α7 nicotinic acetylcholine receptor agonist attenuates the cerebral injury in a rat model of cardiopulmonary bypass by activating the Akt/GSK3β pathway

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Abstract. α7 nicotinic acetylcholine receptor (α7nAchR) agonist treatment may provide a promising therapeutic effect for cerebral injuries. However, it is unclear whether the activation of α7nAchR agonist may reduce cerebral injuries induced by cardiopulmonary bypass (CPB). A total of 96 male Sprague-Dawley rats were randomly divided into four groups (n=24/group): i) Sham operation group; ii) CPB group; iii) CPB + α7nAchR agonist group; and iv) CPB + α7nAchR agonist + α7nAchR antagonist group. Following treatment, 24 rats from each group were sacrificed and the serum and hippocampal tissues were collected. The serum expression levels of S100β, interleukin 6 and tumor necrosis factor α were evaluated by ELISA, hippocampal tissues were analyzed by histopathological examination using hematoxylin & eosin and terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) staining and Caspase 3 expression in the hippocampal tissues was evaluated by immunohisto-chemistry. In addition, Caspase 3, Akt and glycogen synthase kinase 3β (GSK3β), as well as phosphorylated (p)-Akt and (p)-GSK3β were examined by western blot assay. The present study demonstrated that α7nAchR agonist treatment was able to alleviate pathological damage and inhibit hippocampal cell apoptosis and inflammatory response. α7nAchR agonist treatment also increased the expression levels of p-Akt and p-GSK3β, which indicated an upregulation in Akt/GSK3β signaling. These data suggested that α7nAchR agonist may provide a promising new therapeutic approach for cerebral injury caused by CPB.

Introduction

Cardiopulmonary bypass (CPB) is considered indispensable during heart operations, but the potential adverse effects on sensitive organs, such as the brain or the kidneys, cannot be ignored (1). In particular, many of the patients who undergo CPB surgery suffer from adverse cerebral outcomes, which may include stroke, postoperative cognitive dysfunction and transient ischemic attacks (2). The underlying molecular mechanism of cerebral injuries induced by CPB is unknown; however, the pathological changes may in part be due to micro-emboli and impaired cerebral perfusion, as well as cerebral ischemia and inflammatory damage (3,4).

It has been previously reported that CPB may initiate systemic inflammatory reaction syndrome (SIRS) owing to the blood comprehensive contact with non-biological mate-rials (5); CPB may also activate cerebral inflammation in the presence of blood-brain barrier injury or disruption (3,6). Therefore, inflammatory responses serve important roles in the progression of cerebral injures induced by CPB, and reducing inflammation would be of great benefit for CPB surgery of (5,7). For example, ulinastatin treatment exhibited neuroprotective effects on an animal model of CPB, possibly through beneficial effects on anti-inflammatory systems (8).

The cholinergic anti-inflammatory pathway (CAP) is an endogenous neural feedback regulation mechanism and can regulate peripheral inflammatory responses (9). Therefore, the physiological regulation of CAP has been used to treat infectious or inflammatory animal models (9-14). Stimulation of the efferent vagus nerve releases the important neurotransmitter acetylcholine, which acts through the α7 nicotinic acetylcho-line receptor (α7nAchR) expressed in the macrophages and the brain. Notably, it has been revealed that activation of α7nAchR may effectively decrease the expression of proinflammatory cytokines and inhibit the inflammation process (10,15-18). In addition, the α7nAchR agonist PHA568487 has been used to

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treat neuroinflammation following tibia fracture and endotoxemia in mice (15), as well as ischemic stroke injury (16) and brain injury in a subarachnoid hemorrhage model rats (19). Therefore, the α7nAchR agonist may provide promising therapeutic effects for cerebral injuries. However, it is still unclear whether activation of the α7nAchR agonist is able to reduce cerebral injuries induced by CPB.

The present study evaluated the therapeutic effects and the molecular mechanisms of the α7nAchR agonist on CPB-induced brain injury in a rat model. The results indicated that the α7nAchR agonist may effectively inhibit the inflammatory response and reduce apoptosis by activating the Akt/GSK3β signaling pathway.

Materials and methods

Animals and ethical approval. A total of 96 adult male Sprague-Dawley rats (age, 8-9 weeks; weight, 350-450 g) were obtained from Shenyang Military Region General Hospital Laboratory Animal Center [Shenyang, China; license no. SCXK (Liao) 2012-00022012-0002]. Animals were housed at a constant temperature ($22 \pm 1°C$), with 50% relative humidity and a12-h light/dark cycle. The rats had access to food and autoclaved water *ad libitum*. All animal procedures were approved by the Animal Experiments Ethics Committee of the General Hospital of Shenyang Military Region (Shenyang, China).

