Role of HMGB1 translocation to neuronal nucleus in rat model with septic brain injury

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Abstract

High-mobility Group Box-1 (HMGB1) is a central late proinflammatory cytokine that triggers the inflammatory response during sepsis. However, whether HMGB1 is involved in the pathogenesis of septic brain damage is unknown. In this study, we investigated the role of HMGB1 in regulating brain injury in a rat model of sepsis. Wistar rats were subjected to cecal ligation and puncture (CLP) to induce septic brain injury. Hematoxylin and eosin staining was used to detect pathological changes in the cortex. The cellular localization of HMGB1 was determined using immunostaining. Cortical levels of HMGB1, its receptor for advanced glycation end-products (RAGE), and downstream effector, nuclear factor kappa-B (NF- κ B) subunit p65, were detected via western blot. HMGB1 was increased in the cytoplasm via translocation from the nucleus predominantly in neurons. Moreover, RAGE and NF- κ B p65 were upregulated after septic brain injury. Ethyl pyruvate, an inhibitor of HMGB1, down-regulated the expression of RAGE and NF- κ B p65 via inhibiting HMGB1 expression in the cytoplasm. Collectively, our findings suggest that HMGB1 and its signaling transduction have critical roles in the pathogenesis of septic brain injury. HMGB1 inhibition might be a potential new therapeutic target for septic brain injury.

Key words: HMGB1; sepsis; brain injury; RAGE

1. Introduction

Sepsis-induced brain injury is a complex syndrome caused by a systemic inflammatory response to infectious or noninfectious factors. Sepsis is characterized by seizures, focal neurological signs, and an altered mental status, ranging from delirium to coma [1]. Septic brain injury is a common complication of severe sepsis in the pediatric intensive care unit, representing a life-threatening disease that leaves survivors with prolonged behavioral impairments [2,3]. Several mechanisms have been proposed, including oxidative stress [4,5], blood-brain barrier deterioration [6], and cell death [7], but the exact underlying pathophysiology for septic brain injury is still poorly understood. During the progression of the disease, anti-inflammatory cytokines may help to prevent brain damage [8].

High-mobility Group Box-1(HMGB1), a member of the HMG family, is a highly evolutionarily conserved non-histone DNA-binding protein [9]. Its functions are related to its localization in cells. In the nucleus, HMGB1 is involved in the stabilization of the nuclear bodies, the regulation of gene transcription, and the targeting of gene specific binding transcription factors [10]. Extracellular HMGB1 serves as a crucial late proinflammatory cytokine that participates in inflammation and infection processes [11]. Some stimulating factors, such as trauma, inflammation, and stress, can lead to the translocation of HMGB1. HMGB1 is released into the extracellular space by active immune cells, including macrophages, monocytes, and neutrophils [12]. Once released, extracellular HMGB1 binds with specific receptors, such as the receptor for advanced glycation end-products (RAGE), toll-like receptor-2(TLR-2), and TLR-4, to participate in the pathophysiological response [13]. HMGB1 binds to RAGE and activates NF- κ B signaling, which contributes to the regulation of inflammation, apoptosis, proliferation, and autophagy [14]. Still unknown, however, is whether HMGB1 translocation could occur in septic brain dysfunction to drive the activation of RAGE and NF- κ B signaling.

Ethyl pyruvate (EP), an inhibitor of HMGB1, has great therapeutic potential. It has neuroprotective effects against Zn^{2+} toxicity [15], and in other models, it inhibits the expression of HMGB1 [16-18].

In the current study, in order to determine whether HMGB1 was involved in septic brain injury, we determined whether HMGB1 translocation occurs in cortical neurons and whether EP inhibits HMGB1 translocation to alleviate septic brain injury. As studies have shown that the cortex is most frequently injured during the progression of septic brain damage [19], we evaluated the pathological changes in the cortex after the onset of sepsis in a rat model.

