ORIGINAL RESEARCH

Overexpressed HspB6 Underlines a Novel Inhibitory Role in Kainic Acid-Induced Epileptic Seizure in Rats by Activating the cAMP-PKA Pathway

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Abstract

Epilepsy is a commonly occurring neurological disease that has a large impact on the patient's daily life. Phosphorylation of heat shock protein B6 (HspB6) has been reported to protect the central nervous system. In this investigation, we explored whether HspB6 played a positive effect on epilepsy with the involvement of the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway. The epileptic seizure was induced in rats by intraperitoneal injection of kainic acid (KA). The extent of HspB6 phosphorylation and expressions of HspB6, PKA, and inflammatory factors TNF-α, IL-1β, and IL-6 were quantified along with neuronal apoptosis. To further understand the regulatory mechanism of the HspB6 in the hippocampus, we altered the expression and the extent of HspB6 phosphorylation to see whether the cAMP-PKA pathway was inactivated or not in hippocampal neurons of rats post KA. Results showed that HspB6 was poorly expressed, resulting in the inactivation of the cAMP-PKA pathway in rats post KA, as well as an aggravated inflammatory response and hippocampal neuronal apoptosis. HspB6 overexpression and the cAMP-PKA pathway activation decreased the expression of inflammatory factors and inhibited hippocampal neuronal apoptosis. Additionally, HspB6 phosphorylation further augments the inhibitory effects of HspB6 on the inflammatory response and hippocampal neuronal apoptosis. The cAMP-PKA pathway activation was found to result in increased HspB6 phosphorylation. HspB6 decreased apoptosis signal-regulating kinase 1 (ASK1) expression to inhibit inflammatory response and hippocampal neuronal apoptosis. Collectively, our findings demonstrate that activation of the cAMP-PKA pathway induces overexpression and partial phosphorylation of HspB6 lead to the inhibition of ASK1 expression. This in turn protects rats against epilepsy and provides a potential approach to prevent the onset of epileptic seizure in a clinical setting.

Keywords Epilepsy · Heat shock protein B6 · cAMP-PKA signaling pathway · Phosphorylation · Inflammatory response · Hippocampal neuronal apoptosis

Introduction

Epilepsy is a common neurological disease with neurological disorder. Monogenic forms of epilepsy tend to be led by neurotransmitter receptors or ion channels encoded by gene's internal mutations (Ishiura et al. 2018). As many as

80% of patients with epilepsy come from low-income and middle-income countries while underdeveloped and developing countries have a 10~40% epilepsy prevalence, while developed countries have a prevalence rate of 4.9% (Yu et al. 2017). The typical symptoms of children and teenagers who suffer from epilepsy include anxiety of higher degree, leading a reduced life quality (Schraegle and Titus 2017). It has been demonstrated that individuals suffering both emotional and sexual maltreatment from childhood are much more susceptible to developing epilepsy in their later years compared to other individuals (Labudda et al. 2017). Approximately 15% of epileptic episodes occurrences are caused by a direct injury to the central nervous system which includes encephalitis, stroke, or traumatic brain injury (Klein and Tyrlikova 2017). A former study

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has documented that 61–71% of children with epilepsy achieve seizure freedom while 7–20% of children with epilepsy are found to be drug-resistant (Aaberg et al. 2018). These statistics drastically call for an urgent need for the development of novel therapeutic targets to treat epilepsy. It has been reported that heat shock protein B6 (HspB6) (also known as P20 or HSP20) can not only mediate cardioprotective signaling but also can protect the central nervous system from injury (Li et al. 2017a). To develop more on previous findings, our current study aims to focus on investigating the potential role of HspB6 in epilepsy.

HspB6 belongs to the human small Hsp family, which can bind and prevent unfolded proteins from aggregating as a conserved group of molecular chaperones (Heirbaut et al. 2014). HspB6 has also been identified as a regulator for hepatocellular carcinoma apoptosis via direct association with Bax (Nagasawa et al. 2014) In addition, overexpression of HspB6 has been observed in many types of muscles, including uterine smooth muscle, airway, skeletal muscle, bladder, cardiac muscle, colonic and vascular muscles, and can be phosphorylated by cyclic adenosine monophosphate (cAMP)-dependent protein kinases, like protein kinase A (PKA) for example (Fan and Kranias 2011). The cAMP-PKA pathway delivers extracellular stimuli and alters cell responses by the cAMP signaling cascade (Guo et al. 2016). As reported in a previous study, activation of the cAMP-PKA pathway has been found to prevent neurons from undergoing apoptosis (Wang et al. 2005). Moreover, a previous study revealed that when the cognitive impairment of epileptic rats was inhibited, rats exhibited increased phosphoactivation of PKA (Zhen et al. 2016). Since HspB6 and the cAMP-PKA pathway have both been revealed to be of great importance in the nervous system, we hypothesized that HspB6 might interact with the cAMP-PKA pathway which exerts the regulatory effects on epilepsy. Therefore, in the present study, we set out to explore whether cAMP-PKA pathway-mediated HspB6 could serve as a new target choice for treatment of epilepsy.

Methods

Ethics Statement

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Laiwu Hospital Affiliated to Taishan Medical University (No, 201703006; Date, March, 6th, 2017).

Model Establishment

A total of 30 healthy adult male Sprague–Dawley (SD) rats (weighing 232 ± 10 g) were evenly assigned into post-KA group (rats intraperitoneally injected with kainic acid [KA, 12 mg/kg] to induce epileptic seizure) (Hsieh et al. 1999) and normal group (rats intraperitoneally injected with normal saline). KA is a seaweed extract that mediates glutamic kainate receptors in the central nervous system of vertebrate by directly exciting neurons, and enhances the sodium permeability as well as depolarizing neuronal cells, inducing acute epileptic seizure. Due to its properties, KA is widely used to induce epileptic seizure in rats (Sato and Woolley 2016; Ryan et al. 2012). In the post-KA group, epileptic seizures in 12 rats were successfully induced; 10 rats were randomly respectively selected from the post-KA and normal groups for subsequent experiments.

Epileptic seizures were classified according to classical Racine Stages. A successful model of epileptic seizure was confirmed by the presence of epileptic seizures of $\geq$ stage IV and a duration of ≥ 30 min. Rats that had epileptic seizure for more than 1 h were intraperitoneally injected with 3% pentobarbital sodium (P3761, Sigma–Aldrich Chemical Company, St Louis, MO, USA) to terminate their epileptic seizures. If necessary, injection was repeated every 6–8 h. After KA treatment for 24 h, rats in both groups were given intraperitoneal injections of 5 mL normal saline and intragastric administration of food on the next day until they were able to eat on their own. One week later, the rats in the post-KA group were euthanized.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Trizol assay was used to extract total RNA. cDNA was then synthesized according to the instructions provided by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). RT-qPCR was carried out using a Power SYBR® Green Master mix (Applied Biosystems, Carlsbad, CA, USA) and StepOne™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR reaction conditions used were set as 95 °C for 15 min, and 30–40 cycles of 94 °C for 15 s, 60 °C for 20 s and 72 °C for 10 s. Each pair of primers had three duplicate wells. The primers for RT-qPCR are shown in Table 1.

