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ORIGINAL ARTICLE

Probenecid Relieves Cerebral Dysfunction of Sepsis by Inhibiting Pannexin 1-Dependent ATP Release

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Abstract— Acute brain dysfunction and the following neurological manifestation are common complications in septic patients, which are associated with increased morbidity and mortality. However, the therapeutic strategy of this disorder remains a major challenge. Given the emerging role of a clinically approved drug, probenecid (PRB) has been recently identified as an inhibitor of pannexin 1 (PANX1) channel, which restrains extracellular ATP release-induced purinergic pathway activation and inflammatory response contributing to diverse pathological processes. In this study, we explored whether PRB administration attenuated neuroinflammatory response and cognitive impairment during sepsis. In mice suffered from cecal ligation and puncture (CLP)-induced sepsis, treatment with PRB improved memory retention and lessened behavioral deficits. This neuroprotective effect was coupled with restricted overproduction of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and interleukin (IL)-1β in the hippocampus. Since this damped neuroinflammation was replicated by inhibition of ATP release, it suggested that PANX1 channel modulates a purinergic-related pathway contributing to the neurohistological damage. Therefore, we identified PRB could be a promising therapeutic approach for the therapy of cerebral dysfunction of sepsis.

KEY WORDS: probenecid; pannexin 1; cecal ligation and puncture; neuroinflammation; cognitive impairment.

INTRODUCTION

Sepsis is a life-threatened medical event encountered and triggered by infection and following organ dysfunction [33]. Despite constant advances in antibiotics and supportive care interventions, sepsis remains a major cause of death from infection. Overwhelming systemic inflammatory response under sepsis can lead to the organ damages, including the brain injury [16, 33]. Sepsis-associated encephalopathy (SAE) is mentioned to represent diffuse cerebral dysfunction of sepsis with neurological symptoms varying from lethargy and delirium to coma [33]. Unfortunately, SAE is considered as an independent predictor of mortality. Moreover, many septic survivors have long-term cognitive deficits and psychiatric disorders [10, 20, 34]. Although imbalanced neuroinflammation is generally considered to be the key cause leading to SAE, therapy against neuroinflammation is still ineffective even lacking. Deeper research of the pathogenesis of SAE and effective therapy strategy against SAE remain to be explored.

Probenecid (PRB) is a clinically approved drug mainly used to cure gout and hyperuricemia for many years with a limited adverse effect profile [28]. The initial intent of this drug was to decrease or retard the renal excretion of penicillin [6]. Recently, PRB exerted a promising role *in vivo* studies focusing on its anti-

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inflammatory effect, which inhibiting the activation of PANX1 channel [31, 32]. A burgeoning body of researches reported that PANX1 channels contributed to the progression of pathophysiology in nervous system disease, such as stroke, epilepsy, and hepatic encephalopathy [8, 9, 13, 36]. In these studies, PANX1 channels were shown as a danger signal in neuroinflammation *via* ATP purinergic receptor. Additional research suggested that the activation of ATP purinergic signaling and pro-inflammatory factors might contribute to the deteriorative mitochondria function and cellular injury in sepsis [1]. However, it was not yet clear whether PRB possessed some neuroprotective effect on sepsis-induced brain injury. Therefore, we aimed to determine the therapeutic efficacy of PRB in the outcome of SAE.

Here, we hypothesized that PRB administration could inhibit PANX1-mediated release of cellular ATP and ameliorate cognitive impairment. Our results showed that PRB administration rescued behavioral deficits and reduced neuroinflammation after septic injury. Further investigation was also performed to explore the role of extracellular ATP in the neurological consequence after sepsis. In the present study, our findings suggested that PRB could be developed as a potential therapeutic drug for SAE.

MATERIALS AND METHODS

Animals

C57BL/6 mice (male, 6~8-week old, weighing 20~22 g) were obtained from Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and housed in pathogen-free cages at a temperature of 22 °C and a 12-h light dark cycle (8:00 am–8:00 pm, lights on) with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committees of Xi'an Jiaotong University (Xi'an, China).

CLP Model

Mice were subjected to CLP as previously reported with some additional modifications [27]. In brief, after anesthetized using an inhalation of isoflurane, the cecum was exteriorized *via* a 1-cm abdominal midline incision and ligation using a 4-0 silk ligature at midway between the distal pole and the base of cecum. The cecum was tightly ligated with 4-0 silk suture at half of the distance between the base of cecum and the distal pole. Then a 21-gauge needle was used to puncture the cecum once through both surfaces at the middle of the ligation and the tip of the cecum. In the sham group, mice underwent the same procedures without being ligated and punctured. Survival rate and body weight ($n = 8$ for each sham-treated group and $n = 16$ for each CLP-treated group) were evaluated over a 10-day period with assessment every 12 h after treatment. Additionally, after surgery, septic mice received "basic support" (saline at 50 ml/kg subcutaneously immediately and ceftriaxone at 30 mg/kg and clindamycin at 25 mg/kg every 12 h for a total of 3 days) [2, 23]. The sham-operated mice received saline at the constant volume of antibiotic.

