Overexpression of phosphoprotein enriched in astrocytes 15 reverses the damage induced by propofol in hippocampal neurons

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Abstract. Propofol is a general anesthetic used in surgical operations. Phosphoprotein enriched in astrocytes 15 (PEA15) was initially identified in astrocytes. The present study examined the role of PEA15 in the damage induced by propofol in hippocampal neurons. A model of hippocampal neuron damage was established using 50 µmol/l propofol. Cell viability, proliferation and apoptosis of hippocampal neurons were tested by Cell Counting Kit-8 and flow cytometry. Western blotting and reverse transcription-quantitative polymerase chain reaction analysis were performed to measure the expression levels of PEA15, and additional factors involved in apoptosis or in the signaling pathway downstream of PEA15. The present results suggested that propofol significantly decreased PEA15 expression levels in hippocampal neurons. Furthermore, overexpression of PEA15 significantly increased the cell viability and cell proliferation of cells treated with propofol. Additionally, PEA15 overexpression decreased apoptosis, which was promoted by propofol. Treatment with propofol significantly decreased the protein expression levels of pro-caspase-3, B-cell lymphoma-2, phosphorylated extracellular signal-regulated kinases (ERK)1/2, ribosomal S6 kinase 2 (RSK2) and phosphorylated cAMP responsive element binding protein 1 (CREB1). However, propofol upregulated active caspase-3 and Bax expression levels. Notably, PEA15 overexpression was able to reverse the effects of propofol. Collectively, overexpression of PEA15 was able to attenuate the neurotoxicity of propofol in rat hippocampal neurons by increasing proliferation and repressing apoptosis via upregulation of the ERK-CREB-RSK2 signaling pathway.

Introduction

Annually, in the USA, approximately six million children require general anesthesia (1,2). Furthermore, in China, the number of children requiring anesthetics is increasing (3). The human brain develops rapidly between the embryonic stage and 2 years after birth. During this period the number of neurons increases, and large quantities of axons, dendrites and synapses are formed (4-6). Previous clinical studies have demonstrated that anesthesia and surgery may lead to cognitive impairment (7,8). Therefore, decreasing the brain neuronal damage caused by narcotic drugs is becoming an increasingly popular research topic.

A number of previous studies identified that inhalable narcotic drugs, including midazolam, propofol, isoflurane and sevoflurane, are used in newborns (9-12). Propofol is a general anesthetic used in surgical operations. Previous studies demonstrated that propofol may induce neurotoxicity in the brains of newborn animals (13-16). In addition, previous studies identified that propofol-induced apoptosis in numerous types of cells associated with neurons (17-19). Therefore, it is crucial to identify novel effective strategies to decrease the neurotoxicity of propofol in the developing brain.

Phosphoprotein enriched in astrocytes 15 (PEA15) is a phosphoprotein that was initially identified in astrocytes (20). PEA15 is known to mediate the signal transduction pathway; PEA15 serves as a signal connection point, thus possessing the potential to directly regulate cell behavior (21). Previous studies demonstrated that PEA15 serves roles in neuronal processes, including tumor progression, diabetes, metabolic disorders and nervous system diseases (22-26). However, at present, to the best of the authors' knowledge, the effect of PEA15 on propofol-induced hippocampal nerve injury has not been investigated.

In the present study, propofol was used to damage rat hippocampal neurons. Furthermore, the effect and potential molecular mechanism of PEA15 on propofol-induced hippocampal neuronal cell damage were examined by Cell Counting Kit-8 (CCK-8), flow cytometry, western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Materials and methods

Animals. Pregnant Sprague-Dawley (SD) rats (3 months old; embryonic age E18; weight: 200-230 g) were purchased from...
Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were housed in cages at 22±1˚C. The animals had free access to food and water, with a constant humidity (50±10%) and with a 12 h light/dark cycle. The animal experimental research was approved by the Ethics Committee of The First People's Hospital of Changzhou, The Third Affiliated Hospital of Soochow University (Changzhou, China).