Western Blot Analysis

Total proteins of tissues and cells were extracted. Bicinchoninic acid (BCA) kit (20201ES76, Yeasen Company, Shanghai, China) was used to determine protein

Table 1 The primers for RT-qPCR

Gene	Sense (5′–3′)	Anti-sense (5′–3′)
HspB6	GGATTCATTGCTCGAGAGTTCC	TGTGGCCTGGATAGACAGAA

RT-qPCR reverse transcription quantitative polymerase chain reaction, *Hspb6* heat shock protein B6

concentration. Quantification of samples was conducted based on different concentrations with the amount of protein per lane adjusted to 30 µg. Polyacrylamide gel electrophoresis (PAGE) was applied for protein separation before transferring proteins onto a polyvinylidene fluoride (PVDF) membrane using the wet transfer method. Membranes were sealed by 5% bovine serum albumin (BSA) at room temperature for 1 h, and subsequently incubated with rabbit primary monoclonal HspB6 antibody (1:1000, ab68977), p-HspB6 antibody (1:5000, ab58522), PKA antibody (1:1000, ab26322), and apoptosis signal-regulating kinase 1 (ASK1) antibody (1:1000, ab131506) at 4 °C overnight. All the above-mentioned antibodies were used were purchased from Abcam Inc. (Cambridge, MA, USA). The membranes were rinsed with tris-buffered saline with tween 20 (TBST) 3 times (5 min each time), incubated with corresponding secondary antibodies at room temperature for 1 h, and followed by three-time rinse (5 min each time). The membrane was immersed in a chemiluminescence reaction solution and allowed to develop. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as internal reference. Bio-rad Gel Dol EZ imager (Bio-Rad, Richmond, Cal., USA) was employed to develop the membranes. The intensity values of the target bands were analyzed using Image J software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Growth medium was removed from cell culture wells, and the adherent cells were collected by scraping. Cells were washed with cold phosphate buffer saline (PBS), and then re-suspended in 10 mM Tris–Cl (pH = 7.4). Cells were lysed by freeze-thawing 3 times, and protein concentration was measured using a Bradford Assay. Next, 100 µg of protein lysate was analyzed using ELISA according to the instructions provided by the Quantikine Rat tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA).

Immunohistochemical Method

The hippocampus tissues were embedded with paraffin, and cut into sections. Sections were then dewaxed and dehydrated, followed by antigen retrieval in water bath. Normal goat serum sealing fluid (C-0005, Haoran Biotechnology Co., Ltd., Shanghai, China) was added for blocking and left to incubate at room temperature for 20 min. Primary rabbit anti-human glial fibrillary acidic protein (GFAP) antibody (1:5000, ab7260, Abcam Inc., Cambridge, MA, USA) was added to sections for incubation at 4 °C overnight. On the next day, goat anti-rabbit immunoglobulin G (IgG) (1:1000, ab6785, Abcam Inc., Cambridge, MA, USA) was added and incubated at 37 °C for 20 min. Sections was further incubated with horse radish peroxidase (HRP)-labeled streptavidin (0343-10000U, Imunbio Co., Ltd., Beijing, China) at 37 °C for an additional 20 min. Next, 3,3′-diaminobenzidine (DAB) (ST033, WHIGA Co., Ltd., Guangzhou, Guangdong, China) was added for coloration. After that, the sections were counterstained with hematoxylin (PT001, Shanghai Bogoo Biological Technology Co., Ltd., Shanghai, China) for 1 min before rinsing. Sections were then placed in 1% ammonia to allow color to return blue, followed by rinsing with water, sealed with neutral gum, and observed under a light microscope to be photographed. Five high-power fields with approximately 100 cells were randomly selected from each section to be photographed. The number of positive cells < 10% was regarded to be negative, 10% ≤ the number of positive cells < 50% to be positive, and the number of positive cells > 50% to be strongly positive (Atkins et al. 2004).

Nissl Staining

Hippocampus was fixed in 4% paraformaldehyde for 24 h, rinsed with water, dehydrated with gradient ethanol, and cleared with xylene, followed by wax dipping, paraffin embedding, and slicing. The sections were dried at room temperature, and then submerged in distilled water. The gradient alcohol dehydration was conducted with the concentration of alcohol increasing from 70 to 100% (2 min each time). After that, sections were treated with a decreasing concentration of alcohol from 100% back to 70%, 2 min in each concentration. Sections were then washed with distilled water twice, 10 min each time, and stained with 0.5% toluidine blue at 60 °C for 60 min. This was proceeded by rinsing in water, differentiation with gradient alcohol and clearing with xylene. The sections were mounted with neutral gum, and observed under the microscope. Finally, Image-pro Plus 6.0 software (Media Cybernatics, Inc., Silver Spring, MD, USA) was used to analyze the sum of pixels for positive staining, which reflects live neurons in the brain slices.

Recombinant Adenoviral Constructs

Rat HspB6 cDNA and its mutants of TCA-GCA or TCA-GAC (replaced Ser16 with Ala to block or with Asp to mimic phosphorylation) were separately inserted into an E1/E3 deleted adenoviral vector and designated as Ad.HspB6, Ad.S16A, and Ad.S16D, respectively. The mutated nucleotides were identified in bold, and underlined. Ad.HspB6, Ad.S16A, and Ad.S16D encoded the wild-type HspB6, the non-phosphorylatable form of HspB6, and the constitutively phosphorylated form of HspB6, respectively (Fig. 3a).

In Vitro Culture of Hippocampal Neurons

Treatment of culture plate: 24 h before the experiment, 6-well plastic culture plate was coated with 50 µg/mL polylysine. When cells were ready to be inoculated, the inner liquid was discarded, and the plate was rinsed with sterile PBS 2–3 times for subsequent use.