Drug Administration

Mice were randomly allocated (computer-based randomization) to sham, sham + PRB, CLP, and CLP + PRB groups for intraperitoneal (i.p.) injection. The PRB (Sigma-Aldrich, St. Louis, MO) injection solution (pH 7.3) was prepared in 0.9% NaCl solution containing 0.1 M Tris and 0.1 M NaOH, and for pH adjustment with 2 M HCl. Then mice in sham + PRB and CLP + PRB groups received 50 mg/kg PRB immediately and 1 day later by i.p. injection after sham or CLP surgery. Doses of the PRB treatments employed were chosen on the basis of previous studies [4, 5]. Mice in sham and CLP groups received equal volumes of solvent only at the same time points sham or CLP surgery. In addition, mice were randomly divided into the CLP and CLP + apyrase groups for intracerebroventricular (icv) injection. Apyrase (10 units in 5 μl) or equal solvent [4] was injected into the left lateral ventricle (0.2 mm posterior, 0.9 mm lateral, and 2.4 mm ventral to the bregma) after sham or CLP surgery [21].

Behavioral Procedures

The survivors underwent open-field test ($n = 17$ mice per group) and step-down inhibitory avoidance test ($n = 12$ mice per group) 10 days after CLP [18, 25]. All the two behavioral tests were performed between 9:00 am and 4:00 pm during the light cycle. To avoid interference by fecal boli and urination of other mice, the trial site was cleaned up after each test.

Open-Field Test

This test mainly evaluated motor performance in the training session and non-associative memory in the test session. This task was performed in an arena measuring 50 cm × 50 cm surrounded by 50-cm high walls. The arena

was subdivided into 25 squares by lines. Mice were gently put into the left rear quadrant and left to explore the arena for 5 min (training session). After 24 h, mice were submitted again to the same quadrant (test session). The decreases in the number of crossing and rearing were taken as a measure to assess the retention of habituation by an open-field system (Chengdu Taimeng Software Co. Ltd., Sichuan, China).

Step-down Inhibitory Avoidance Test

This task mainly evaluated the aversive memory. The training apparatus was a 150 × 25 × 30 cm acrylic box with six channels. The floor consisted of parallel caliber stainless steel bars (1 mm diameter) with 1 cm apart. 4 cm-wide, 5 cm-high platforms were placed in the left corner. In the training test, mice were put on the platform that faced the corner and received a 0.3 mA, 2.0 s foot shock once stepping down on the grid. The latency of step-down on the grid with all four paws was recorded. A retentional long-term memory test was performed 24 h after training. The retention test trial was procedurally similar to the training except the foot shock. Test trial latency (maximum, 180 s) was used to measure the inhibitory avoidance retention by a step-down inhibitory avoidance system (Chengdu Taimeng Software Co. Ltd.).

ATP Determination

To determine extracellular ATP concentration in the hippocampus, mice ($n = 5$ mice per group) were sacrificed at 24 h after CLP or sham surgery and brains were taken out on ice immediately. Then hippocampi were separated from the brain tissues and homogenized into single-cell suspensions by a gentleMACS™ dissociator (Miltenyi Biotec, Teterow, Germany). The supernatants were obtained to determine ATP concentration. Cerebrospinal fluids (CSF) of mice ($n = 5$ mice per group) were also collected to determine ATP concentration at 24 h after CLP or sham surgery. ATP levels were determined using a firefly luciferin luciferase assay-based ATP determination kit (Thermo Fisher Scientific, Inc., Rockford, IL). Each reaction contained 1 mM dithiothreitol, 0.5 mM D-luciferin, and 1.25 μg/ml firefly luciferase in reaction buffer. After 15-min incubation with brain samples, luminescences were measured using a Varioskan® Flash microplate reader (Thermo Fisher Scientific, Inc., Rockford, IL). ATP levels were expressed in percent of control [4].

Enzyme-Linked Immunosorbent Assay

The samples of brains ($n = 5$ mice per group) were rapidly harvested at 48 h after CLP or sham surgery. TNF-α, IL-6, and IL-1β levels in the hippocampal homogenates were quantified with specific enzyme-linked immunosorbent assay kits for mice according to the manufacturer's instructions (eBioscience, San Diego, CA).

Immunoblotting

The hippocampus was rapidly isolated on ice at 0, 4, 12, 24, 48, and 72 h after septic injury. The hippocampal homogenate ($n = 5$ mice per group) preparation and protein concentration determination by bicinchoninic acid method were performed. Equal amounts of protein (60 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA). Primary antibodies to pannexin 1 (1:2000, GTX31510, GeneTex, USA), cleaved caspase 3 (1:1000, GTX22302, GeneTex, USA), cytochrome c (1:1000, 10993-1-AP, Proteintech, China), and GAPDH (1:10,000, 60004-1-Ig, Proteintech, China) were used. The immunoreactive bands were detected by enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Images were captured on ChemiDoc™ XRS+ system (Bio-Rad, Hertfordshire, UK) and analyzed with quantity one software (Bio-Rad).

Immunofluorescence of Ionized Calcium-Binding Adapter Molecule 1 (Iba1)-Positive Microglial Cells

For immunofluorescence detection, sections ($n = 3$ mice per group) were probed overnight with the anti-Iba-1 mouse monoclonal antibody to assess microglial activation. Then the slides were incubated with goat anti-mouse IgG conjugated with Alexa Fluor 488 (Millipore, Billerica, MA) for 1 h at room temperature. As a negative control, the primary antibody was replaced by nonimmune mouse serum. The fluorescence intensity of Iba-1^+ cells in the hippocampus and cortex were measured using the ImageJ software [29].

