependent manner. In addition, inhibition of PARP-1 with PJ34 or knockdown of PARP-1 with SiRNA rescued glutamate-induce HT22 cell death and decreased the upregulation of PARP-1 and the excessive synthesis of PAR polymer. All these results support that PARP-1 played an important role in the damage of HT22 cells induced by glutamate.

PAR polymer has toxic effect on cells. Toxicity of PAR is related to the length and complexity of the PAR polymer [11, 28]. The size of PAR polymer greater than 60 ADP-ribose units are more toxic than the less complex polymers [11]. PAR is a negatively charged polymer, which can change the internal biochemical characteristics of the receptor and regulate its structure and function [29]. PAR is synthesized in the nucleus and then transferred to mitochondria resulting in phosphatidylserine turnover to the ectoplasmic membrane which induces mitochondrial depolarization and MPT pore opening resulting in release of un-truncated AIF from mitochondrial [11, 30].

AIF is a widely expressed mitochondrial protein which has been observed to translocate from the mitochondria to the nucleus to induce chromatin condensation and DNA fragmentation [31]. Our results found that glutamate caused mitochondrial membrane potential collapse in HT22 cells and induced translocation of AIF from the mitochondria to the nucleus leading to cell death. Pretreatment with PARP-1 inhibitor PJ34 and knockdown of PARP-1 with siRNA inhibited mitochondrial membrane potential collapse and translocation of AIF to nucleus as well as rescued glutamate-induced HT22 cell death. All these results illustrated that PAR polymer was involved in glutamate-induced mitochondrial depolarization and subsequent AIF translocation to nucleus. So far, it is not clear how does AIF induce DNA fragmentation. A recent study identified a PARP-1-dependent AIF-associated nuclease (PAN) called macrophage migration inhibitory factor (MIF) which was recruited by AIF to the nucleus where MIF cleaves genomic DNA into large fragments [32].

PARP-1 is directly activated by chromosomal DNA strand breaks [27]. ROS is an important mediator of DNA damage and induce DNA damage by oxidation of nucleoside bases (such as the formation of 8-oxoguanine) [33]. Ma et al. found that deoxypodophyllotoxin (DPT) triggered excessive expression of PARP-1 and synthesis of PAR polymer in glioma cells and confirmed that production of ROS induced by DPT contributed to the activation of PARP-1 [21]. In epilepsy, excessive production of ROS can be induced in the process of epileptic discharge. The evidence of ROS production increase in epileptic model in vivo was initially confirmed in brain homogenate [34]. Subsequently, it was also supported by fluorescence staining in vitro [35, 36]. Kumari et al. found that the level of ROS was increased in HT22 cell injury model induced by glutamate, and the oxidative damage of glutamate was in a concentration dependent manner [37]. Our study showed that glutamate induced HT22 cells death in a time dependent manner, accompanied with the production of intracellular ROS, generation of 8-OHdG, and the decreased level of GSH. Pretreatment of NAC can rescue HT22 cell injury induced by glutamate, and decrease the production of intracellular ROS, generation of 8-OHdG, and increase the level of GSH as well as inhibit the level of PARP-1, accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in HT22. Therefore, these results indicated that ROS may be the initiating factor in the mechanism of glutamate induced parthanatos death in HT22 cells. NAC, as an antioxidant, may play a neuroprotective role by increasing intracellular GSH levels.

5. Conclusion

In conclusion, we demonstrated that parthanatos was involved in the damage of hippocampal neurons in rats following epileptic seizure and ROS might be the initiating factor in the process of parthanatos. Our work highlights the robust neuroprotective potential of pharmacological interventions targeting PARP activity as promising intervention strategies in epilepsy, and provides multiple potential targets which are needed to verify in future studies.

Declarations

Conflicts of Interest

The authors state no conflict of interest.

Acknowledgement

National Natural Science Foundation of China and is gratefully acknowledged.

Funding

This study was supported in part by National Natural Science Foundation of China (81871008) and National Key R&D Program of China (2017YFC0110304).

Author contributions

XW performed the cell experiment part and wrote the manuscript. WQ performed the animal experiment part. MM contributed to analysis of this study. PF and HM contributed the design of this study. All authors read and approved the final paper.

Ethics approval

The experimental protocols were approved by the Institutional Animal Care and Animal Ethics Committee of Jilin University of China.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Pj34 alleviated hippocampal neuron loss in epileptic rats induced by KA. (A) Representative hippocampal sections stained with FJB showed degenerating neurons from different groups. (B) Quantitative analysis showed significant numbers of FJB-positive neurons present in the CA1 and CA3 regions after SE induction and can be reduced in number by PJ34. Scale bar, 50 μ m (n= 6 per group, **p < 0.01 versus control group, ## P<0.01 versus KA + vehicle group)

Figure 2

PJ34 modulated the expression of PARP-1, PAR and AIF in epileptic rats induced by KA. Proteins from rat hippocampus in sham, KA+vehicle, and KA+PJ34 groups were determined by western blot. β -actin, COX IV and histone were used as cytoplasmic, mitochondrial and nuclear loading controls respectively. (A-B) The expression level of PARP-1 and PAR polymer were significantly increased after SE induction and can be decreased by PJ34. (C-D) The expression level of AIF was decreased in mitochondrial but increased in nucleus after SE induction, which can be reversed by PJ34. (n= 6 per group, **p < 0.01 versus control group, ## P<0.01 versus KA + vehicle group)

Figure 3

Effects of glutamate on HT22 cells. (A) The viabilities of HT22 cells induced by glutamate were in a dose and time dependent manner. (B) The cell death ratio of HT22 cells induced by glutamate for different incubation was progressively increased. (C) The decline percentage of mitochondrial membrane potentials in HT22 cells induced by glutamate for different incubation was progressively increased. The data were expressed as the mean $\pm$ S. Representative results of six experiments are shown (**p < 0.01 versus control group).