Culture of brain hippocampal neurons: 24-h or 1-day old newborn rats were disinfected with ethanol with a volume fraction of 0.75. On a clean bench, the ophthalmic scissors were used to cut the skull and brain tissue in order to remove the hippocampus after exposing the cerebral cortex. The hippocampus was placed in a glass culture dish containing PBS. After the removal of the meninges and blood vessels with ophthalmic forceps, the hippocampus was rinsed in another culture dish, and cut into blocks with dimensions of 1 mm × 1 mm × 1 mm on ice. The tissues were incubated with equal volume of 0.125% trypsin an incubator with 5% CO_2 at 37 °C for 20 min, and shaken once every 10 min. Following the addition of culture medium to terminate cell detachment, detached tissues were transferred into a centrifuge tube, and added with 2 mL cultivation medium. The centrifuge tube was then gently triturated several times with a flame-polished glass tube, and allowed to stand for 2–3 min. The upper cell suspension was aspirated. Cell concentration was adjusted to 5×10^8 cells/L with culture medium, and the cells were inoculated to polylysine-coated plates (2 mL per well), and incubated in a 5% CO_2 incubator at 37 °C. After 24 h, culture medium in the 6-well plates was removed and replaced with a maintenance medium for further culture. In the following days, half of the medium was replaced twice every 7 days. On the 5th day of inoculation, 50 µg/mL cytosine arabinoside (Ara-C) was added to the medium to prevent over-proliferation of non-neuronal cells. After 48 h, the cells were further cultured in fresh maintenance medium. Hippocampal neurons were later identified by immunofluorescence cytochemistry to confirm that hippocampal neurons had high purity.

Grouping and Transfection of Hippocampal Neurons

Hippocampal neurons were classified into groups as shown: oe-HspB6 group (transfected with HspB6 overexpression plasmid) and oe-negative control (oe-NC) group (transfected with empty plasmid); si-HspB6 group (transfected with siRNA against HspB6) and si-NC group (transfected with scramble siRNA); H89 group (treated with 10 uM of H89, an inhibitor of PKA; Sigma–Aldrich Chemical Company, St Louis, MO, USA) and dimethyl sulfoxide (DMSO) group (treated with DMSO solvent as a control compound-carrier); Ad.HspB6 group (treated with overexpressed HspB6 adenovirus), Ad.S16A group [treated with overexpressed HspB6 (S16A) adenovirus], and Ad.S16D group [treated with overexpressed HspB6 (S16D) adenovirus]. HspB6 (S16A) is a mutant that 16th serine mutated to alanine, while HspB6 (S16D) is a mutant that 16th serine mutated to aspartic acid.

On the 5th day of cell culture, 250 µL of OPTI-MEM was mixed with 4 µL adenoviral particles, and allowed to stand for 20 min. Then, the original culture solution for hippocampal neurons was collected. Samples were rinsed twice with antibiotic-free Neurobasal medium, followed by the addition of 1.5 mL antibiotic-free Neurobasal medium and well-incubated adenovirus particles for 5 h. The solution was replaced by the original culture solution for further cell culture.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

After transfection for 24 h, cells were fixed with 2% formaldehyde at room temperature for 1 h, and permeabilized with 0.1% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) on ice for 2 min. After being rinsed with PBS three times, the cells were incubated with 50 µL TUNEL mixtures in a humid and dark environment at 37 °C for 1 h according to the instructions provided by the kit. After incubation, cells were observed under an inverted fluorescence microscope and images were taken (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All experimental data were analyzed by a normal distribution and variance test. Measurement data in accordance with normal distribution were expressed as mean ± standard deviation. Differences between two groups of data were compared by an independent sample t test. Comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA). Pairwise comparison within one group was performed using the least-significant difference (LSD) t test. Two-factor combined effects

were analyzed at the same time by two-way ANOVA. Values of $p < 0.05$ were considered significant.

Results

Downregulated HspB6 Participates in the Development of Epilepsy by Promoting the Inflammatory Response

Rat models were established through intraperitoneal injection of KA. Following model establishment, eight rats were found to exhibit the onset of epileptic seizure of a grade IV or above. Symptoms included bilateral forelimb clonus, hind limb standing, falling down, and imbalance. Moreover, one rat did not exhibit a seizure; and one rat twitched to death. The rats in the normal group exhibited normal daily activities without any epileptic symptoms during the whole duration of the experiment.

When investigating the effect of HspB6 on epilepsy, RT-qPCR assay and western blot analysis were used to determine the expression of HspB6 in the hippocampus of rats with epileptic seizure post KA. The results showed that compared with the normal group, the expression of HspB6
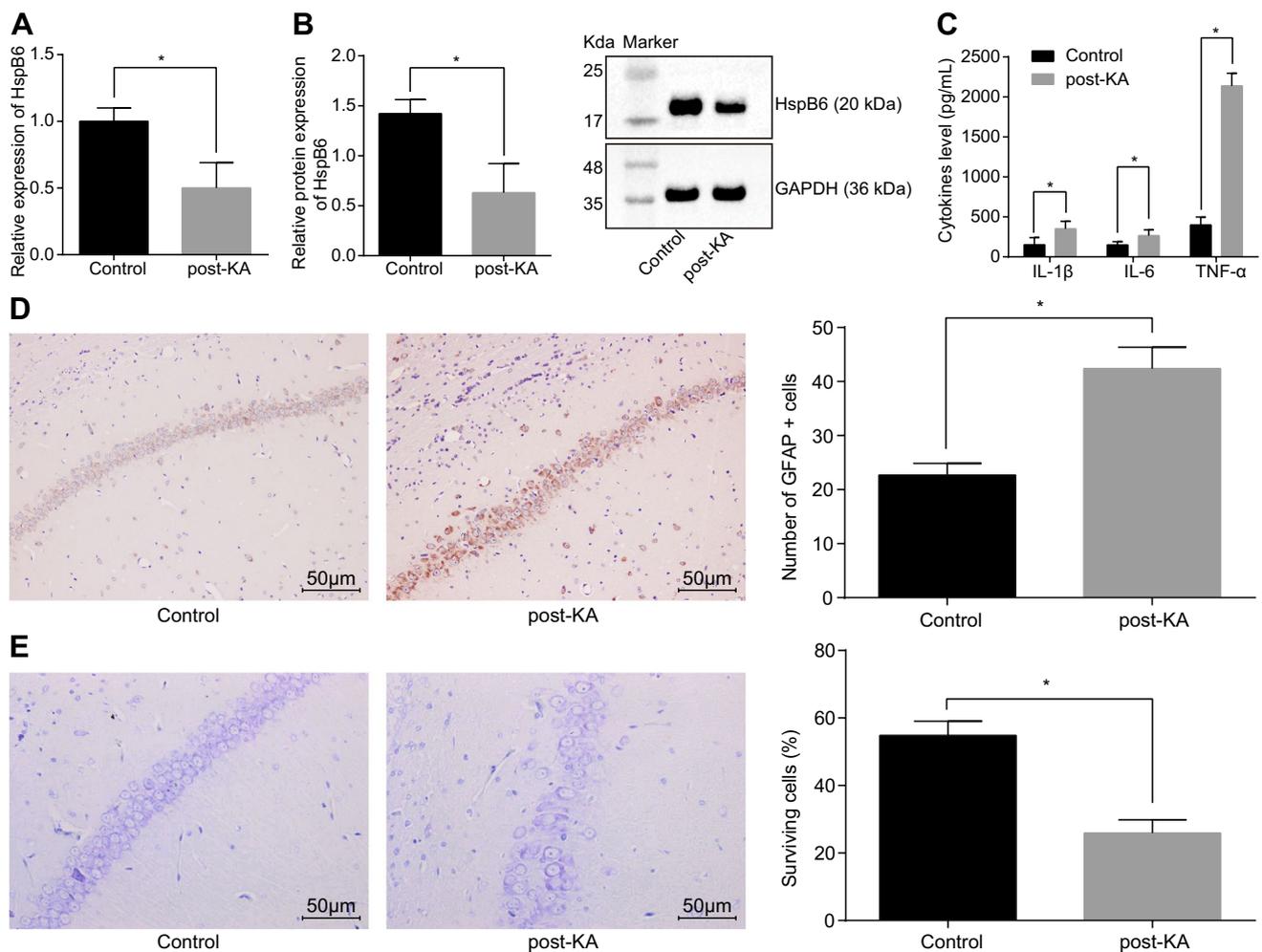