CPB animal model establishment. CPB surgery was performed as previously reported (7), with minor modifications. Briefly, rats received an intraperitoneal (i.p.) injection of 10% chloral hydrate (300 mg/kg; Shanghai Ziyuan Pharmaceutical Co., Ltd., Shanghai, China) for anesthesia. Photopic oral intubation was performed using a 16 G intravenous (i.v.) catheter, and animals were mechanically ventilated with a small animal ventilator (settings: Frequency, 60 beats/min; tidal volume, 3 ml/kg; inspiratory to expiratory ratio, 1:1.5) connected to a monitor to observe the heart rate, oxygen saturation and rectal temperature of the rats. During surgery, anesthesia was maintained with i.v. injection of pipecuronium bromide (0.1 mg/kg; Hangzhou Minsheng Pharmaceutical Co., Ltd., Hangzhou, China).

The puncture site was sterilized with iodophor (Shandong Lierkang Disinfection Technology Co., Ltd., Dezhou, China), followed by exposure and puncture of the vein. Right femoral vein catheterization (24G) was performed to open the fluid path, which was transfused with 6% hydroxyethyl starch (Guangdong Jiabao Pharmaceutical Co., Ltd., Qingyuan, China) and connected to a microinfusion pump. The left femoral artery was catheterized (22G) and used to monitor blood pressure. Coccygeal artery catheterization (22G) and right internal jugular vein catheterization (18G) were performed to drain blood for CPB. The drainage tube, a homemade blood storage device, a constant peristaltic pump (Baoding Longer Precision Pump Co., Ltd., Baoding, China), silicone tubing (internal diameter, 4 mm) and a rat membrane oxygenator (Guangdong Kewei Medical Instrument Co. Ltd., Dongguan, China) were installed between the two puncture sites to establish the CPB circuit. Heparin sodium (300 IU/kg; Shenyang Haitong Pharmaceutical Co., Ltd., Shenyang, China)

was injected into the left femoral vein once the activated clotting time reached 480 sec.

CPB was performed with the membrane oxygenator to supply oxygen. The low-flow CPB velocity was 35 ml/kg/min, which was later increased to 100-120 ml/kg/min at full-flow bypass. To prevent air embolism, 1-2 ml of blood was retained in the blood storage device. Mean arterial pressure was maintained at >60 mmHg, partial CO_2 pressure at 35-45 mmHg, base excess at -3-3 mmol/l mmHg, pH at 7.35-7.45 and hematocrit at >0.25. Rats were treated with 2-20 μg/100 g epinephrine hydrochloride (Wuhan Grand Pharmaceutical Group Co., Ltd., Wuhan, China) and fluids during surgery to maintain a stable circulation.

Groups and treatments. Rats were randomly divided into four groups (n=24/group): i) The Sham group (S group), in which intubation and mechanical ventilation were performed in the right femoral artery only and the right internal jugular vein was catheterized without bypass; ii) the CPB surgery group (C group), which received the CPB surgery aforementioned; iii) the α7nAchR agonist group (P group), which received an i.p. injection of the α7nAchR agonist PHA568487 (0.8 mg/kg; Tocris Bioscience; Bio-Techne, Minneapolis, MN, USA) 30 min prior to CPB establishment; and iv) the PHA568487 + α7nAchR antagonist group (M group), which were also pretreated with PHA568487 (0.8 mg/kg) for 30 min, followed by i.p. injection of the α7nAchR antagonist methyllycaconitine (MLA; 6 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). CPB surgery was performed 60 min after MLA injection.

Specimen collection and processing. Arterial and venous blood samples were collected prior to CPB (T0), upon completion of CPB surgery (T1), 2 h post-CPB (T2) and 6 h post-CPB (T3); subsequently, rats were sacrificed with 2% pentobarbital sodium (40 mg/kg by i.p. injection; Merck Sharp & Dohme, Shanghai, China). The systemic circulation system of the rats was infused with saline (250-400 ml), and the whole brain was collected on the ice and divided into two halves along the median sagittal line. The hippocampus was isolated from each of the two halves, the right half was fixed in 4% paraformaldehyde (PFA) at room temperature for 24 h, and the left side was stored at -80°C for western blot analysis. Sera were separated by centrifugation at 1,000 x g for 10 min at 4°C, and stored at -80°C.

Histopathological assessment. Fixed hippocampal tissues were gradually dehydrated with ethanol and embedded in paraffin. Paraffin blocks were subsequently sectioned (5 μm) and stained with a Hematoxylin & Eosin (H&E) staining kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Double-blind evaluation of hippocampal injury was performed by two expert pathologists. Images of the histopathological examination were captured by a light microscope (Olympus Corporation, Tokyo, Japan) at x400 magnification.

Tissue apoptosis assay. A terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) Assay kit (Shanghai Fusheng Industrial Co., Ltd., Shanghai, China) was used, according to the manufacturer's protocol, to determine the effects of α7nAchR agonist treatment on apoptosis in the

fixed and mounted hippocampal sections. DAPI was used as a nuclear stain, with sections stained with 100 ng/ml DAPI for 5 min. Apoptotic rates were examined and images captured using a light microscope (Olympus Corporation, Japan) at a magnification of x400, and the densitometric scanning was finally analyzed by using the MetaMorph BX41 Image Analysis System (Olympus Corporation, Japan). A total of 5 images were captured randomly for each section at x400 magnification and integral optical density was calculated using Microscopic Image Analyzer (MetaMorph BX41 Image Analysis System). Percentages of TUNEL-positive cells above untreated controls were calculated as follows: %apoptosis = (number of TUNEL-positive cells / number of total cells) x 100.