Statistical Analysis

Comparisons of differences in continuous variables were conducted with unpaired Student's t test or one-way ANOVA test with Bonferroni correction for multiple comparisons where appropriate (data are presented as mean ± SD or mean ± SEM). Survival curves (Kaplan–Meier plots) were analyzed using the log-rank test. Body weight changes were determined by two-way analysis of variance

(ANOVA) with two-factor repeated measures ANOVA. The mean latency of step-down inhibitory avoidance test was expressed as median and interquartile ranges using the Mann–Whitney *U* test. SPSS 20.0 for (SPSS, Inc., Chicago, IL, USA) was used to conduct the statistical analyses. *P* value less than 0.05 was considered to be statistically significant.

RESULTS

PRB Treatment Did Not Improve Body Weight and Mortality After Sepsis

A severe CLP model with "basic support" was prepared. The survival rates and body weight were monitored for consecutive 10 days. Figure 1 shows the survival rate and body weight changes of sham and CLP mice treated with vehicle or PRB. Mice from sham and sham + PRB groups both exhibited 100% survival rates during the 10-day follow-up. Mice from CLP and CLP + PRB groups presented 81.25% and 87.5% survival rates respectively 10 days after sepsis induction, and there was no statistical difference between the two septic groups (Fig. 1a). There was significant difference in body weight changes when sham *vs.* CLP or sham *vs.* CLP + PRB ($P < 0.01$). However, we did not observe significant difference in body weight changes between CLP and CLP + PRB on days 1–10 after CLP surgery (Fig. 1b).

PRB Treatment Ameliorated Behavioral Deficits After Sepsis

The survivors were conducted behavioral analysis using the open-field test and step-down inhibitory avoidance test. The open-field test has been adopted to evaluate the habituation to novel environment which is believed to be one of the most common forms of learning. The number of rearing and crossing in the training session has been used to examine the ability to explore a new environment. Compared with mice in the sham group, mice exhibited significant decreased rearing and crossing numbers indicating depressive-like and apathetic behavior after CLP surgery (Fig. 2a). There were no meaningful differences in the number of crossing and rearing between groups after CLP in the habituation to the open-field training session ($P > 0.05$), demonstrating no difference in motor and exploratory activity between groups after CLP. Decreased exploration in the test session has been used as an index of memory. However, only the crossing and rearing numbers after CLP surgery decreased in the test session as compared to those obtained in the training session in the CLP + PRB group ($P < 0.05$) suggesting memory amelioration(Fig. 2a). The latency in the inhibitory avoidance task was considerably decreased in the CLP group when compared to the sham group, suggesting impaired memory. PRB administration dramatically lengthens the latency when suffering from CLP challenged (Fig. 2b). To eliminate the impact inducing by the diversity in the foot shock sensitivity, the flinch or jump nociceptive thresholds were evaluated with no significant differences between groups (data not shown). Therefore, we concluded that PRB had a

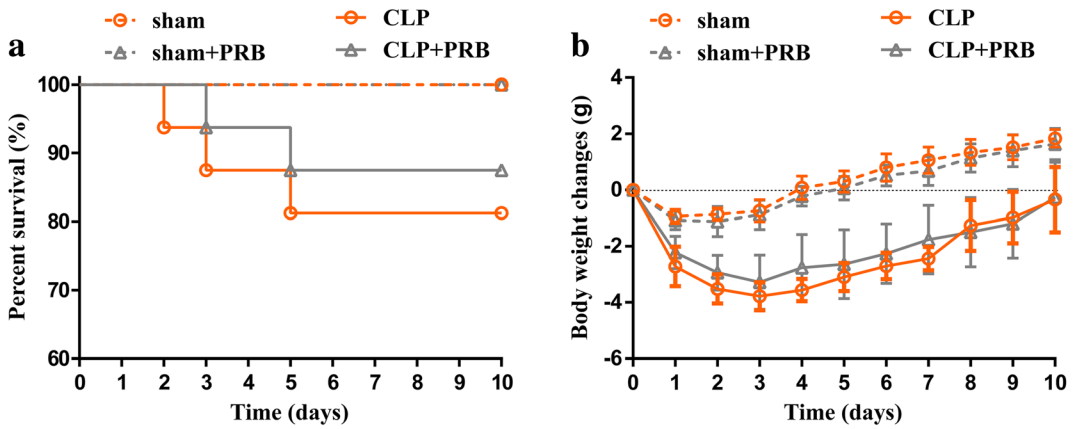

Fig. 1. Changes in the survival rate and body weight. There were 8 animals in sham group and sham + PRB groups, and 16 animals in CLP group and CLP + PRB group respectively. **a** The survival rates were analyzed by the Kaplan–Meier method and compared by log-rank test. **b** Body weight changes were determined by two-way analysis of variance (ANOVA) with two-factor repeated measures ANOVA.