2Methods

2.1 Animals and treatments

Thirty-day-old male Wistar rats were purchased from Sichuan Jianyang Dashuo Animal Science and Technology Co., Ltd. The Research Animal Care Committee of Sichuan University approved all research protocols, and the methods were carried out in accordance with approved guidelines and regulations. All animals were given free access to water and food and housed in cages with a 12-h light/dark cycle. The environmental humidity was 50-55%, and the temperature was kept at 22-25°C. All efforts were made to minimize the number of rats used and their suffering. All rats were randomly distributed to either a sham-operated group, a group that received cecal ligation and puncture (CLP), or rats receiving CLP followed by EP treatment (CLP+EP group). In the CLP groups, sepsis was induced as previously reported [20]. Briefly, all animals were provided water but not food for 6h before undergoing CLP surgery. Rats were anesthetized with 10% chloral hydrate (300mg/1 ml, intraperitoneally), followed by a 1-cm midline laparotomy. The cecum was isolated, disinfected, and tightly ligated before being gently punctured twice with an 18-gauge needle. The exact position of ligation comprises 75% of the cecum. The abdomen was then closed in two layers to position the cecum. The same procedure was performed on the sham-operated group, but without the ligature and puncture. In the CLP+EP group, EP at 40mg/kg (Sigma Aldrich, St. Louis, MO) was dissolved in 2ml of Lactate Ringer Solution and was intraperitoneally administered at 30 min after the CLP operation [21]. The sham group and CLP group received the same volume of Lactate Ringer Solution at the same time. After the operation, rats had free access to

food and water. In our experiments, we found the mortality within 24 hours is around 20%. Around 12h after the procedure of CLP operation, animals begin to show malaise, fever, chills, and reduced motor activity.

2.2 Histological examination

After sequential perfusion with 0.9% normal saline and 4% paraformaldehyde (100ml each), the brain tissues were fixed in a 4% paraformaldehyde solution for 24-36 h at 4°C. Tissues were paraffin-embedded, and sectioned (5µm) before staining with hematoxylin and eosin. The brain tissues were examined using a Leica inverted optical microscope to observe the pathological changes (Leica, Mannheim, Germany).

2.3 Immunofluorescence staining

To delineate the localization of HMGB1, the neuronal marker NeuN, the astrocyte marker glial fibrillary acidic protein (GFAP), and the microglia marker ionized calcium binding adaptor molecule 1 (Iba-1) were used for fluorescence double immunolabelling. Brain tissues were embedded in 2.5% agarose, and samples were cut into 40µm sections by using an oscillating tissue slicer (Leica). Sections were washed three times in phosphate-buffered saline (PBS) at room temperature, then incubated in 0.3% TritonX-100 for 30min, and blocked for 1h in fetal calf serum. Sections were incubated with the primary HMGB1 polyclonal antibody, 1:1,000 (Abcam, Cambridge, MA, USA); anti-NeuN monoclonal antibody, 1:500 (Millipore, Danvers, MA USA); anti-GFAP monoclonal antibody, 1:500 (Millipore); and anti-Iba-1 monoclonal antibody, 1:300 (Millipore) at 4°C overnight. Next, sections were washed three times in PBS for 15 min in total. Sections were incubated for 2h in the dark with the appropriate secondary antibodies, which were a mixture of Dylight 488-conjugated donkey anti-rabbit IgG, 1:500 (Jackson ImmunoResearch, West Grove, PA, USA) and Cy3-conjugated donkey anti-mouse IgG, 1:500 (Jackson ImmunoResearch). Afterwards, the sections were washed another three times in PBS prior to being counterstained with 4, 6-diamidino-2-phenylindole (DAPI; 1:500, Sigma, St. Louis, MO, USA) for 10 min at room temperature in the dark. Sections were mounted onto glass slides in antifade mounting medium (Beyotime, Haimen, China). Fluorescence microscopy imaging was performed using a confocal laser scanning

microscope (Olympus, Tokyo, Japan) and FV-ASW-3.1 software (Olympus).