**Isolation of hippocampal neurons.** The extraction and cultivation methods of the neurons were performed as previously described (27,28). In total, 5 newborn SD rats (3 male and 2 female; weight, 5.0-6.0 g) were sacrificed by cervical dislocation, within 24 h following birth. A solution of 75% ethanol was used to disinfect the fetal rats in sterile trays. Subsequently, the rats were guillotined. The brain was removed using bent tweezers and placed in a precooled Hank's balanced salt solution and the hippocampus was separated from the brain. Subsequently, the hippocampus was digested using 2% trypsin for 15 min at 37˚C in a cell incubator with 5% CO₂. The digestion was stopped by adding neurobasal medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Cellbio, Shanghai, China). The hippocampal neurons were obtained and maintained in an incubator at 37˚C with 5% CO₂. The morphology of the cells was observed daily using a TMM-220 inverted light microscope (magnification, x200; Shanghai Tianxing Instrument Co., Ltd., Shanghai, China).

**Cell treatment and transfection.** Propofol was purchased from Fresenius Kabi Austria GmbH (Graz, Austria). As previously described (29,30), the hippocampal neurons were exposed to 50 µmol/l propofol for 0, 6, 12 and 24 h. Subsequently, cells were transfected with 50 nM PEA15 cloned in pcDNA3.1 or empty pcDNA3.1 vectors (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) and cultured for 48 h at 37˚C. A total of six experimental groups were considered in the present study: Control group (0.1% PBS), empty-vector group (transfected with pcDNA3.1 vector), PEA15 group (transfected with PEA15, propofol group (treated with 50 µmol/l propofol), empty-vector + propofol group (transfected with pcDNA3.1 empty vector and treated with 50 µmol/l propofol), and PEA15+propofol group (transfected with PEA15 and treated with 50 µmol/l propofol).

**Cell viability assay.** A CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) assay was conducted to detect the cell viability. A total of 2x10⁴ cells/ml in logarithmic phase were inoculated in 96-well plates and maintained in an incubator at 37˚C with 5% CO₂ for 24 h. Subsequently, cells were treated with propofol at 12.5, 25 and 50 µmol/l for 6 h at 37˚C and divided into the aforementioned experimental groups, and transferred into the incubator for 6, 12 and 24 h. CCK-8 reagent was added into each well. Cells were cultured in the incubator at 37˚C with 5% CO₂ for 2.5 h. An HBS-1096A microplate reader (Nanjing Dettie Experimental Equipment Co., Ltd., Nanjing, China) was used to measure the absorbance at 450 nm.

**Flow cytometry assay.** Cell apoptosis was determined using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. The cells were digested with 0.25% trypsin (Beyotime Institute of Biotechnology). The supernatant was removed and cells were resuspended in an incubation buffer at a density of 2x10⁶ cells/ml. Annexin V-FITC and PI were added to the cell suspension in the dark at room temperature for 20 min. The CytoFLEX flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) was used to detect cell apoptosis. Cell proliferation was assessed using the CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were digested with 0.25% trypsin (Beyotime Institute of Biotechnology). The supernatant was removed and cells were resuspended in an incubation buffer at a density of 2x10⁵ cells/ml. The CFSE solution was added to the cell suspension and incubated for 15 min at room temperature. A CytoFLEX flow cytometer (CytExpert software v1.2; Beckman Coulter, Inc.) was used to measure the cell proliferation.