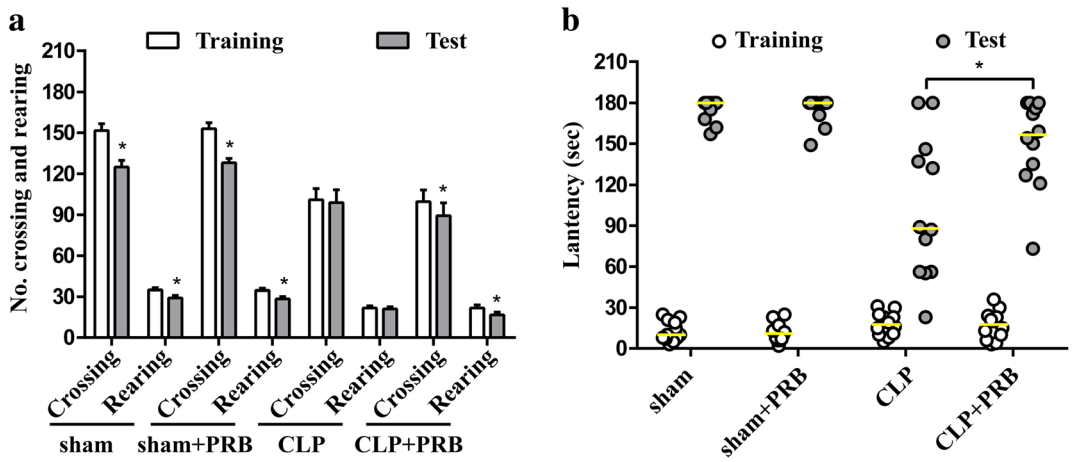

Fig. 2. Effect of PRB treatment on cognitive impairment after sepsis. Ten days after sham or CLP surgery, mice were submitted to open-field or step-down inhibitory avoidance test. **a** Open-field test. Data were represented as mean ± SEM of crossings and rearings of training (white columns) and test (gray columns) sessions ($n = 17$ mice per group). Significant differences were indicated between the training and test sessions per group (Student's t test, $^*P < 0.05$). **b** Step-down inhibitory avoidance test. Data were expressed as median and interquartile ranges ($n = 12$ mice per group) represent the mean latency in seconds. Significant differences of latency in the test sessions were found between CLP and CLP + PRB groups (Mann–Whitney U test, $^*P < 0.05$).

protective effect on the brain following septic injury as improved memory retention.

Microglial Activation and Neural Apoptosis After Sepsis Was Accompanied by PANX1 Upregulation

In the hippocampus, we observed that the expression levels of PANX1 began to increase significantly at 12 h when suffering from CLP challenge (Fig. 3a, b). In addition, compared with the sham group, the elevated level of cleaved caspase 3 and cytochrome c went up sharply from 24 h after CLP (Fig. 3c, d). Microglial activation is an indication of neuroinflammation in sepsis. Microglia-evoked neuroinflammation played a dominant role in the neuronal consequent of various brain injuries including sepsis-induced brain injury [12]. Early microglial activation preceded even accelerated neuronal loss and behavioral impairment [3]. Accordingly, we examined the extent of microglial accumulation (Iba1, a microglial marker) in the cortex and hippocampus by immunofluorescence analysis 12 h after sepsis. As shown in Fig. 3e, f, Iba1 immunoreactivity was significantly elevated within the hippocampus, indicating that microglia are activated after septic injury. The fluorescence intensity of Iba1 was also increased significantly in the cortex, consistent with microglial activation in hippocampus (Fig. 3f). The increased PANX1 expression might involve in earlier microglial activation and subsequent neural apoptosis.

PRB Suppressed Microglial Activation and Lessens Neuroinflammation After Sepsis

After submitted to severe polymicrobial sepsis and treated with PRB twice, expressions of PANX1 in the hippocampus were detected. PRB treatment could decrease the expression of PANX1 in the hippocampus after septic injury as it presents in the sham-treated mice (Fig. 4a, b). The elevated expressions of PANX1 in septic mice and fall back after PRB treatment were also observed in the hippocampus (Fig. 4a, b). Neuroinflammation was an important aspect of SAE, which contributed to the ongoing brain dysfunction associated with poor outcome prognosis and increased mortality. The levels of pro-inflammatory cytokines were significantly upregulated and play a crucial role in mediating the neuroinflammatory response and cognitive dysfunction in response to SAE. To further investigate whether PRB-mediated suppression of microglial activation could alleviate neuroinflammation in SAE, hippocampi harvested from vehicle or PRB-treated SAE mice were subjected to ELISA assays to determine the expression of pro-inflammatory cytokines. We found that the expressions of TNF-α, IL-6, and IL-1β were elevated in the hippocampus 48 h after septic injury, but their expressions were suppressed in the hippocampus of PRB-treated septic mice (Fig. 4c–e). The above results indicated that PRB could suppress microglia-activated neuroinflammation in the CLP-induced cerebral dysfunction model.