Fig. 1 HspB6 is poorly expressed and inflammatory factors are highly expressed in rats with epileptic seizure post KA. **a** results of RT-qPCR assay showed that relative mRNA of HspB6 was lowered in the hippocampus of rats post KA when compared with the normal rats; **b** results of western blot analysis showed that relative protein level of HspB6 was decreased in hippocampus of rats post KA when compared with the normal rats; **c** results of ELISA assay showed that cytokine levels of inflammatory factors (TNF-α, IL-1β, and IL-6) were higher in hippocampus of rats post KA than in hippocampus of normal rats; **d** results of immunohistochemistry showed that astrocytes were more in hippocampus of rats post KA indicated by the larger number of GFAP-positive cells (scale bar: 50 μm); **e** results of Nissle staining showed that the neuronal survival was significantly reduced in rats post KA (scale bar: 50 μm); *$p < 0.05$ versus the normal group; $n = 10$; the measurement data were expressed as mean ± standard deviation, and analyzed by independent sample t test, *HspB6* heat shock protein B6, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *GFAP* glial fibrillary acidic protein

significantly decreased in the post-KA group (Fig. 1a, b, $p < 0.05$). In addition, an ELISA assay was used to determine the expression of inflammatory factors TNF-α, IL-1β, and IL-6 in the hippocampus of rats post KA. The results revealed that the expressions of TNF-α, IL-1β, and IL-6 significantly increased in the post-KA group compared to the normal group (Fig. 1c, $p < 0.05$). Through immunohistochemistry, the morphological structure of astrocytes in the hippocampus of rats with epileptic seizure post KA was analyzed and compared. Results demonstrated that the number of GFAP-positive cells in the post-KA group was significantly higher than that in the normal group (Fig. 1d, $p < 0.05$). Nissl staining showed that the hippocampal neurons in the normal group were orderly arranged in a certain direction, and that neuronal cells were round or elliptical with a lavender colored cytoplasm. In the post-KA group, the hippocampal neurons appeared disordered and disoriented, while neuronal cells were irregular in morphology with a wrinkled and dark purple cytoplasm. Compared with the normal group, neuronal survival in the post-KA group was significantly lower (Fig. 1e, $p < 0.05$). All results above suggest that HspB6 was poorly expressed, while inflammatory factors were upregulated with more astrocytes present and more apoptotic hippocampal neurons in rats with epileptic seizure post KA.

HspB6 Inhibits the Inflammatory Response and Hippocampal Neuronal Apoptosis in Rats post KA

In order to examine the role of HspB6 in rats with epileptic seizure post KA, we intervened the expression of HspB6 with oe-HspB6 (transfected with HspB6 overexpression) and si-HspB6 (transfected with siRNA against HspB6). RT-qPCR assay was used to determine the expression of HspB6 in hippocampal neurons in the oe-HspB6, the oe-NC, the si-HspB6, and the si-NC groups following *in vitro* culture and transfection. The result showed that the expression of HspB6 in the oe-HspB6 group was significantly higher compared with that in the oe-NC group ($p < 0.05$). However, the si-HspB6 group exhibited significantly lower HspB6 expressions in comparison to the si-NC group (Fig. 2a, $p < 0.05$). ELISA assay was adopted to measure the expression of the inflammatory factors TNF-α, IL-1β, and IL-6 in hippocampal neurons following transfection. Results revealed that the expression of TNF-α, IL-1β, and IL-6 was significantly reduced in the oe-HspB6 group compared with that in the oe-NC group ($p < 0.05$) but higher in the si-HspB6 group than that in the si-NC group (Fig. 2b, $p < 0.05$). TUNEL staining was performed to assess cell apoptosis of hippocampal neurons. Compared with oe-NC group, cell apoptosis of hippocampal neurons in oe-HspB6 group was significantly attenuated ($p < 0.05$) compared with the si-NC group,

whereas apoptosis of hippocampal neurons was promoted in the si-HspB6 group (Fig. 2c, $p < 0.05$). Thus, our results suggested that overexpressed HspB6 could notably inhibit the initiation of the inflammatory pathways and hippocampal neuronal apoptosis in rats with epileptic seizure post KA.

Elevated Extent of HspB6 Phosphorylation Augments the Inhibitory Effects on Inflammatory Response and Hippocampal Neuronal Apoptosis in Rats post KA

Adenovirus vectors of wild-type HspB6, the non-phosphorylated form of HspB6, and the constitutively phosphorylated form of HspB6 were established (Fig. 3a) in order to alter the phosphorylation status of HspB6 in hippocampal neurons cultured *in vitro*. This was done with the aim of detecting the regulation of inflammatory responses and epilepsy by phosphorylation of HspB6. Hippocampal neurons were assigned into Ad.HspB6 group (treated with overexpressed HspB6 adenovirus), Ad.S16A group [treated with overexpressed HspB6 (S16A) adenovirus], and Ad.S16D group [treated with overexpressed HspB6 (S16D) adenovirus]. Western blot analysis was used to analyze the phosphorylation status of HspB6 in hippocampal neurons. Results showed that the Ad.S16A group had a lower phosphorylation level of HspB6 ($p < 0.05$), whereas the Ad.S16D group had an obviously higher phosphorylation level of HspB6 when both were compared with the Ad.HspB6 group (Fig. 3b, $p < 0.05$). An ELISA was then performed to determine the expressions of inflammatory factors TNF-α, IL-1β, and IL-6 in hippocampal neurons which showed that compared to the Ad.HspB6 group, the Ad.S16A group exhibited an obviously higher expression levels of TNF-α, IL-1β, and IL-6 ($p < 0.05$), whereas the Ad.S16D group displayed the opposite trend (Fig. 3c, $p < 0.05$). Furthermore, the hippocampal neuronal apoptosis that took place following transfection was detected by TUNEL staining. We found that compared with the Ad.HspB6 group, the apoptosis rate of hippocampal neurons was significantly elevated in the Ad.S16A group ($p < 0.05$) while the Ad.S16D group exhibited an opposite trend (Fig. 3d, $p < 0.05$). According to our results, we can conclude that overexpression of HspB6 could inhibit the onset of the inflammatory response and hippocampal neuronal apoptosis while phosphorylation of HspB6 could help strengthen these inhibitory traits.