**Western blotting.** Subsequent to being treated, cells were lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The protein content was analyzed using the Bradford method. A 10% SDS-PAGE was used to separate (20 µg/lane) proteins, that were transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was sealed and blocked using 5% non-fat milk at 37˚C for 1.5 h. Subsequently, the PVDF membrane was incubated with primary antibodies at 4˚C for 24 h. The following antibodies were used in the present study: Anti-PEA15 [1:800; Cell Signaling Technology (CST), Inc., Danvers, MA, USA; cat. no. 2780], anti-pro-caspase-3 (1:1,000; Abcam, Cambridge, UK; cat. no. ab32135), anti-ERK1/2 (1:200; CST, Inc.; cat. no. 9102), anti-ribosomal S6 kinase 2 (RSK2; 1:500; Cellbio, Shanghai, China); anti-B-cell lymphoma-2 (Bcl-2; 1:700; Abcam; cat. no. ab32150), anti‑active caspase‑3 (1:600; Abcam; cat. no. ab3847), anti-B-cell lymphoma-2 (Bcl-2; 1:700; Abcam; cat. no. ab59348), anti-apoptosis regulator BAX (Bax; 1:1,000; Abcam; cat. no. ab32503), anti-phosphorylated (p)-extracellular signal-regulated kinases 1/2 (ERK1/2; 1:1,000; CST, Inc.; cat. no. 4370), anti-ERK1/2 (1:600; CST, Inc.; cat. no. 9102), anti-ribosomal S6 kinase 2 (RSK2; 1:500; Abcam; cat. no. ab32133), anti-p-CAMP-response element binding protein 1 (CREB1; 1:800; Abcam; cat. no. ab32096), anti-CREBI (1:700; Abcam; cat. no. ab32515) and anti-β-actin (1:5,000; Abcam; cat. no. ab179467). The PVDF membrane was subsequently incubated with Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; cat. no. ab205718; 1:2,000) at room temperature for 1 h. The proteins were detected using enhanced chemiluminescent reagents (Thermo Fisher Scientific, Inc.). The density of bands was determined using the Quantity One software version 2.4 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**RT-qPCR.** Total RNA from the neurons was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using an RT kit (HaiGene, Harbin, China) at 37˚C for 15 min followed by 85˚C for 5 sec. The ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to amplify the cDNA with AceQ qPCR SYBR-Green Master Mix (Vazyme, Piscataway, NJ, USA). qPCR conditions were set as follows: 10 min pretreatment at 95˚C, 96˚C for 15 sec, 63˚C for 45 sec.
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(35 cycles) and a final extension at 75˚C for 10 min. β-actin was used as the reference gene. The primers used were purchased from Shanghai ShineGene Molecular Biotech, Inc. (Shanghai, China) and were the following: Pea15 (forward, 5'-ccT Gac caa caa CT cac cc-3' and reverse, 5'-GaT cTT caG cac acG GGT Tc-3'), Bcl-2 (forward, 5'-Gcc TTc TTT GaG TTc GGT GG‑3' and reverse), Bax (forward, 5'‑GAG ACA CCT GAG cTG acc TT-3' and reverse, 5' -cGT cTG caa aca TGT ca G cT-3') and β-actin (forward, 5'-caa caT GGa TGa GcG Ga a GG-3' and reverse, 5'-Gca GTG TaG caG caT cGa aa-3').

The 2 −ΔΔCq method was used to calculate gene expression levels (31).

Statistical analysis. SPSS (version 20; IBM Corp., Armonk, NY, USA) software was used for statistical analysis. The data are presented as the mean ± standard deviation. The experiments were independently repeated at least three times. The experimental data were analyzed by one-way analysis of variance with Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Propofol inhibits the expression level of PEA15 in hippocampal neurons. Following hippocampal neuron isolation, the cells were cultured for 2 days. The neurons presented 2-3 neurites, and were flat and multilateral. Following 6 days of incubation, the number of neurites increased, and the cells interconnected with each other through the neurites. Notably, the majority of the cells aggregated and exhibited a visible halo surrounding the cells (Fig. 1A).

Cell viability decreased following treatment with propofol at 12.5, 25 and 50 µmol/l for 6 h. The decrease in cell viability was significant in the 50 µmol/l group (Fig. 1B). Therefore, 50 µmol/l propofol was selected as the working concentration. Western blotting and RT-qPCR assays were performed to examine the effects of propofol on the expression level of PEA15. The protein expression level of PEA15 was determined by (C) western blotting and (D) densitometric analysis. (E) mRNA expression level of PEA15 was measured by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. 0 h. PEA15, phosphoprotein enriched in astrocytes 15.
of PEA15 in hippocampal neurons. The western blotting suggested that the protein expression level of PEA15 was decreased following treatment with propofol for 6, 12 and 24 h. Notably, the protein expression level of PEA15 was lower at 12 h compared with 24 h; however, the difference was not significant (Fig. 1C and D). Furthermore, propofol significantly decreased the mRNA expression level of PEA15 in a time-dependent manner (Fig. 1E).