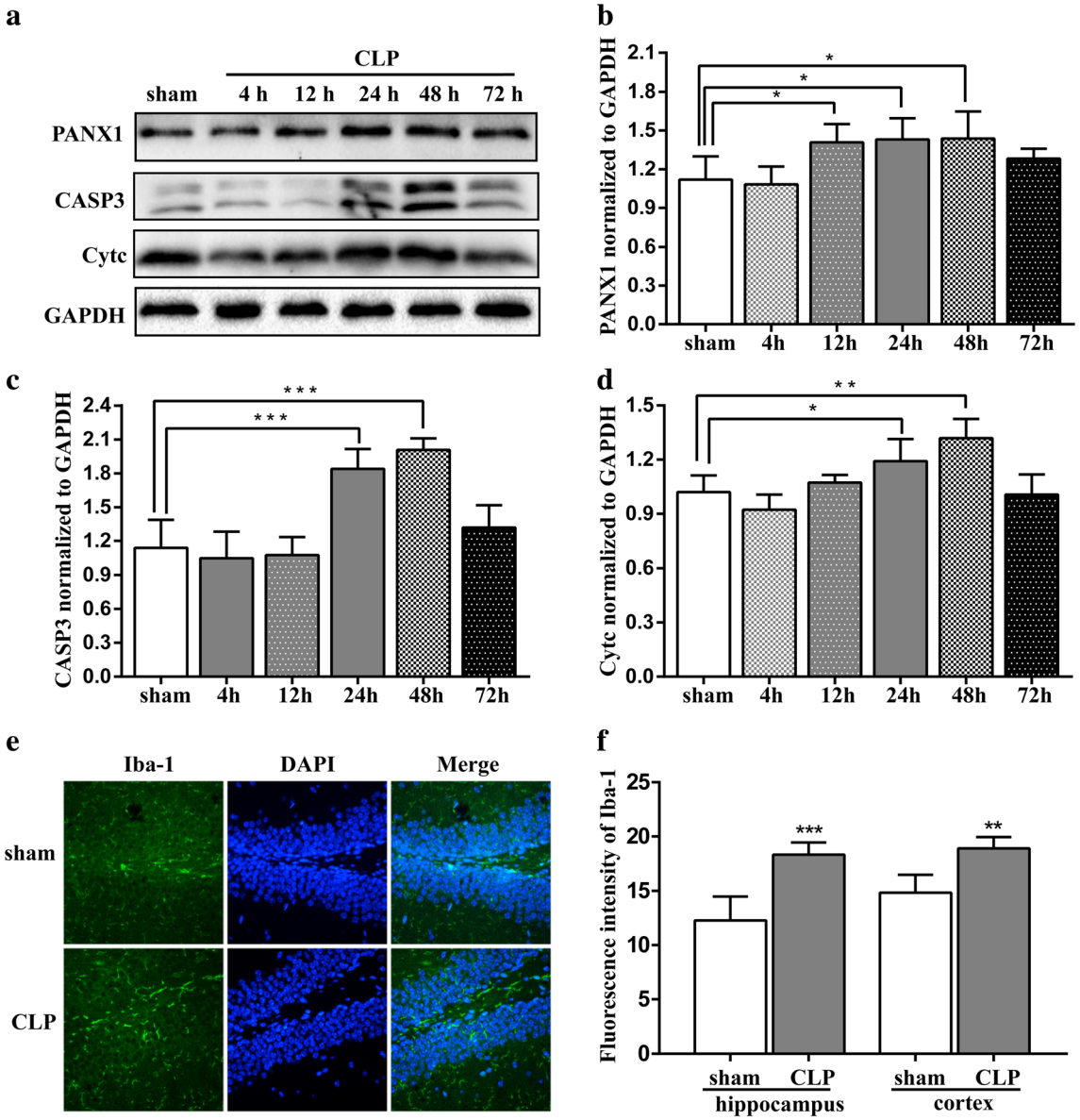

Fig. 3. The expression of PANX1 in the hippocampus was accompanied by microglial activation and neural apoptosis after sepsis. **a** Western blot bands of PANX1, CASP3, Cytc, and GAPDH proteins were displayed. GAPDH glyceraldehyde-3-phosphate dehydrogenase; CASP3 cleaved caspase 3; Cytc cytochrome c. **b**–**d** The relative expressions of PANX1, CASP3, and Cytc in the hippocampus at 0, 4, 12, 24, 48, and 72 h were analyzed after septic injury ($n = 5$ per group). GAPDH was used as a loading control. **e**, **f** Iba1 immunofluorescent staining was observed in the hippocampus and cortex at 12 h after septic injury in mice ($n = 3$). Iba1 was used as a microglia marker. Data were expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

PRB Treatment Decreased the Pro-inflammatory Cytokine Production Through Inhibition of Extracellular ATP Release

Because PANX1 activation was an essential cause of ATP release, we asked whether PANX1-mediated ATP release occurred when suffering from CLP surgery. For this purpose, we tested the content of ATP in the CSF and found that the level of ATP was markedly higher in CLP-treated mice than sham-treated mice (Fig. 5a). Similar higher extracellular ATP concentration in the hippocampus was also observed after CLP surgery (Fig. 5c). Next, we assessed the correlation between the amount of ATP in the