Inhibition of ASK1 Expression by HspB6 Inhibits Hippocampal Neuronal Apoptosis in Rats post KA

Bioinformatics website (http://string-db.org/cgi/input.pl) was used to predict and demonstrated that an interaction between HspB6 and ASK1 existed (Fig. 4a). Online literature search provided evidence suggesting that ASK1
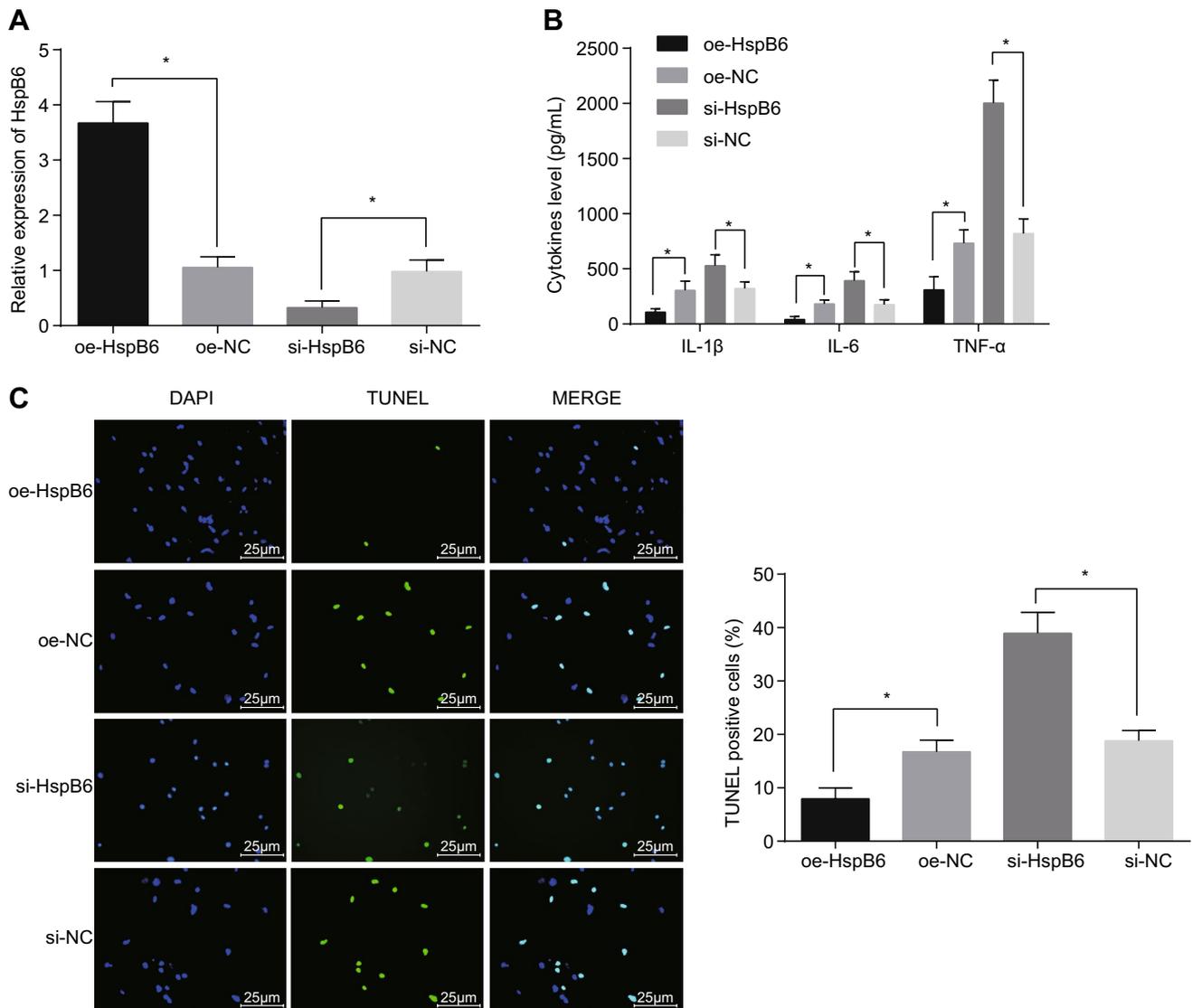
Fig. 2 Inflammatory response and hippocampal neuronal apoptosis are effectively inhibited by overexpression of HspB6 in rats with epileptic seizure post KA. **a** RT-qPCR assay was used to determine the relative mRNA expression of HspB6 following transfection; **b** results of ELISA showed that upregulated HspB6 inhibited the cytokine levels of inflammatory factors (TNF-α, IL-1β and IL-6); **c** results of TUNEL staining showed that upregulated HspB6 decreased hippocampal neuronal apoptosis (×400); *$p < 0.05$ versus the oe-NC or si-NC group; the measurement data were expressed as mean ± standard deviation, and analyzed by one-way analysis of variance; *HspB6* heat shock protein B6, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay, *TUNEL* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *NC* negative control