Immunohistochemistry. To further determine the effects of α7nAchR on apoptosis in the hippocampus, expression levels of the cellular apoptosis maker Caspase 3 was examined by immunohistochemical analysis. Briefly, dimethylbenzene was used to remove the paraffin from the hippocampal sections, followed by immersion in distilled water. Subsequently, antigen retrieval was conducted by placing the slides in a microwave in 10 mmol/l citrate buffer, pH 6.0, for 15 min. The slides were washed with 0.01 mmol/l PBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) every 5 min for 3 times, followed by incubating in TBS + 0.3% H_2O_2 + 0.1% saponin at room temperature for 15 min to block the endogenous peroxidase. The slides were blocked with goat serum (Sigma-Aldrich; Merck KGaA) in TBS + 0.1% saponin for 20 min at room temperature, followed by incubating with polyclonal rabbit anti-Caspase 3 (1:300; ab13847; Abcam, Cambridge, UK) overnight at 4˚C. The slides were incubated with biotin-conjugated secondary antibody (1:2,000; ab6720; Abcam) for 30 min, and 3,3'-diaminobenzidine stain (8 min at room temperature) was used to visualize Caspase 3 expression in the hippocampus. Images of Caspase 3 expression were captured with a light microscope (Olympus, Japan) at a magnification of 400x. A total of 5 images were captured randomly for each section at x400 magnification and integral optical density was calculated using Microscopic Image Analyzer (MetaMorph BX41 Image Analysis System).

ELISA determination of S100β, tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in rat serum. Serum expression levels (in 100 μl) of S100β, TNF-α and IL-6 were determined by ELISA kits (S100β, JM-E10007507; TNF-α, JM-E10009363; IL-6, JM-E10004387; TSZ Biosciences, San Francisco, CA, USA), according to the manufacturer's protocol. Optical density was measured at 450 nm using a Spectra Max M5 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Western blot analysis. The frozen hippocampal tissues (100 mg) were ground with a glass homogenizer and subsequently homogenized with Radioimmunoprecipitation Assay Buffer (1 ml; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) using an IKA T10 homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany), followed by centrifugation at 12,000 x g for 15 min at 4˚C. The supernatant was collected and protein quantification was performed by bicinchoninic acid assay, and equal amounts of protein lysate (40 μg) were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes in transfer buffer [12 mM Tris base, 96 mM glycine (pH 8.3) and 15% methanol]. Membranes were blocked for 2 h in TBS + 0.5% Tween-20 (TBST) with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at room temperature and subsequently probed with polyclonal rabbit anti-Akt (1:500; ab8805; Abcam), polyclonal rabbit anti-GSK3β (1:1,000; ab115774; Abcam), monoclonal rabbit anti-p-Akt (1:500; 13038; Cell Signaling Technology, Inc. Danvers, MA, USA), polyclonal rabbit anti-p-GSK3β ser9 (1:500; ab131097; Abcam), polyclonal rabbit anti-Caspase 3 (1:300; ab13847; Abcam) or monoclonal rabbit anti-β-actin antibody (1:100; 8457; CST, USA) overnight at 4˚C. Membranes were washed with TBST buffer three times, followed by incubating with monoclonal goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:4,000; HS101; Beijing TransGen Biotech Co., Ltd., Beijing, China) for 1 h at room temperature. ECL chemiluminescence was used to detect protein expression levels, which were visualized by scanning densitometry (170-8070 Molecular Imager ChemiDoc XRS System; Bio-Rad Laboratories, Inc. Hercules, CA, USA) using ImageJ Software (version 1.37; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Quantitative data were expressed as the mean ± standard deviation. Statistical analyses were performed with GraphPad Prism software, (version 6.00; GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were analyzed with one-way analysis of variance, followed by an appropriate multiple comparison test (Tukey's procedure). P<0.05 was considered to indicate a statistically significant difference.

Results

α7nAchR agonist alleviates pathological injury caused by CPB. To determine the protective effects of α7nAchR agonist on the morphological alterations of the hippocampus, sections were evaluated at 6 h post-CPB, the T3 time point, by H&E staining. There was no detectable morphological damage to the hippocampal tissues in the S group (Fig. 1A), whereas clear cellular degeneration and abnormal cell arrangements were observed in the samples of CPB-injured rats (Fig. 1B), which indicated that the rat model of cerebral injury caused by CPB was successfully established. Following pretreatment with the α7nAchR agonist, only a slight morphological change was observed in the P group as compared to those in the C group (Fig. 1C), which suggested that the α7nAchR agonist may have alleviated the pathological injury of the CPB-injured rats; however, the typical vacuolated degenerations in hippocampal neurons were observed in those co-treated with α7nAchR antagonist (Fig. 1D), indicating the protective effects of α7nAchR agonist may be inhibited by MLA treatment. These results suggested that activation of α7nAchR may alleviate CPB-induced pathological injury.