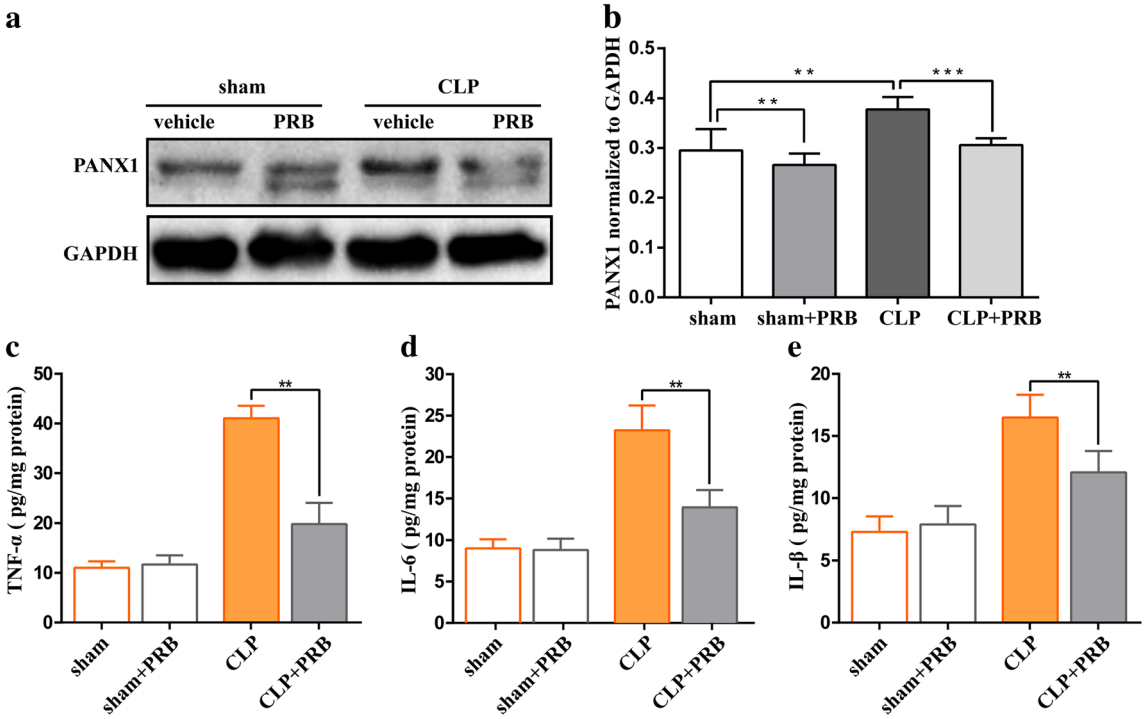

Fig. 4. PRB inhibited PANX1 expression and downregulated pro-inflammatory cytokine levels in the hippocampus. **a** PRB treatment inhibited PANX1 expression in the hippocampus. GAPDH was served as a loading control. **b**–**d** PRB downregulated TNF-α, IL-6, and IL-1β levels in the hippocampus. Data were expressed as mean ± SD, $n = 5$ mice per group, $^{**}P < 0.01$, $^{***}P < 0.001$.

CSF and the step-down latency. A regression analysis indicated that the latencies of the CLP mice were inversely correlated with the extracellular ATP concentration in the CSF or hippocampus (Fig. 5b, d). A larger amount of ATP in the CSF signified worsened neurological outcomes which with a lower step-down latency. As a consequence of the established proof that PANX1 was crucially involved in the outcomes of SAE, we tested the clinically used broad-spectrum PANX1 inhibitor, PRB, on the effects of ATP release. In CLP-treated mice, systemic administration of PRB significantly reduced the release of extracellular ATP both in the CSF and hippocampus (Fig. 5a, c).

PRB Improved Memory Retention Following Septic Injury by Inhibiting ATP Release

In CLP-treated mice, we tested this possibility by icv administration of apyrase, an ATP-degrading enzyme depleting extracellular ATP [4]. Hippocampi harvested from vehicle or apyrase-treated septic mice were subjected to ELISA assays. We found that the levels of TNF-α, IL-6, and IL-1β were suppressed after apyrase administration (Fig. 6a–c). Next we reasoned whether degradation of extracellular ATP in the brain was necessary for the improved cognitive behaviors. Then septic survivors were conducted behavioral analysis using the open-field test and step-down inhibitory avoidance test. In the open-field test, there was a significant reduction in both crossing and rearing numbers ($P < 0.05$) after apyrase administration suggesting memory amelioration (Fig. 6d). In the test session, the step-down latency in the inhibitory avoidance task was considerably lengthened after apyrase administration (Fig. 6e), suggesting restoring memory. Therefore, we concluded that ATP was a key substrate for SAE progress. Inhibiting PANX1-mediated ATP release might be a reason of improved memory retention following septic injury by PRB.

DISCUSSION

Most research studies reported that sepsis presented brain dysfunction and long-term cognitive impairment by activating microglia and inducing the release of pro-inflammatory cytokines [11, 23, 24]. The SAE injury is characterized by complex, dormant, and difficulty in