2.4 Western blotting analysis

Cortical tissues were homogenized and nuclear and cytoplasmic proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA). A bicinchoninic acid (BCA) assay was used to measure the protein concentration (Sigma). Equal amounts of each sample were subjected to electrophoresis on 12% SDS-polyacrylamide gels for 40 min at 70 V followed by 120 min at 120V, and then transferred onto a polyvinylidene difluoride membrane for 60min (Millipore). After blocking with 5% skim milk in Tris-buffered saline and Tween20 (TBS-T) for 1 h at room temperature, the blots were incubated with the primary antibodies in 2.5% fetal bovine serum at 4°C overnight. The antibodies included: rabbit anti-HMGB1 polyclonal antibody (1:1,000, Abcam), rabbit anti-RAGE polyclonal antibody (1:1,000, Abcam), mouse anti-NF- κ B p65 monoclonal antibody (1:500, Millipore), rabbit anti-Histone 3 monoclonal antibody (1:2,000, Millipore), and rabbit anti- β -tubulin polyclonal antibody (1:10,000, Sigma). After washing for three times in TBS-T, the blots were incubated with the appropriate horseradish-peroxidase conjugated anti-rabbit or anti-mouse secondary antibodies (1:3,000, Santa Cruz, Dallas, TX, USA) at room temperature for 1 h. Bands were visualized with enhanced chemiluminescence (Millipore) and exposed to film. The films were scanned on an Image Scanner by using a gel imaging analysis system (Bio-Rad, Berkeley, CA, USA). Band density was analyzed using Quantity One software (Bio-Rad). The density ratio was measured as the relative intensity of each band against Histone 3 or β -tubulin (as internal controls).

2.5 Statistical analysis

The data are shown as the mean $\pm$ standard deviation (SD). Comparisons between groups were performed using a one-way analysis of variance (ANOVA) and the Student-Keuls test. Analysis was performed using SPSS software (version 21.0). A *P*-value less than 0.05 was considered statistically significant.

3Results

3.1 Histopathologic evaluation of the cortex

In the sham group, the cortex structure was clear at both the macroscopic and light microscopic levels. Cortical neuron arrangement and numbers were both normal. At 24h after the CLP surgery, the cortical structure was irregular, and the morphologies of the neurons were diverse. The numbers of cortical neurons were reduced, and edema was present. However, at 24h after CLP with EP treatment, the cortical structure was more organized compared to that of the CLP group. In addition, the number of neurons no longer appeared reduced, and the edema within the cortex was alleviated (Figure 1).

3.2 Cellular localization of HMGB1 in the cortex of the sham and CLP groups

To demonstrate the cellular localization of HMGB1, double immunofluorescent staining was performed for HMGB1, the neuronal marker NeuN, the astrocyte marker GFAP, and the microglia marker Iba-1. In the sham group, HMGB1 was expressed in the nucleus of neurons, astrocytes, and microglia, but was predominantly expressed in neuronal nuclei. Twenty-four hours after CLP, HMGB1 translocated from the nucleus to the cytoplasm predominantly in cortical neurons, whereas a small number of cortical astrocytes and microglia expressed HMGB1 (Figure 2).

3.3HMGB1, RAGE, and NF-κB p65 expression in the sham and CLP groups

Western blot analysis showed that nuclear HMGB1 expression decreased from 6h to 48h after the CLP operation compared to the sham group ($P<0.05$; Figure 3A). Cytosolic HMGB1 correspondingly followed the opposite trend, where the level of cytosolic HMGB1 increased within the cortex from 6h post-CLP, peaked at 24h, and remained high at 48h post-surgery ($P<0.05$; Figure 3B). Compared to the sham group, the level of RAGE increased within the cortex from 6h post-CLP, peaked at 24h, and remained high at 48h post-surgery ($P<0.05$; Figure 3C). Nuclear NF-κB p65 expression increased from 3h to 24h after the CLP operation compared to the sham group. At 12h and 24h post-CLP, the expression of nuclear NF-κB p65 was significantly higher than that of the sham group ($P<0.05$; Figure 3D).