Figure 4

Glutamate induced the expression of parthanatos-related proteins. (A) Western blot analysis of PARP-1, PAR polymer, mitochondrial AIF and nucleus AIF levels in HT22 cells following treatment with glutamate for different incubation time (6h, 12h, 24h). β-actin, COX IV and histone were used as cytoplasmic, mitochondrial and nuclear loading controls respectively. (B) Immunofluorescent analysis of the accumulation of PAR polymer (red) in HT22 cells treated with glutamate compared with the control. Nuclei were stained with DAPI (blue). (C) Immunofluorescent analysis of the translocation of AIF (green) from mitochondrial to nucleus in HT22 cells treated with glutamate compared with the control. Nuclei were stained with DAPI (blue). Scale bars: 50 μm. Data are expressed mean ± SD. Representative results of six experiments are shown (* P<0.05 and ** P<0.01 versus control group).

Figure 5

PARP-1 inhibitor PJ34 ameliorated glutamate-induced parthanatos in HT22 cells. (A) LDH release assay showed that pretreatment with PJ34 made the death ratio of HT22 cells decrease from $66.7 \pm 5.2\%$ to $50.0 \pm 4.3\%$ ($p < 0.01$). (B) JC-1 staining showed that pretreatment with PJ34 inhibit the decline percentage of mitochondrial membrane potentials in HT22 cells form $55.3 \pm 3.6\%$ to $39.7 \pm 3.1\%$ ($p < 0.01$). (C) Western blotting analysis showed that glutamate-induced upregulation of PARP-1, accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in HT22 cells were all significantly inhibited by pretreatment with PJ34. Data are expressed mean $\pm$ SD. Representative results of six experiments are shown (* $P < 0.05$ and ** $P < 0.01$ versus control group, # $P < 0.05$ and ## $P < 0.01$ versus GLU group).

Figure 6

Knockdown of PARP-1 with SiRNA attenuated glutamate induced HT22 cell death. (A) LDH release assay showed that knockdown of PARP-1 with SiRNA rescued glutamate induced HT22 cell death, compared those treated with scramble SiRNA. (B) JC-1 staining showed that knockdown of PARP-1 with SiRNA inhibit the decline percentage of mitochondrial membrane potentials in HT22 cells induced by glutamate, compared those treated with scramble SiRNA. (C) Western blotting analysis showed that transfection with PARP-1 SiRNA in HT22 cells inhibited the up-regulated levels of PARP-1, accumulation of PAR polymer, and translocation of AIF from mitochondria to nucleus in HT22 cells, compared those treated with scramble SiRNA. Data are expressed mean $\pm$ SD. Representative results of six experiments are shown (* $P < 0.05$ and ** $P < 0.01$ versus Scrambled siRNA group, # $P < 0.05$ and ## $P < 0.01$ versus Scrambled siRNA + GLU group).

Figure 7

NAC decreased the level of ROS induced by glutamate in HT22 cells. (A) Flow cytometry showed that the level of ROS increased progressively after HT22 cells incubated with glutamate for 6h, 12h and 24h, compared with the control group. (B) Pretreatment of antioxidant NAC attenuated the overproduced ROS induced by glutamate in HT22. (C) Fluorescence microscopy with DCFH-DA staining showed that the green fluorescence in HT22 cells that were treated 24 h with glutamate was much brighter than that in the control group, and the green fluorescence can be decreased by antioxidant NAC. Scale bars: 10 μ m Data are expressed mean $\pm$ SD. Representative results of six experiments are shown (** P<0.01 versus control group, ## P<0.01 versus GLU group).

Figure 8

ROS contributed to glutamate-induced parthanatos in HT22 cells. (A) The level of 8-OHdG increased progressively after HT22 cells incubated with glutamate for 6h, 12h and 24h, compared with the control group. Antioxidant NAC effectively inhibit the increased level of 8-OHdG in HT22 cells induced by glutamate. (B) The level of GSH decreased progressively after HT22 cells incubated with glutamate for 6h, 12h and 24h, compared with the control group. Antioxidant NAC effectively increased the level of GSH in HT22 cells induced by glutamate. (C) Pretreatment of Antioxidant NAC decreased the cell death ratio of HT22 cells induced by glutamate. (D) Western blot analysis showed that glutamate-induced upregulation of PARP-1, accumulation of PAR polymer, and translocation of AIF from mitochondrial to nucleus in HT22 cells were all significantly inhibited by antioxidant NAC. Data are expressed mean $\pm$ SD. Representative results of six experiments are shown (* P<0.05 and ** P<0.01 versus control group, ## P<0.01 versus GLU group).