inhibitor may be a potential therapeutic target used to treat major inflammatory lesions including kidney, lung, and liver diseases in clinical practices (Lovering et al. 2018). Neuronal protection against cerebral ischemia–reperfusion injury could be promoted through the inhibition of the AT1/ASK1/MKK4/JNK3 signaling pathway in the CA1 zone in the hippocampus of rats (Zhang et al. 2012). To determine the interaction between HspB6 and ASK1, the expression of ASK1 was measured by western blot analysis after the hippocampal neurons were transfected with lentivirus vectors: Ad.HspB6, Ad.S16A, and Ad.S16D. The results showed that compared to the Ad.HspB6 group, ASK1 expression was significantly lower in the Ad.S16A group ($p < 0.05$) but was significantly higher in the Ad.S16D group (Fig. 4b, $p < 0.05$). These results suggested that the inhibitory effects of HspB6 phosphorylation on the inflammatory response and hippocampal neuronal apoptosis in rats with epileptic seizure post KA occurred as a result of suppression of ASK1 expression.
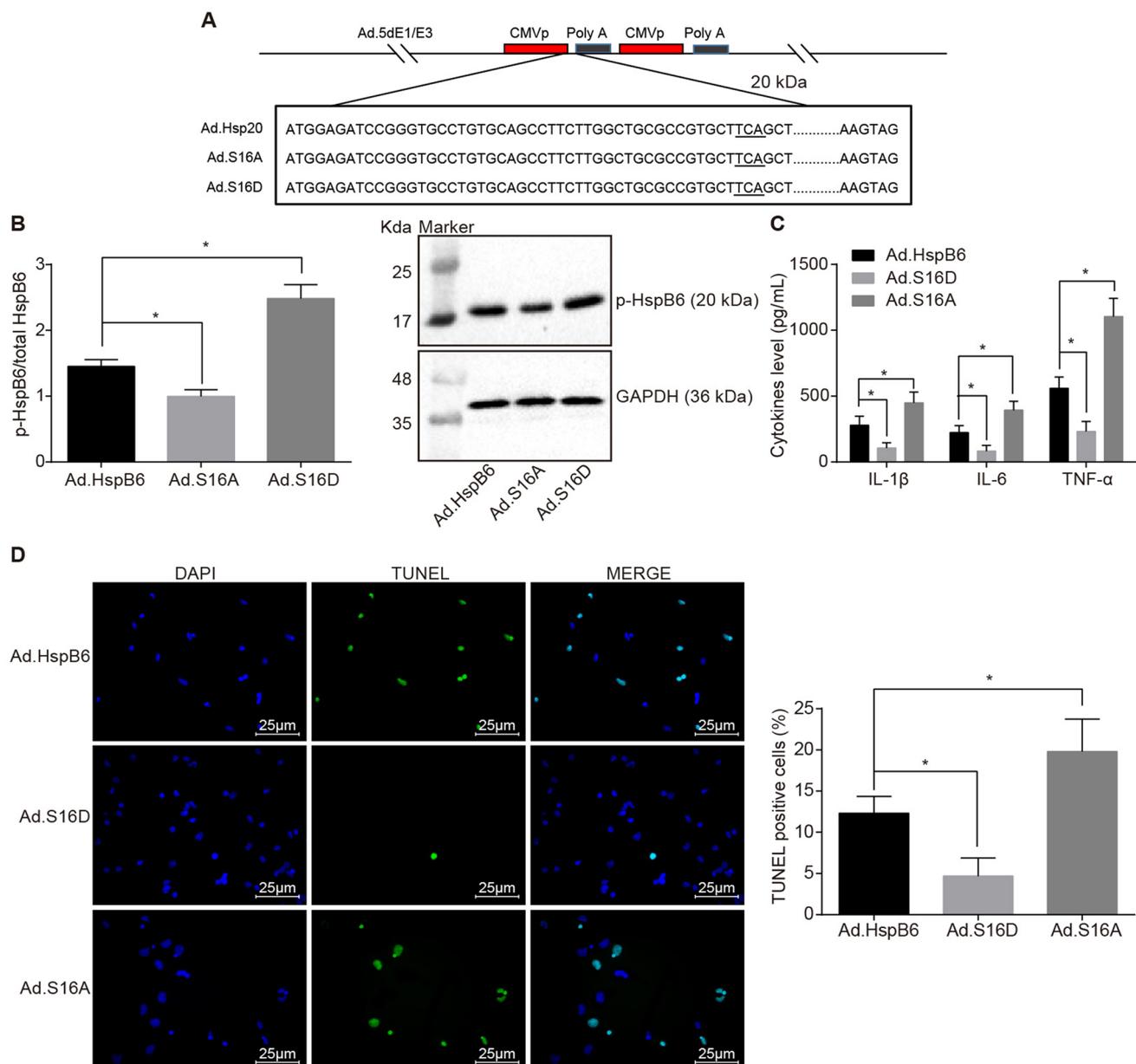
Fig. 3 The phosphorylation of HspB6 can further strengthen the inhibitory effects of HspB6 on the inflammatory response and hippocampal neuronal apoptosis in rats with epileptic seizure post KA. **a** recombinant adenoviral vectors was presented in a diagram; **b** results of western blot analysis showed that phosphorylation level of HspB6 was decreased by Ad.S16A but elevated by Ad.S16D; **c** results of ELISA assay showed that expression of inflammatory factors (TNF-α, IL-1β, and IL-6) was elevated by Ad.S16A but decreased by Ad.S16D; **d** results of TUNEL staining revealed that hippocampal neuronal apoptosis was promoted by Ad.S16A but suppressed by Ad.S16D; *$p < 0.05$ versus the Ad.HspB6 group; the measurement data were expressed as mean ± standard deviation, and analyzed by one-way analysis of variance; *HspB6* heat shock protein B6, *ELISA* enzyme-linked immunosorbent assay, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *TUNEL* terminal dexynucleotidyl transferase-mediated dUTP nick end labeling

The Activation of the cAMP-PKA Pathway Promotes the Extent of HspB6 Phosphorylation in Hippocampal Neurons

It has been previously shown that HspB6 may be phosphorylated by PKA/PKG (Beall et al. 1999; Flynn et al. 2003). HspB6 or the phosphorylated form of HspB6 could potentially lead to protective effects on apoptosis (Fan et al. 2004). Western blot analysis was applied to analyze the phosphorylated state of HspB6 in hippocampal neurons that were cultured in vitro and respectively transfected with oe-HspB6 and oe-NC, the results of which showed that the
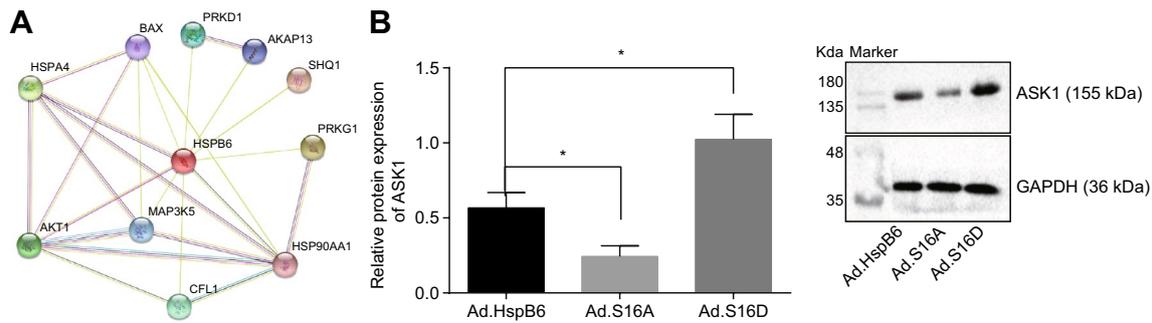
Fig. 4 HspB6 inhibits ASK1 expression in hippocampal neuron of rats with epileptic seizure post KA. **a** results of bio-information predication showed that HspB6 interacted with ASK1 in hippocampal neurons; **b** results of western blot showed that ASK1 expression was reduced by Ad.S16A in hippocampal neurons; the measurement data were expressed as mean ± standard deviation, and analyzed by one-way analysis of variance; $*p < 0.05$ versus the Ad.HspB6 group; *HspB6* heat shock protein B6, *ASK1* apoptosis signal-regulating kinase 1

concentration of the phosphorylated state of HspB6 in the oe-HspB6 group was significantly higher compared with that in the oe-NC group (Fig. 5a, $p < 0.05$). When we further assessed the phosphorylation status of HspB6 in hippocampal neurons of rats with epileptic seizure post KA using western blot analysis, we found that the extent of HspB6 phosphorylation in the post-KA group was significantly lower than that in the normal group (Fig. 5b, $p < 0.05$).