α7nAchR agonist inhibits CPB-induced apoptosis of hippocampal neurons. To determine the effects of α7nAchR agonist treatment on apoptosis in hippocampal neurons, the T3

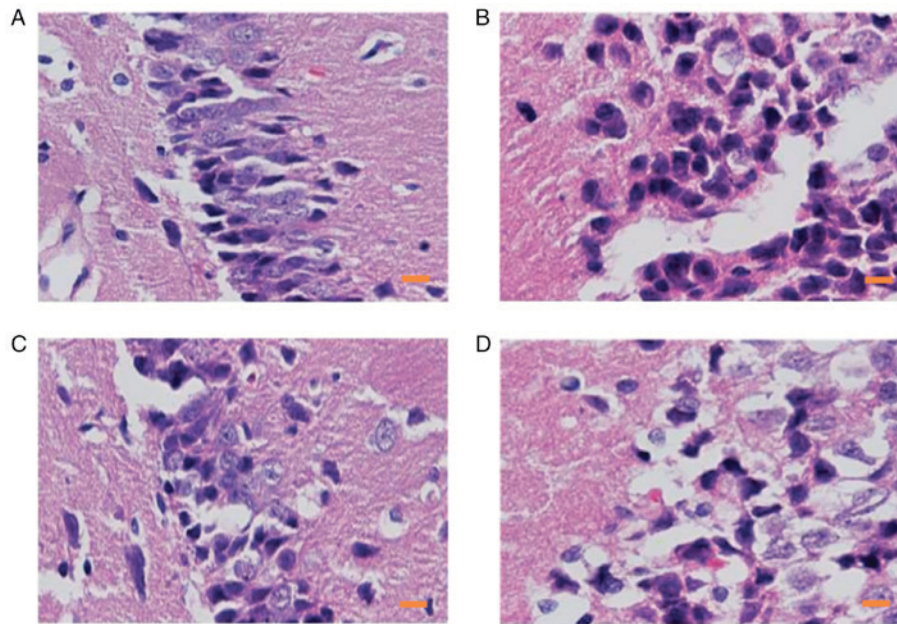Figure 1. α7nAchR agonist pretreatment alleviates pathological injury in the hippocampus. Hippocampal tissues were examined at T3 by hematoxylin & eosin staining to evaluate the protective effects of α7nAchR agonist. The hippocampal samples collected from the (A) Sham control group exhibited no evidence of morphological damage, whereas (B) hippocampal tissues of the rats with CPB model group exhibited signs of obvious cellular degeneration and abnormal cell arrangements. (C) Rats pretreated with α7nAchR agonist prior to CPB surgery exhibited only slight morphological alterations compared with the CPB group; however, more obvious pathological changes were observed in rats (D) co-treated with the α7nAchR antagonist. Magnification, x400; scale bar, 20 μm. α7nAchR, α7 nicotinic acetylcholine receptor; CPB, cardiopulmonary bypass; T3, 6 h post-CPB.

sections were also evaluated by TUNEL staining. Compared with the control neurons in the S group, the neurons in the C group exhibited typical signs of apoptosis (Fig. 2A); neuronal apoptosis appeared to be lower in the P and M groups when compared with the C group (Fig. 2A). To further determine the effects of the α7nAchR agonist on hippocampal neuron apoptosis, the integrated OD average of apoptosis positive area was quantified in captured images from all experimental groups. Compared with the S group, apoptosis was significantly increased in CPB-injured rats in groups C, P and M, which suggested that hippocampal cell apoptosis may be induced following CPB surgery. Notably, a lower rate of neuronal apoptosis was observed in rats pretreated with the α7nAchR agonist compared with the C group ($P<0.05$; Fig. 2B); however, apoptosis was significantly increased in rats co-treated with the α7nAchR antagonist compared with the P group (Fig. 2B). These results indicated that CPB-induced apoptosis of hippocampal neurons may be effectively reduced by pretreatment with the α7nAchR agonist.

In the light of the inhibition of α7nAchR agonist on apoptosis of hippocampal neurons, the protein expression levels of Caspase 3, a key downstream inducer of apoptosis (20), was evaluated by western blot assay. In the T0 and T1 tissue specimen, no significant differences were detected in Caspase 3 expression between any of the groups, which suggested that apoptosis was not induced at this period in time. Conversely, tissues at T2 and T3 exhibited increased Caspase 3 expression in the CPB-injured rats in groups C, P and M compared with expression in the S group rats ($P<0.05$; Fig. 3A and B), which implied that apoptosis was activated 3-6 h post-CPB surgery. Caspase 3 expression was significantly decreased in P group rats following pretreatment with α7nAchR agonist compared

with the C group ($P<0.05$), whereas this effect was reversed in M group rats co-treated with the α7nAchR antagonist ($P<0.05$; Fig. 3A and B).