3.4 Effect of EP on HMGB1, RAGE, and NF- κ B p65 expression in the cortex

Twenty-four hours after the administration of EP, brain cortical tissues were harvested, and western blot and double immunofluorescent staining was carried out to measure the expression levels nuclear and cytosolic HMGB1 and RAGE, and nuclear NF- κ B p65. EP administration significantly increased nuclear HMGB1 and decreased cytosolic HMGB1 levels at 24h post-CLP compared to the CLP group ($P<0.05$; Figure 4A, 4B, and 4E). RAGE and nuclear NF- κ B p65 levels were decreased significantly in the CLP+EP group compared to the CLP group ($P<0.05$; Figure 4C and 4D). According to these findings, we concluded that EP might partly inhibit HMGB1 translocation, resulting in a reduction in the activation of RAGE and NF- κ B p65 signaling.

4. Discussion

In the present study, we detected HMGB1 expression predominantly in the nuclei of cortical neurons, whereas a small number of astrocytes and microglia expressed HMGB1. After the CLP, HMGB1 translocated to the cytoplasm where it may have acted as a crucial late proinflammatory cytokine to induce sepsis and brain dysfunction. However, the group receiving EP showed reduced levels of brain damage. This reduction was associated with significant increases in nuclear HMGB1, and decreases of its downstream effectors, RAGE and nuclear NF- κ B p65.

Sepsis most frequently induces brain damage [22], and cytokines are crucial factors in this pathology. Early proinflammatory cytokines are involved in the development of sepsis; however, some anti-inflammatory agents have limited effects against sepsis, suggesting roles for other factors [23-25]. Compared with early inflammatory cytokines, after exposure to endotoxin, HMGB1 shows an increase at 12-18h, which is delayed compared to TNF- α [26]. Thus, HMGB1 is considered a late proinflammatory factor [27,28]. Clinical and experimental studies have detected the release of HMGB1 during the inflammatory response [29,30], suggesting that targeting this molecule may be an alternative therapeutic avenue for treating sepsis.

As a significant mediator of inflammatory disease, HMGB1 could trigger or amplify the inflammatory response to result in cell injury and necrosis. In animal experiments, drugs that inhibit the activation of HMGB1, such as antibodies or inhibitors, are effective in alleviating the inflammatory response [11]. For example, in a model of subarachnoid hemorrhage, HMGB1 translocates at an early phase after hemorrhage onset, and extracellular HMGB1 acts as a cytokine to contribute to the subsequent brain damage [31]. We observed similar translocation of HMGB1 at 24h following sepsis-induced brain injury. Translocation may involve two pathways, i.e. via active nerve cell secretion or via the loss of cellular membrane integrity, which could passively release HMGB1 into the extracellular environment. Activated microglia plays a neuroprotective role via up-regulating expression of neurotrophic factors; however, activated microglia can also release a large amount of inflammatory factors [32, 33]. Excessive inflammatory cytokines may participate in the activation of neurons microglia and astrocytes, and could lead to the death of cells in septic brains. Although translocation of HMGB1 was observed in neurons, microglia, and astrocytes, it occurred primarily in neurons, especially in the later phase of brain damage.

The inflammatory activity of HMGB1 is mainly mediated by its binding to the RAGE receptor. Banger et al. found that HMGB1 or RAGE knockout mice showed decreased physiological signs of cardiac impairment after suffering from an autoimmune response to cardiac troponin I [34], suggesting a role for this signaling pathway in inflammatory and immune responses. Our research found that expression of both RAGE and the nuclear NF- κ B p65 subunit in the rat cortex increased significantly at 12h and 24h after CLP. We speculate that higher amounts of cytoplasmic HMGB1 may play an important role in facilitating the action of HMGB1 release. Once released, HMGB1 evokes innate immune response via its interaction with cell surface receptors. The binding of HMGB1 to RAGE activates a signaling pathway through NF- κ B activation, indicating that the activation of this signaling pathway could be heightened by sepsis-induced brain inflammation. In our study, immunofluorescence staining showed that most HMGB1 positive cells were

colocalized with NeuN positive cells. Therefore, neurons are the main source of HMGB1. In parallel, we think that RAGE is mainly localized in neurons. On the other hand, we found that a low level of HMGB1 was expressed in microglia and astrocytes. Therefore, we think that microglia and astrocytes might express low level of RAGE receptor.