H89, an inhibitor of PKA, was applied to block the cAMP-PKA pathway (Song et al. 2015). In order to determine how the cAMP-PKA signaling pathway affected the extent of HspB6 phosphorylation, hippocampal neurons were treated with H89 and DMSO following in vitro culture, and PKA expression was quantified using western blot analysis. The results revealed that expression of PKA in the H89 group was significantly lower compared to the DMSO group (Fig. 5c, $p < 0.05$). The extent of Hsp86 phosphorylation was also evaluated by western blot analysis, which showed that the extent of HspB6 phosphorylation in the H89 group was significantly lower than that in the DMSO group (Fig. 5d, $p < 0.05$). Thus, our data suggest that HspB6 was hypo-phosphorylated in rats with epileptic seizure post KA, which could be promoted through the activation of the cAMP-PKA signaling pathway.
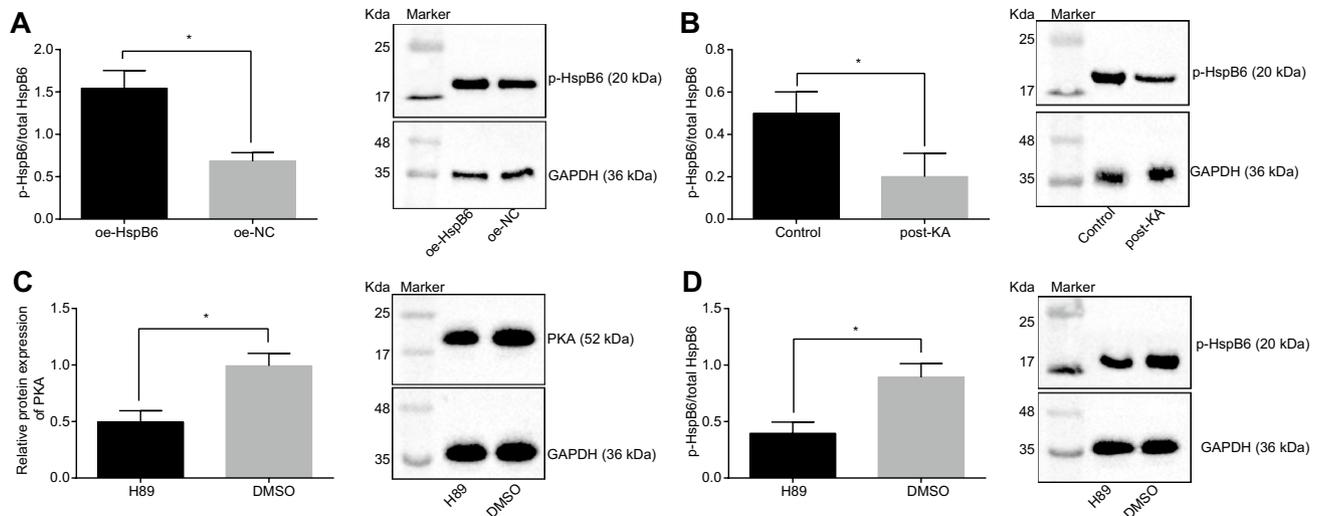

Fig. 5 Activated cAMP-PKA signaling pathway increases HspB6 phosphorylation in hippocampal neurons. **a** western blot analysis revealed that forced expression of HspB6 increased the extent of HspB6 phosphorylation in hippocampal neurons; **b** western blot analysis revealed that HspB6 was hypo-phosphorylated in rats post KA, $n = 10$; **c** western blot analysis revealed that hippocampal neurons treated with H89 had an obviously lower PKA expression than those treated with DMSO; **d** hippocampal neurons treated with H89 had an obviously lower extent of HspB6 phosphorylation; $*p < 0.05$ versus the oe-NC, normal, or DMSO group; the measurement data were expressed as mean ± standard deviation, and analyzed by independent sample *t* test; *HspB6* heat shock protein B6, *cAMP-PKA* cyclic adenosine monophosphate-protein kinase A, *DMSO* dimethyl sulfoxide

The cAMP-PKA Signaling Pathway Suppresses the Development of Epilepsy Through Inhibition of Inflammatory Response

In the last part of the investigation, RT-qPCR assay was utilized to characterize the PKA expression in hippocampus of rats with epileptic seizure post KA so as to elucidate the mechanism of the cAMP-PKA signaling pathway as well as the involvement of inflammatory response in epilepsy. We found that compared with the normal group, PKA expression was significantly lower in the post-KA group (Fig. 6a, $p < 0.05$). ELISA was then employed to analyze the expression of inflammatory factors TNF-α, IL-1β, and IL-6 after the cAMP-PKA pathway was inhibited. This demonstrated that compared with the DMSO group, the H89 group had an obviously higher expression of TNF-α, IL-1β, and IL-6 (Fig. 6b, $p < 0.05$). Meanwhile, TUNEL staining was applied to measure the rate of hippocampal neuronal apoptosis, which highlighted that the rate was higher in the H89 group compared to the DMSO group (Fig. 6c, $p < 0.05$). Our results have demonstrated that the cAMP-PKA signaling pathway could inhibit the onset of inflammatory response that may further lead to anti-epilepsy effects.

Discussion

Epilepsy is characterized by a persistent tendency to generate epileptic seizures (Fisher et al. 2014; Mohler 2006). The HspB6 protein has been confirmed as a mediator in central nervous system (Li et al. 2017a). Due to its involvement in the CNS, we selected HspB6 as the research objective to determine the extent of its role in epilepsy treatment. Our results showed that HspB6 was able to interact with the cAMP-PKA pathway in hippocampal neurons of rats with epileptic seizure post KA. When activated, cAMP-PKA pathway leads to upregulated HspB6 in order to inhibit inflammatory response and hippocampal neuronal apoptosis in rats post KA through repressing ASK1 expression.