To confirm the location of Caspase 3 expression in the hippocampus, immunohistochemical analysis was used to determine the expression at T2, as the Caspase 3 expression reached a peak in the CPB-injured rats at T2 according to the western blotting data aforementioned. Caspase 3 expression was detected in the neurons of hippocampus (Fig. 3C), and Caspase 3 expression was significantly inhibited in the P group compared with the C group ($P<0.05$; Fig. 3D), which was consistent with western blotting results. Therefore, these results indicated that the α7nAchR agonist may effectively inhibit apoptosis in hippocampal neurons, which may partly be accomplished by suppressing the expression of Caspase 3.

α7nAchR agonist pretreatment reduces serum levels of S100β, TNF-α and IL-6 in CPB-injured rats. Serum expression levels of S100β, TNF-α and IL-6 were measured to evaluate the inflammatory response in rats with CPB injury. Compared with the control S group, rats in the CPB groups C, P and M exhibited significantly increased levels of S100β, TNFα and IL6 at experimental time points T1-T3 ($P<0.05$; Fig. 4A-C, respectively), which was considered as an indicator of serious cerebral injury. The levels of S100β, TNFα and IL6 were significantly decreased rats in the P group following pretreatment with 7nAchR agonist compared with the expression levels in CPB model rats in the C group at the T1-T3 experimental time points ($P<0.05$); however, rats in the M group exhibited an increase in serum expression levels compared with the P group ($P<0.05$; Fig. 4A-C). The apparent improvement of
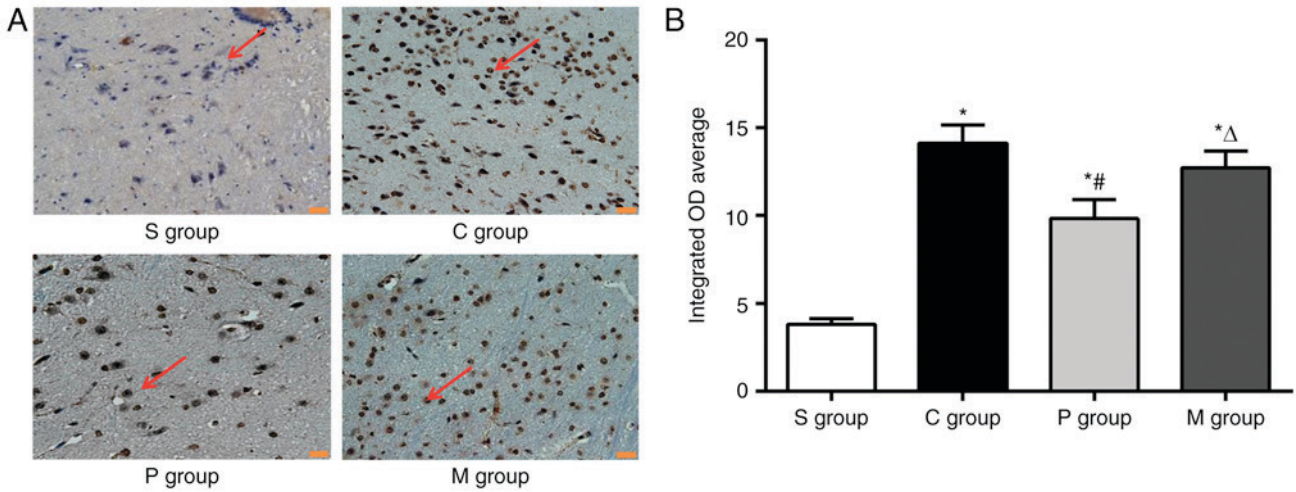
Figure 2. α7nAchR agonist pretreatment inhibits neuronal apoptosis in the hippocampus. Hippocampal tissues at T3 were examined by TUNEL assay to evaluate the effects of α7nAchR agonist on apoptosis. (A) Hippocampal neurons exhibited typical apoptosis, whereas a lower neuronal apoptosis can be observed after pretreatment of α7nAchR agonist. Magnification, x400; scale bar, 20 μm; red arrows indicated positive expressions. (B) Quantitative results of TUNEL assay from part A. Data are presented as the mean ± standard deviation; n=24/group; *P<0.05 vs. S group; #P<0.05 vs. C group; ΔP<0.05 vs. P group. α7nAchR, α7 nicotinic acetylcholine receptor; C group, CPB surgery only; CPB, cardiopulmonary bypass; M group, CBP + α7nAchR agonist PHA568487 + α7nAchR antagonist methyllycaconitine; P group, CBP + α7nAchR agonist PHA568487; S group, Sham operation; T3, 6 h post-CPB.
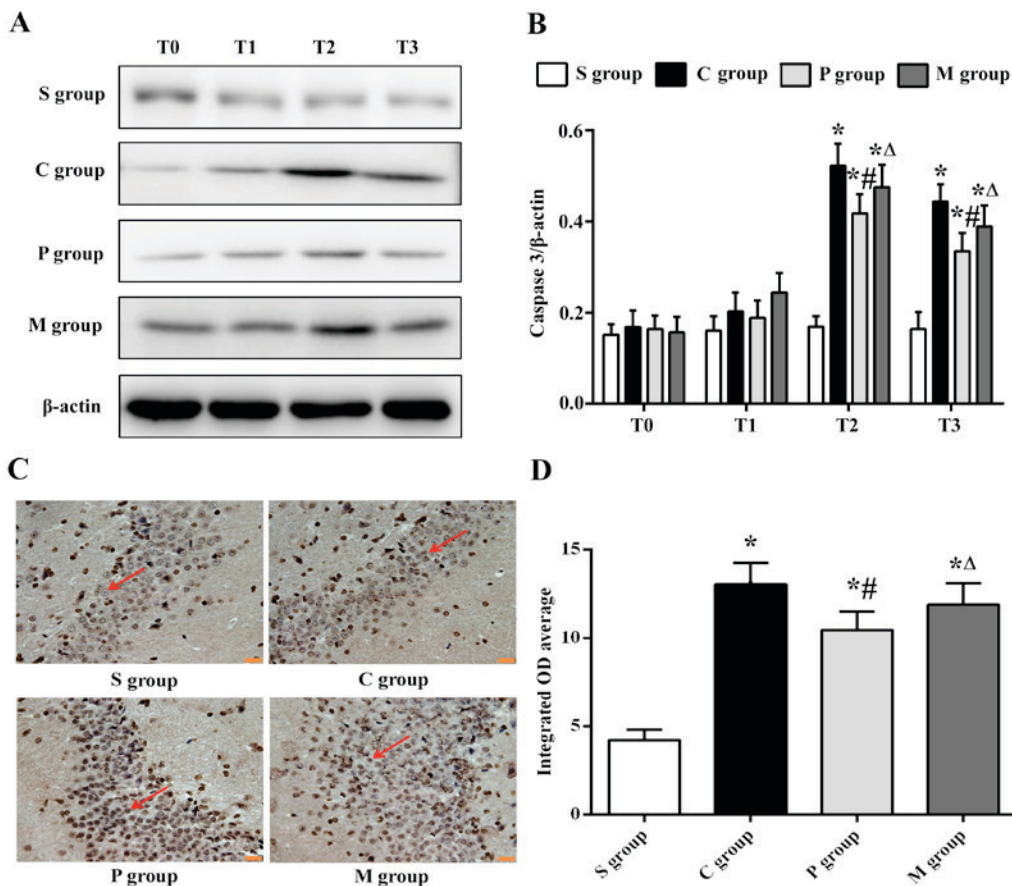