Previous studies have reported that EP is an anti-inflammatory agent in clinical conditions, such severe pancreatitis, hepatitis, and sepsis [21, 35, 36]. Recent research found that EP could alleviate post-ischemic brain damage by inhibiting HMGB1 phosphorylation and secretion [37]. Other research has found that EP can regulate the HMGB1-RAGE axis to decrease the expression and secretion of HMGB1 from the nucleus into the cytoplasm [18]. As shown in our immunofluorescence and western blot assays, EP treatment significantly reduces HMGB1 translocation and release at 24h after CLP, and the expression of RAGE and nuclear NF- κ B p65 were decreased significantly at the same time point. These results might suggest that EP blocks HMGB1 translocation, reducing extracellular HMGB1 and the production of its key signaling components, and thereby reducing HMGB1-induced activation of downstream signaling pathways.

Several limitations to our study should be noted. First, different doses of EP were not tested in the current study. To determine the full dose-response curve, different concentrations of EP should be administered after CLP. Second, we did not clarify the pathway of secretion for HMGB1. A comprehensive understanding of the regulation of HMGB1 during sepsis-induced brain injury is desirable to better design interventions that counter HMGB1 activity.

5. Conclusions

In conclusion, our study demonstrated that CLP could evoke HMGB1 translocation from the nucleus to the cytoplasm predominantly in the cortical neurons for rats with septic brain injury. EP administration inhibited HMGB1 subcellular translocation and

reduced the expression of RAGE and nuclear NF- κ B p65. These EP-mediated changes may be protective against septic brain injury, thus supporting a potential therapeutic role for EP in sepsis-induced brain damage.

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Author Contributions: YL wrote the manuscript and analyzed the data; XL and YQ designed the study; JH performed western blotting experiments; TZ performed immunofluorescence staining; FZ participated in the pathology examinations; SL conducted the statistical analysis; and DM revised the manuscript. All authors have approved the final article.

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Figure legends

Figure 1. Histopathologic evaluation of the cortex. Brain tissues were stained using hematoxylin and eosin to detect the pathological changes in the cortex following the induction of sepsis. Original magnification: $\times 400$. Data are represented from 3 independent experiments, $n=6$ for each group in each experiment.

Figure 2. Localization of HMGB1 in cortical NeuN-, GFAP- and Iba-1-positive cells. A high degree of co-localization within cortical cells was observed for the high-mobility group box-1 protein (HMGB1; green), neuron-specific nuclear protein (NeuN; red), glial fibrillary acidic protein (GFAP; red), and ionized calcium binding adaptor molecule 1 (Iba-1; red). Images were obtained from the cortex of the sham-operated rats and the experimental rats at 24h after cecal ligation and puncture (CLP24h). Cell nuclei were labeled with 4, 6-diamidino-2-phenylindole (DAPI; blue). Results show that HMGB1 translocated from the nucleus to the cytoplasm predominantly in cortical neurons, whereas a small number of cortical astrocytes and microglia expressed HMGB1. (D) The ratio of HMGB1 volume was significantly higher in neurons. $*P < 0.05$ vs. GFAP group. $^{\#}P < 0.05$ vs. Iba-1 group. Original magnification: $\times 400$. Data are represented from 3 independent experiments, $n=6$ for each group in each experiment.