The major finding of our study was that inhibition of the cAMP-PKA pathway and overexpression and phosphorylation of HspB6 led to an increase in hippocampal neuronal
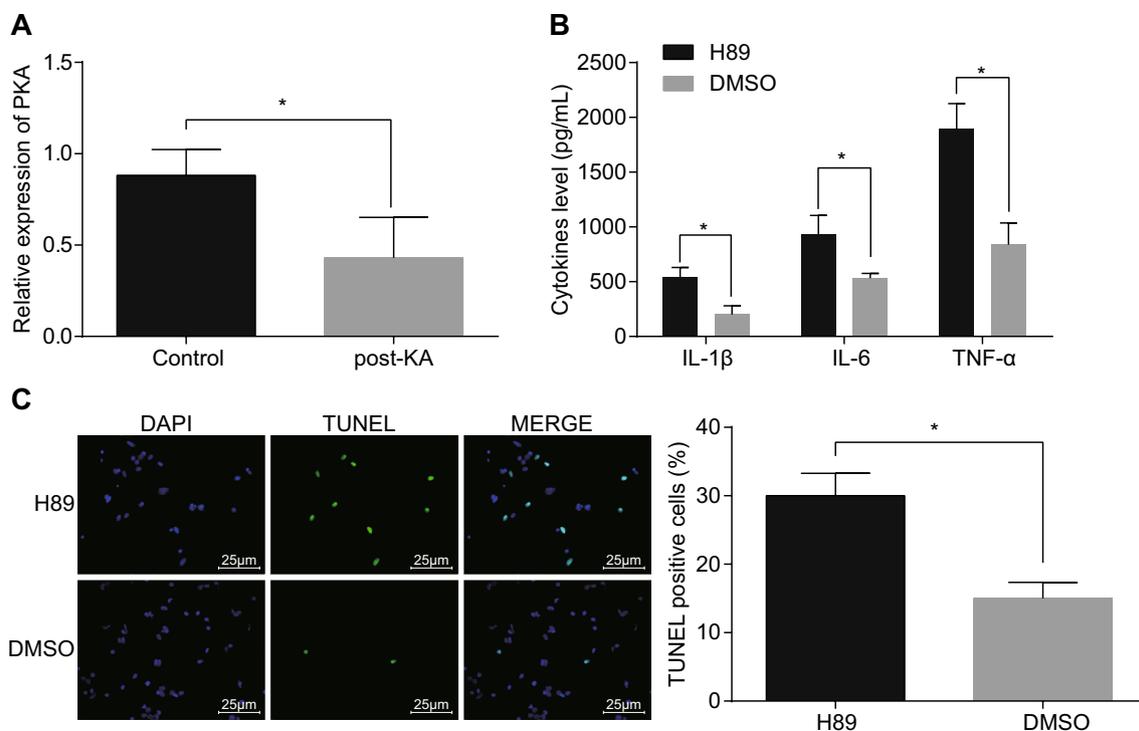

Fig. 6 The cAMP-PKA signaling pathway participates in the attenuation of epilepsy by repressing the inflammatory response. **a** results of RT-qPCR showed that PKA expression was significantly lowered in hippocampal tissues of rats post KA; **b** results of ELISA showed that expression of TNF-α, IL-1β, and IL-6 was elevated by H89; **c** results of TUNEL staining showed that hippocampal neuronal apoptosis rate was increased by H89; $n = 10$; the measurement data were expressed as mean ± standard deviation, and analyzed by independent sample t test; *$p < 0.05$ versus the normal or DMSO group; *cAMP-PKA* cyclic adenosine monophosphate-protein kinase A, *HspB6* heat shock protein B6, *RT-qPCR* reverse transcription quantitative polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay, *TUNEL* terminal dexynucleotidyl transferase-mediated dUTP nick end labeling, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *DMSO* dimethyl sulfoxide

apoptosis and inflammatory response. This was reflected in the increased levels of inflammatory factors TNF-α, IL-1β, and IL-6, in rats post KA. It has been reported that the pathophysiology of epilepsy involves inflammation (Gao et al. 2018). Seizure activity is considered a type of inflammation in the brain, which can result from physiologic, behavioral as well as environmental stressors (Mazarati et al. 2017). Neuronal excitability as well as seizure susceptibility has been documented to be promoted by inflammation (Xiao et al. 2017). Glia cells, such as microglia and astrocytes, and neurons can be attributed to epilepsy, which has overexpression of IL-1β, IL-6 as well as TNF-α (Tombini et al. 2013). In addition, it is reported that hippocampal neuronal apoptosis can be induced by acquired epilepsy (Xie et al. 2016). Serious hippocampal neuronal loss tends to be a pathological feature for temporal lobe epilepsy (Tan et al. 2015). Similarly, it has been reported that in rats post KA, the progression of epilepsy can be inhibited by attenuated inflammatory response and hippocampal apoptosis as well as cognitive impairments by chronic trigeminal nerve stimulation (Wang et al. 2016). A study conducted by Lin Li et al. revealed that the cAMP-PKA pathway was involved in pathways such as neuroplasticity, learning and memory ability, and neuronal regeneration. Therefore, the activation of the cAMP-PKA pathway caused by Chinese medicines could rescue central nervous system function (Li et al. 2017b). Interestingly, another study showed that when epilepsy was suppressed, rats exhibited an increased phosphoactivation of PKA (Zhen et al. 2016). Besides, HspB6 not only regulates cardioprotective signaling but also protects central nervous system from injury (Li et al. 2017a).

In another portion of our research, we found that inhibition of ASK1 expression by HspB6 resulted in suppressed hippocampal neuronal apoptosis in rats post KA. We also observed that the activated cAMP-PKA pathway promoted high expression and increased phosphorylation of HspB6 protein. Increased HspB6 and activated cAMP-PKA pathway lead to a decrease in inflammatory factors that are able to inhibit inflammation and attenuate hippocampal neuronal apoptosis. It has been demonstrated that cAMP-dependent protein kinase can phosphorylate HspB6 as a small heat shock protein (Ba et al. 2009), which was in line to what we found in the present study. Moreover, HspB6 overexpression has been demonstrated to decrease expression of TNF-α and NF-κB pathway-related genes (Nagasawa et al. 2015). Furthermore, Xuemin Wang et al. observed that inactivated cAMP-PKA pathway decreased neuron survival rate (Wang et al. 2005). ASK1, also named mitogen-activated protein kinase kinase kinase 5 (MAP3K5), has been identified as a potential apoptotic inducer in the context of many kinds of physiological conditions (Sakauchi et al. 2017). Short duration epilepsy has been reported to recruit a molecular scaffold complex that comprises of a receptor interacting protein and a death domain protein associated with tumor necrosis factor receptor (TNFR). The components of the molecular scaffold could serve to explain why ASK1 was activated (Shinoda et al. 2003). Likewise, higher expression level of ASK1 has also been discovered in the brain of patients with temporal lobe epilepsy (Yamamoto et al. 2006). Thus, we have illustrated that HspB6 was able to inhibit ASK1 expression, suggesting that the function of HspB6 in rats post KA was significantly related to the repression of ASK1.

In conclusion, our results demonstrate that overexpressed HspB6 caused by an activated cAMP-PKA pathway inhibits the inflammatory response and hippocampal neuronal apoptosis in rats with epileptic seizure post KA by down-regulating ASK1. These findings may open novel avenues for future epilepsy therapies. However, our study has focused on SD rat models, which warrants additional research in other animal models, as we are unsure of the unwanted side effects in a clinical setting. Furthermore, we will continue to explore the mechanism of cAMP-PKA pathway in human cells.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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