Figure 3. α7nAchR agonist pretreatment inhibits Caspase 3 protein expression in the hippocampus. (A) Western blot analysis for Caspase 3 in hippocampus at different time points in the different experiments groups. (B) Densitometric analysis Caspase 3 expression presented in (A); decreased expressions of Caspase 3 were observed in rats in the P group following pretreatment of α7nAchR agonist compared with those in the C group at T2 and T3. (C) Immunohistochemistry for Caspase 3 in hippocampus at T2. Magnification, x400; scale bar, 20 μm; red arrows indicate positive expressions. (D) Integrated OD average analysis indicated the decreased expression levels of Caspase 3 in P group rats pretreated with the α7nAchR agonist compared with expression in the C group model rats. Data are presented as the mean ± standard deviation; n=24/group; *P<0.05 vs. S group; #P<0.05 vs. C group; ΔP<0.05 vs. P group. α7nAchR, α7 nicotinic acetylcholine receptor; C group, CPB surgery only; CPB, cardiopulmonary bypass; M group, CBP + α7nAchR agonist PHA568487 + α7nAchR antagonist methyllycaconitine; P group, CBP + α7nAchR agonist PHA568487; S group, Sham operation; T0, prior to CPB; T1, upon completion of CPB; T2, 3 h post-CPB; T3, 6 h post-CPB.
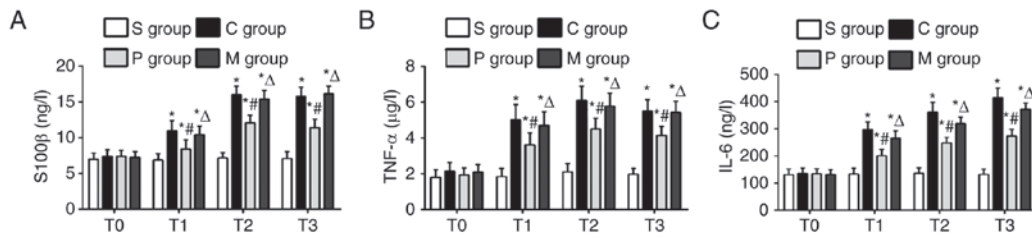
Figure 4. α7nAchR agonist pretreatment reduces the serum expression levels of S100β, TNF-α and IL-6 in CPB-injured rats. The serum levels of (A) S100β, (B) TNF-α and (C) IL-6 were measured to evaluate the anti-inflammation effects of α7nAchR agonist on CPB-injured rats. Serum levels of S100β, TNFα and IL6 were significantly decreased in P group rats following pretreatment with the α7nAchR agonist compared with levels in the C group model rats. Data are presented as the mean ± standard deviation; n=24/group; *P<0.05 vs. S group; #P<0.05 vs. C group; ΔP<0.05 vs. P group. α7nAchR, α7 nicotinic acetylcholine receptor; C group, CPB surgery only; CPB, cardiopulmonary bypass; M group, CBP + α7nAchR agonist PHA568487 + α7nAchR antagonist methyllycaconitine; IL, interleukin; P group, CBP + α7nAchR agonist PHA568487; S group, Sham operation; T0, prior to CPB; T1, upon completion of CPB; T2, 3 h post-CPB; T3, 6 h post-CPB; TNF-α, tumor necrosis factor α.
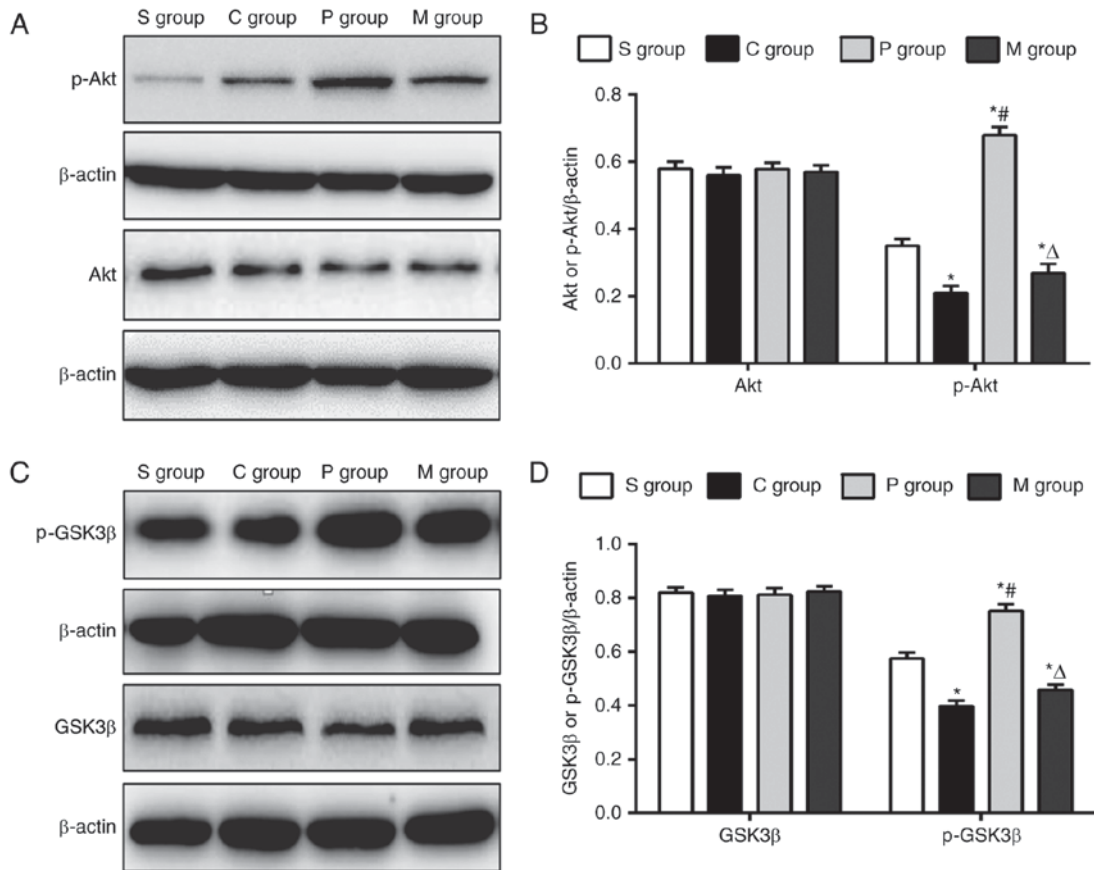