_overexpression of PEA15 reverses the decreased viability of hippocampal neurons induced by propofol._ The cell viability in the PEA15 + propofol group was significantly increased compared with the propofol and empty-vector + propofol groups (Fig. 2D).

Overexpression of PEA15 represses the propofol-induced apoptosis of hippocampal neurons. The flow cytometry results suggested that propofol significantly promoted cell apoptosis. However, PEA15 overexpression was able to decrease the apoptosis of cells treated with propofol compared with the propofol and empty-vector + propofol groups (Fig. 3).

Overexpression of PEA15 reverts the proliferation of hippocampal neurons inhibited by propofol. The cell proliferation assay suggested that propofol significantly decreased the proliferation of hippocampal neurons. In addition, transfection of PEA15 was sufficient to increase the cell proliferation of cells treated with propofol compared with the propofol and empty-vector + propofol groups (Fig. 4).

**PEA15 and propofol regulate apoptosis-associated factors in hippocampal neurons.** Western blotting and RT-qPCR analysis were performed to investigate the effects of PEA15 and propofol on apoptosis-associated factors. The western blotting results...
suggested that treatment with propofol promoted the protein expression levels of active caspase-3 and Bax (Fig. 5A and B), and led to downregulation of pro-caspase-3 and Bcl-2 protein expression levels (Fig. 5A and C). By analyzing the protein and the mRNA expression levels of Bax and Bcl-2, it was identified that the Bax/Bcl-2 ratio was significantly increased following treatment with propofol (Fig. 5D and E). Notably, PEA15 overexpression led to opposite effects.

PEA15 and propofol regulate ERK-CREB-RSK2 signaling in hippocampal neurons. Western blotting was conducted to determine the protein expression levels of ERK1/2, p-ERK1/2, p-CREB1, CREB1 and RSK2, in order to examine the molecular mechanisms of PEA15 and propofol on hippocampal neurons. The present results suggested that propofol decreased the protein expression levels of p-ERK1/2, p-CREB1 and RSK2 (Fig. 6A and B). Additionally, it was identified that the ratios between the phosphorylated proteins p-ERK1/2 and p-CREB1, and the respective non-phosphorylated proteins ERK1/2 and CREB1, were significantly decreased (Fig. 6C and D) when cells were treated with propofol. Furthermore, PEA15 promoted the protein expression levels of p-ERK1/2, RSK2 and p-CREB1 in the hippocampal neurons that were treated with propofol. Notably, the protein

Figure 3. Overexpression of PEA15 represses the propofol-induced apoptosis of hippocampal neurons. (A) An Annexin V-FITC/PI apoptosis detection kit was used to detect apoptosis of the hippocampal neurons. (B) Bar graph indicates the apoptosis of hippocampal neurons among the groups. *P<0.05, **P<0.01 vs. control; ***P<0.01 vs. empty-vector; ^*P<0.01 vs. propofol; ^^^P<0.01 vs. empty-vector + propofol. PEA15, phosphoprotein enriched in astrocytes 15; FITC, fluorescein isothiocyanate; PI, propidium iodide.
expression levels of ERK1/2 and CREB1 among groups were not significantly affected.

Discussion

The majority of cultured hippocampal neurons derive from the hippocampus of neonatal rats, as suckling mice gain an advantage in finding a location to feed (32,33). A previous study identified that hippocampal neurons cultured at day 6 are similar to neurons of a developing human brain (34). During this period, neurons are sensitive to external stimuli, including neurotoxicity caused by anesthetics (34). Therefore, in the present study, primary hippocampal neurons were extracted from neonatal rats and used as a model of developing neurons. Following 6 days of culturing, the cell morphology remained stable and the neurons were treated with propofol.

Accumulating evidence suggested that propofol was neurotoxic in vitro (35-37). Monni et al (36) observed that propofol promoted cell death in rat hypoglossal motoneurons. Yan et al (37) demonstrated that propofol induced apoptosis...
of hippocampal neurons in newborn mice. Furthermore, numerous previous studies demonstrated that propofol is a potent inducer of neuronal damage (35,38,39). Similar to previous studies, the present results suggested that propofol significantly decreased the viability and proliferation of neuronal cells, promoting cell apoptosis. Therefore, in the present study, treatment with propofol was used as a model for damaging neuronal cells.