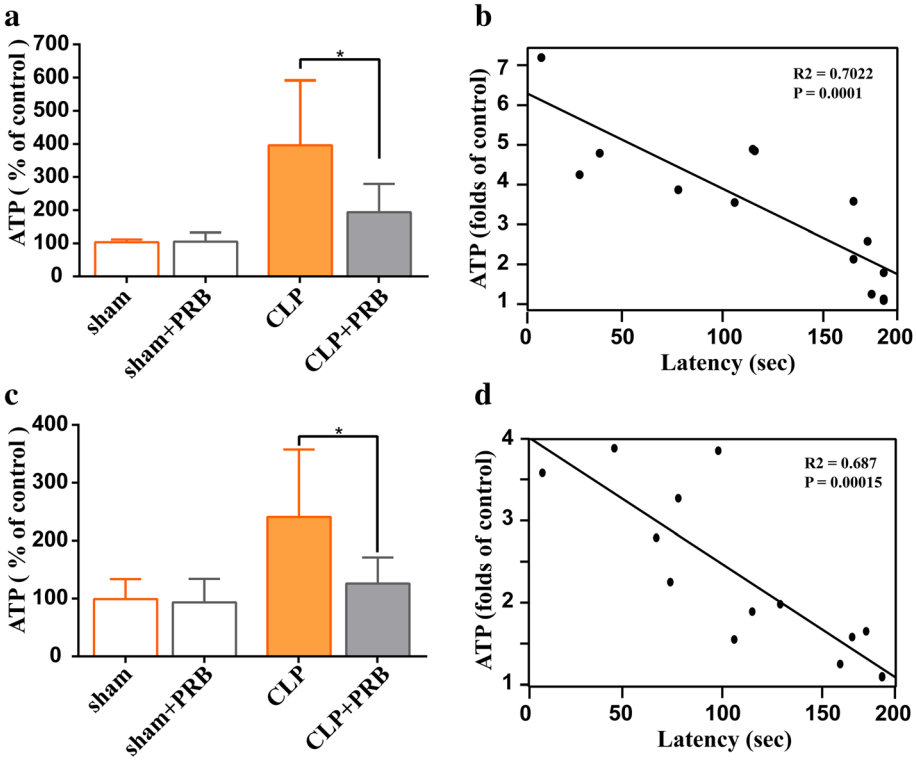

Fig. 5. Extracellular ATP concentrations and its correlation with latency of step-down task were determined after sepsis. **a**, **c** Extracellular ATP concentrations in the CSF (**a**) and hippocampus (**c**) of CLP mice without and with PRB treatment were determined using ATP determination kit ($n = 5$). CSF cerebrospinal fluid. **b**, **d** The regression analysis showed that the ATP levels in the CSF (**b**) and hippocampus (**d**) were inversely correlated with the latency of step-down task. Bars were represented as mean ± SD, $^*P < 0.05$.

diagnosis and therapy. Despite great progress in the treatment of sepsis, the incidence of SAE remained staying at a high level which further also aggravated the morbidity and mortality. Almost all progressive clinical treatments have yielded disappointing outcomes, and no effective treatments for SAE are available to this day [37]. It has been well-demonstrated that PRB could reduce neuroinflammation, which might foster its translational interest. PRB was increasingly recognized as a promising candidate therapy for various neurodegenerative and neurological diseases, including epilepsy, stroke, Alzheimer disease, and experimental autoimmune encephalomyelitis [5, 8, 9, 13, 14, 36]. As the therapeutic effect of PRB in SAE is uncertain, we therefore investigated its function in CLP-induced cerebral dysfunction. The primary novel findings of present study were as follows: (1) microglial activation and neural apoptosis after sepsis were accompanied by PANX1 upregulation; (2) the latencies of step-down inhibitory avoidance test were inversely correlated with the extracellular ATP concentration in the CSF or hippocampus of CLP mice; (3) PRB lessened neuroinflammation after sepsis by inhibiting the release of ATP; and (4) PRB treatment ameliorated behavioral deficits and exerted beneficial effects in the brain following septic injury by inhibiting ATP release. Taken collectively, these findings provided the first line of evidence for a functional role of PRB in playing neuroprotective effect *via* inhibiting ATP release, thereby contributing to the ameliorated SAE severity and preventing SAE progression.

Antibiotics administration is one of the essential strategies for septic treatment. However, overuse of antibiotics is a growing concern in septic patients which increases the occurrence of drug-resistant bacteria. Furthermore, antibiotics cannot effectively inhibit the release of microbial constituents, such as unmethylated CpG dinucleotides of microbial DNA sequences (CpG) or lipopolysaccharide (LPS), which contribute to the expansion of brain dysfunction. Therefore, it is necessary to explore new strategies to control brain dysfunction without impairing the host antimicrobial