Figure 5. α7nAchR agonist promotes expression of p-Akt and p-GSK3β. (A) Western blot analysis for Akt and p-Akt in the hippocampus at T3. (B) p-Akt expression levels were significantly increased in the P group following pretreatment with the α7nAchR agonist compared with expression in the C group model rats. (C) Western blot analysis for GSK3β and p-GSK3β in the hippocampus at T3. (D) p-GSK3β expression levels were significantly increased in the P group following pretreatment with the α7nAchR agonist compared in the C group model rats. Data are presented as the mean ± standard deviation; n=24/group; *P<0.05 vs. S group; #P<0.05 vs. C group; ΔP<0.05 vs. P group. α7nAchR, α7 nicotinic acetylcholine receptor; C group, CPB surgery only; CPB, cardiopulmonary bypass; GSK3β, glycogen synthase kinase 3β; M group, CBP + α7nAchR agonist PHA568487 + α7nAchR antagonist methyllycaconitine; p, phosphorylated; P group, CBP + α7nAchR agonist PHA568487; S group, Sham operation; T3, 6 h post-CPB.

inflammation suggested that α7nAchR agonist pretreatment may have a beneficial effect on anti-inflammatory systems of the rat model of CPB.

α7nAchR agonist pretreatment promotes phosphorylation of Akt and GSK3β. To further explore the underlying mechanisms by which α7nAchR agonist alleviated the cerebral injuries caused by CPB, Akt/GSK3β pathway activation was examined

to determine the protective effects of α7nAchR agonist on the CPB-injured rats, as the Akt/GSK3β pathway was previously identified as a significant cell survival pathway (21). CPB rats in the C group exhibited a significant increase in the expression of p-Akt and p-GSK3β compared with expression levels in the S group (P<0.05; Fig. 5); whereas the expression levels of p-Akt and p-GSK3β were significantly increased in the P group following pretreatment with the α7nAchR agonist,

compared with the S group and C group, which suggested that the α7nAchR agonist may promote the phosphorylation of Akt and GSK3β. Rats in the M group that were co-treated with the α7nAchR antagonist exhibited a significant decrease in p-Akt and p-GSK3β expression levels compared with the P group (Fig. 5). By contrast, no significant differences in the expression levels of total Akt and total GSK3β were identified between the groups (Fig. 5), which implied that the α7nAchR agonist did not affect the expressions of Akt and GSK3β. Therefore, these results indicated that the α7nAchR agonist may effectively upregulate the activation of the Akt/GSK3β signaling pathway in the CPB-injured rats.

Discussion

Cerebral injury is a serious complication following the use of CPB in the cardiac surgery (2,22,23). This pathological lesion may be due to several aspects, including impaired cerebral perfusion and oxygenation, cerebral microemboli and SIRS (3,4). Among these factors, SIRS is one of great significance for CPB; therefore, minimizing SIRS is widely considered as a prerequisite strategy for inhibiting the inflammatory response (5). It is generally accepted that proinflammatory cytokines such as TNF-α may further increase the permeability of the blood-brain barrier and subsequently promote the invasion of inflammatory cytokines and immune cells (24). Results from the present study demonstrated that expression levels of the proinflammatory cytokines, including the TNF-α and IL-6, were significantly increased in CPB-injured rats, which was consistent with previous reports (7,25). Therefore, reducing proinflammatory cytokines levels may alleviate neuronal injury and improve functional recovery.

As a physiological regulation of the innate immune system, CAP has been widely used to inhibit the expression of proinflammatory cytokines for treating infectious and inflammatory diseases (16). According to that report, activation of the main regulatory target, α7nAchR, may aid in the reduction of proinflammatory cytokines. Therefore, the present study hypothesized that the α7nAchR agonist may effectively inhibit the serum levels of TNF-α and IL-6 in the CPB-injured rats, which suggested that the α7nAchR agonist may provide a promising strategy for reducing SIRS post-CPB.

S100β is regarded as a reliable serum maker of cerebral injury following the breakdown of the blood-brain barrier (26-28). In the present study, an increased serum level of S100β was observed in the CPB model rats compared with normal rats, whereas the α7nAchR agonist was able to decrease the serum level of S100β, which demonstrated that the CPB model was successfully established and that the neuroprotective effects may be achieved by pretreatment with the α7nAchR agonist.

Several previous reports suggested that the hippocampus is sensitive to ischemia and reperfusion injury caused by CPB (1,29). In the present study, clear pathological damage and an increase in cell apoptosis and Caspase 3 expression levels in the hippocampus were observed in the CPB-injured rats, which confirmed that pathological changes occur in the hippocampus following CPB surgery. Notably, these pathological injuries were effectively inhibited in rats pretreated

with the α7nAchR agonist, which demonstrated the protective effects of the α7nAchR agonist on CPB rats.

Additional studies have demonstrated that the Akt/GSK3β pathway serves a central role in cell survival in a number of neurological diseases (30-32). In particular, activation of the Akt/GSK3β pathway may attenuate apoptosis, which is closely related to the regulation of Caspase 3 expression (33-35). Based on the present results that demonstrated the inhibitory effects of the α7nAchR agonist on apoptosis and Caspase 3 expression, activation of the Akt/GSK3β pathway was further examined for the protective effects of α7nAchR agonist on CPB. The results indicated that p-Akt and p-GSK3β expressions were upregulated following α7nAchR agonist pretreatment, which suggested that the α7nAchR agonist may be able to inhibit hippocampal cell apoptosis by activating the Akt/GSK3β pathway.

To further determine the protective effects of the α7nAchR agonist on CPB, the α7nAchR antagonist was concurrently administered in the present study. By contrast to pretreatment with the α7nAchR agonist alone, co-treatment with the α7nAchR antagonist resulted in significant increases in the serum levels of S100β, TNF-α and IL-6, as well as the pathological damage, increased apoptosis and increased Caspase 3 expression, and a significant decrease in the expression levels of p-Akt and p-GSK3β. These results further demonstrated the neuroprotective effects of α7nAchR agonist on CPB-injured rats.

In conclusion, the present study demonstrated that the α7nAchR agonist may reduce pathological damage and apoptosis in the hippocampus by upregulating Akt/GSK3β signaling. The α7nAchR agonist may provide a promising therapeutic approach for cerebral injury caused by CPB.

Acknowledgements

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