Figure 3. The expression of the HMGB1, RAGE, and NF- κ B p65 in a septic brain damage rat model. Brains were harvested 3h, 6h, 12h, 24h, and 48h after sham and cecal ligation and puncture (CLP) surgeries. Western blot analyses were performed for the expression of nuclear and cytosolic HMGB1, RAGE, and nuclear NF- κ B p65. The density of each band of interest is expressed relative to Histone 3 or β -tubulin levels per sample. Data are expressed as the mean $\pm$ SD, ($n = 5$, each group) from 3 independent experiments $*P < 0.05$ vs. sham group.

Figure 4. The effect of ethyl pyruvate (EP) on the cortical expression of HMGB1, RAGE, and NF- κ B p65. The effects of EP on the expression of nuclear and cytosolic HMGB1 and RAGE, and nuclear NF- κ B p65, were determined using double immunofluorescent staining and western blot densitometry. The density of each band of interest is expressed relative to Histone 3 or β -tubulin levels per sample. Data are expressed the mean $\pm$ SD, (n = 5, each group) from 3 independent experiments, * P < 0.05 vs. the sham group. # P < 0.05 vs. the sham group or the CLP24h group. (E) EP inhibited HMGB1 translocation in cortical neurons at 24h after CLP. Original magnification: $\times$ 400. Data are represented from 3 independent experiments, n=6 for each group in each experiment.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure legends

Figure 1. Histopathologic evaluation of the cortex. Brain tissues were stained using hematoxylin and eosin to detect the pathological changes in the cortex following the induction of sepsis. Original magnification: $\times 400$. Data are represented from 3 independent experiments, $n=6$ for each group in each experiment.

Figure 2. Localization of HMGB1 in cortical NeuN-, GFAP- and Iba-1-positive cells. A high degree of co-localization within cortical cells was observed for the high-mobility group box-1 protein (HMGB1; green), neuron-specific nuclear protein (NeuN; red), glial fibrillary acidic protein (GFAP; red), and ionized calcium binding adaptor molecule 1 (Iba-1; red). Images were obtained from the cortex of the sham-operated rats and the experimental rats at 24h after cecal ligation and puncture (CLP24h). Cell nuclei were labeled with 4, 6-diamidino-2-phenylindole (DAPI; blue). Results show that HMGB1 translocated from the nucleus to the cytoplasm predominantly in cortical neurons, whereas a small number of cortical astrocytes and microglia expressed HMGB1. (D) The ratio of HMGB1 volume was significantly higher in neurons. $*P < 0.05$ vs. GFAP group. $^{\#}P < 0.05$ vs. Iba-1 group. Original magnification: $\times 400$. Data are represented from 3 independent experiments, $n=6$ for each group in each experiment.

Figure 3. The expression of the HMGB1, RAGE, and NF- κ B p65 in a septic brain damage rat model. Brains were harvested 3h, 6h, 12h, 24h, and 48h after sham and cecal ligation and puncture (CLP) surgeries. Western blot analyses were performed for the expression of nuclear and cytosolic HMGB1, RAGE, and nuclear NF- κ B p65. The density of each band of interest is expressed relative to Histone 3 or β -tubulin levels per sample. Data are expressed as the mean $\pm$ SD, ($n = 5$, each group) from 3 independent experiments $*P < 0.05$ vs. sham group.

Figure 4. The effect of ethyl pyruvate (EP) on the cortical expression of HMGB1,

RAGE, and NF- κ B p65. The effects of EP on the expression of nuclear and cytosolic HMGB1 and RAGE, and nuclear NF- κ B p65, were determined using double immunofluorescent staining and western blot densitometry. The density of each band of interest is expressed relative to Histone 3 or β -tubulin levels per sample. Data are expressed the mean $\pm$ SD, (n = 5, each group) from 3 independent experiments, * P < 0.05 vs. the sham group. # P < 0.05 vs. the sham group or the CLP24h group. (E) EP inhibited HMGB1 translocation in cortical neurons at 24h after CLP. Original magnification: $\times 400$. Data are represented from 3 independent experiments, n=6 for each group in each experiment.