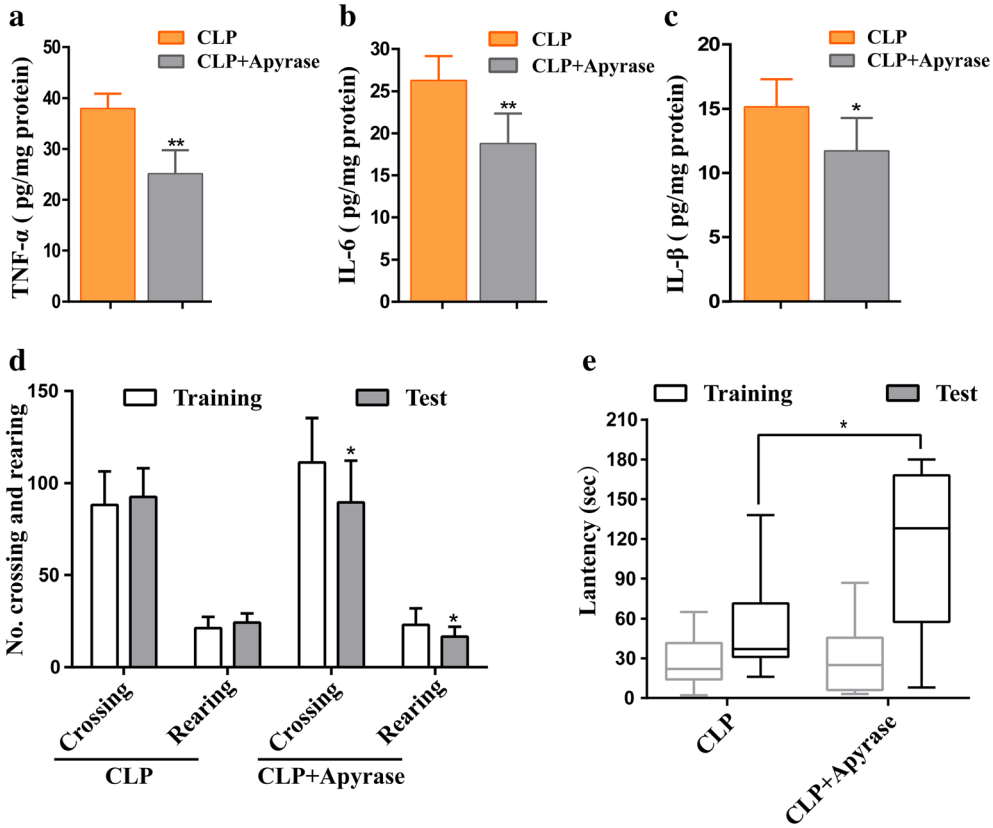

Fig. 6. Removal of extracellular ATP improved cognitive impairment after sepsis. **a**–**c** TNF-α, IL-6, and IL-1β levels in the hippocampus were detected in the presence or absence of apyrase. Data were expressed as mean ± SD, $n = 5$ mice per group, $^{*}P < 0.05$, $^{**}P < 0.01$. **d** Open-field test. Data were represented as mean ± SEM of crossings and rearings of training (white columns) and test (gray columns) sessions ($n = 12$ mice per group). Significant differences were indicated between the training and test sessions per group (Student's *t* test, $^{*}P < 0.05$). **e** Step-down inhibitory avoidance test. Data were expressed as median and interquartile ranges ($n = 12$ mice per group) represent the mean latency in seconds. Significant differences of latency in the test sessions were indicated between CLP *vs.* CLP + apyrase group (Mann–Whitney *U* test, $^{*}P < 0.05$).

defense system. A recent study showed a critical role for PANX1 and P2X7 downstream of caspase 11 for susceptibility to sepsis [38]. Another study also reported that PRB administration was helpful in LPS-induced septic model with skeletal muscle cellular energy crisis and histopathological injury, which was ascribed to the ability of PRB to reduce serum ATP and inflammatory factor (TNF-α, IL-6, and IL-1β) production [17]. However, current research claimed that inhibiting endogenous purinergic signaling by blocking PANX1 channels could inhibit PMN activation and PMN-induced organ damage, while this approach did not improve the outcome of sepsis. Instead, the disruption of PANX1 channels impaired the initial immune defenses, so there were not enough PMNs locating and eliminating bacteria that spread throughout the host and cause full-blown sepsis [22]. These facts emphasized that a more optimal combined treatment was required for the better outcome of sepsis. Here we demonstrated the effects of treatment with PRB in combination with antibiotics in the CLP model. We used 50 mg/kg PRB *via* i.p. injection twice combining with antibiotics after CLP. Interestingly, this dosage was enough to attenuate sepsis-induced neuroinflammatory events without affecting 10-day survival rate suggesting that premature action of PRB was critical in reducing the subsequent progression of the neuroinflammatory response. PRB administration after CLP reduced the levels of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, presented in the brain tissue which lead to neural injury.

One major contributor to SAE progression is the activation of microglia [23]. Microglial activation after injury promoted the release of numerous pro-

inflammatory factors, which in turn could determine neuronal apoptosis [15]. In this study, the activated microglia cells, determined by Iba1, were increased significantly in the hippocampus and cortex after septic injury which was in line with other studies [18, 19]. One important factor mediating the inflammatory mediator action on microglia was the injury-related release of nucleotides (particularly ATP) *via* PANX1 channels [30]. Present evidence indicated that PRB is able to cross the blood-brain barrier [26, 35] and does not block other potential routes (including other pannexin channels and connexin hemichannels) of ATP release that was involved in brain dysfunction [7, 8, 31]. So we examined whether PRB-mediated PANX1 channel inhibition after SAE restricted the neuroinflammatory responses *via* lessening ATP release.

In summary, we showed that PRB attenuated the inflammatory response and improved long-term cognitive impairment when administered with antibiotics. Present study proved the important value of PRB for future therapeutic strategies of SAE—particularly in consideration of approved clinical application. However, we are not in a position to explain the mechanism through which PRB enhances cognitive function completely, and further studies are needed. In addition, further studies are also needed to explore the role of PRB in the acute and chronic activation states of microglia.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were approved by the Institutional Animal Care and Use Committees of Xi'an Jiaotong University (Xi'an, China).

Competing Interests. The authors declare that they have no competing interests.

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