A recent study demonstrated that the expression of PEA15 was downregulated in diabetic SD rats, promoting middle cerebral artery occlusion (25). A previous study demonstrated that overexpression of PEA15 decreased glucose deprivation-induced cell death in neurons (40). Therefore, it was hypothesized that the overexpression of PEA15 may decrease the neurotoxicity of propofol, and that PEA15 and propofol may be associated in hippocampal neurons. As hypothesized, the experimental results suggested that propofol significantly decreased PEA15 expression. Furthermore, the CCK-8 and flow cytometry results suggested that PEA15 overexpression significantly increased the viability and proliferation of neurons, and decreased the apoptosis of neuronal cells, reverting the effects of propofol. The present results suggested that overexpression of PEA15 conferred a neuroprotective effect on propofol-induced neuronal damage by promoting proliferation and decreasing apoptosis.

In order to investigate the mechanisms underlying PEA15 and propofol in neuronal cells, the protein expression levels of apoptosis-associated factors, including pro-caspase-3, active caspase-3, Bcl-2 and Bax, were examined by western blotting and RT-qPCR analysis. Liang et al (35) demonstrated that propofol induced apoptosis of the developing neurons by upregulating the protein expression levels of cleaved-caspase-3 and Bax, and by downregulating the Bcl-2 protein expression level. Similarly, the present results demonstrated that propofol significantly increased the protein expression levels of active caspase-3 and Bax, and decreased pro-caspase-3 and Bcl-2 expression. Furthermore, Ahn et al (22) observed that PEA-15 increased Bcl-2 and pro-caspase-3 protein expression levels, and decreased Bax and cleaved-caspase-3 protein expression levels in neuroblastoma cells treated with 1-methyl-4-phenylpyridinium. The present study demonstrated that the overexpression of PEA15 in neurons treated with propofol, increased the protein expression levels of pro-caspase-3 and Bcl-2, and Bax and cleaved-caspase-3 expression levels were downregulated. The present results suggested that overexpression of PEA15 decreased the apoptotic effects induced by propofol in nerve cells by upregulating pro-caspase-3 and Bcl-2, and by downregulating active caspase-3 and Bax.

ERK-CREB signaling regulates cell growth and apoptosis of rat hippocampal neurons (41-43). Furthermore, RSK2 was observed to be involved in the ERK pathway in the hippocampus (44). Kozinn et al (45) demonstrated that propofol inhibited the ERK-CREB pathway in hippocampal neurons.
neurons. In the present study, it was hypothesized that the mechanisms underlying the PEA15 and propofol effects on the proliferation and apoptosis of hippocampal neurons may be associated with the ERK-CREB pathway. The present results suggested that propofol significantly repressed the phosphorylation levels of ERK1/2 and CREB1, and decreased the protein expression level of RSK2. However, overexpression of PEA15 was able to revert the protein expression levels of p-ERK1/2, p-CREB1 and RSK2, which were suppressed by propofol. The present results suggested that the cell damage caused by propofol was attenuated by PEA15 overexpression. However, testing the effects of PEA15 knockdown may further aid the understanding of the role of PEA15 in neuronal injury.

In conclusion, the present results suggested that PEA15 ameliorated propofol-induced hippocampal nerve damage by promoting cell proliferation, and PEA15 was able to decrease cell apoptosis by upregulating the ERK-CREB-RSK2 signaling pathway. The present study suggested that PEA15 may represent a potential therapeutic agent to treat human diseases characterized by neuronal damage.

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Availability of data and materials

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

Authors’ contributions

FX provided a substantial contribution to conception and design of the present study. QL and ZC were involved in data acquisition, analysis and interpretation. ZC and FX were involved in
drafting the article or critically revising it for important intellectual content. All authors gave final approval of the version to be published. All authors agreed to be accountable for all aspects of the work, and in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal experimental research was approved by the Ethics Committee of The First People's Hospital of Changzhou, The Third Affiliated Hospital of Soochow University (Changzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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