Original Paper

Erythropoietin-Derived Peptide Protects Against Acute Lung Injury After Rat Traumatic Brain Injury

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Key Words

Traumatic brain injury • Acute lung injury • Inflammation • Macrophages • Erythropoietin analogue

Abstract

Background: Traumatic brain injury (TBI) can be complicated by TBI-triggered acute lung injury (ALI), in which inflammation plays a central role. It has been reported that an Erythropoietin-derived peptide (pHBSP) was able to ameliorate TBI; however, its function in TBI-caused ALI has not been reported yet. **Methods:** In this study, we studied the effect of pHBSP on TBI-caused ALI by using a weight-drop induced TBI model. At 8 h and 24 h post-TBI, pulmonary edema (PE) and bronchoalveolar lavage fluid (BALF) proteins were measured, and haematoxylin and eosin (H&E) staining of lung sections was carried out. At 24 h following TBI, the lungs were harvested for immunofluorescence staining and qRT-PCR analysis. **Results:** At 8 h and 24 h post-TBI, pHBSP treatment significantly decreased wet/dry ratios, decreased total BALF protein, and attenuated the histological signs of pulmonary injury. At 24 h post-TBI, pHBSP treatment decreased the accumulation of CD68⁺ macrophages in the lung and reduced the mRNA levels of TNF- α , IL-6, IL-1 β and iNOS in the lung. **Conclusions:** We identified the protective role that pHBSP played in TBI-caused ALI, suggesting that pHBSP is a potent candidate for systemic therapy in TBI patients.

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Introduction

Traumatic brain injury (TBI), a life-threatening syndrome, is the major cause of neurological illness [1]. Due to its high incidence and poor outcome, TBI causes almost half of all trauma-associated mortality in many countries [2], especially in developed countries [3].

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TBI is frequently complicated by an intensive systemic inflammatory response, in which increased infiltration of inflammatory cells is observed in multiple organs, such as the liver [4], kidney [5] and lung [6], further leading to the dysfunction of these organs. Among these organs, the lung is the most vulnerable organ to inflammation triggered by TBI [7]. Clinical studies reported that 81% of TBI patients develop lung dysfunction, which is regarded as the most common organ injury following TBI [8, 9]. Additionally, accumulated studies have demonstrated that the TBI-triggered systemic inflammatory response causes acute lung injury (ALI) [10]. Moreover, in TBI-caused ALI, inflammatory cascade involving Nrf2 and nuclear factor- κ B were significantly induced [11], suggesting that inflammatory response could be a potential therapeutic target of TBI-caused ALI.

Erythropoietin (EPO) is a hormone that serves to regulate the production of red blood cells. Moreover, increasing data have identified the anti-inflammatory functions of EPO in various diseases such as peritonitis [12], experimental autoimmune neuritis (EAN) [13], ischaemia/reperfusion-injury [14] and obesity [15]. However, the long-term application of EPO may cause life-threatening complications, such as increasing the incidence of thrombosis and raising blood pressure, which limits its long-term clinical application [16]. To avoid these side effects of EPO, an EPO analogue containing the amino acids obtained from the aqueous face of helix B of erythropoietin, named pyroglutamate helix B surface peptide (pHBSP), was developed. It is non-erythropoietic but maintains the anti-inflammatory and tissue protective functions of EPO [16, 17]. It has been reported that pHBSP ameliorates TBI; however, the function of pHBSP in TBI-caused ALI has not been reported yet [18]. The current study further investigated the effects of pHBSP on modulating TBI-caused ALI.

Materials and Methods

Animals

Male Lewis rats (8–9-week-old, 350–400g, Vital River, Beijing, China) were housed under a 12-h light and 12-h dark and with free access to food and water. All animal experimental procedures in this study were approved by the Animal Care and Use Committees of Third Military Medical University.

Establishment of TBI-induced ALI model and pHBSP intervention

Rats were grouped randomly and were deeply anaesthetized by intraperitoneal injection of chloral hydrate (7%, 0.5 ml/100 g); a severe TBI model was then induced according to a well-established open-skull weight-drop contusion model [19, 20]. Briefly, rats underwent craniotomy, then a circular region of the skull (3.0-mm diameter) was removed and a 20 g weight was dropped from 20 cm above the removed region of the skull. After injury, the scalp was sutured closed.

For therapeutic treatment, rats with severe TBI-caused ALI were intraperitoneally injected with 60 μ g/kg pHBSP or an equivalent volume of PBS at 3-h and 12-h following injury.

Rat in the sham operation group underwent anaesthesia, scalp incision and craniotomy without TBI.

Measurement of Lung Wet/Dry Ratios

At 24 h following TBI, the rats were deeply anaesthetized, and then the upper and middle lobes of the right lung in each rat were acquired from the thoracic cavity ($n = 6$). The lung samples were weighed immediately and then dried in an oven at 70°C, separately. The wet/dry weight ratio was then calculated.

Measurement of bronchoalveolar lavage fluid (BALF) protein

Rats were deeply anaesthetized, and the left lung of each rat was lavaged by ice-cold PBS 6 times ($n = 6$); using this method, BALF was collected. Then, the BALF was centrifuged at 1300 rpm for 20 minutes at 4°C, and the supernatant was collected for further study. The concentration of BALF proteins were measured following Lowry's method [21], in which bovine serum was applied as a standard.

Histology and analysis of lung injury

To evaluate macrophage accumulation, rats were deeply anaesthetized and perfused intracardially with a 4% paraformaldehyde solution. The lungs were quickly removed and postfixed in 4% paraformaldehyde overnight at 4°C. Tissues were cut into equally long segments, embedded in paraffin, serially sectioned (3 µm) and mounted on silane-covered slides.

The procedures for the haematoxylin and eosin (H&E) and immunofluorescence staining and the evaluation of the immunostaining data were described previously [22]. The CD68 antibody was used to detect macrophages (1:100; Serotec, Oxford, UK).

The analysis of lung injury was carried out by using a semiquantitative scoring system blinded to the sham operation group described by G. Matute-Bello et al. [23]. In brief, the scoring system included 1) the score of neutrophils in the alveolar space and interstitial space, 2) the formation of the hyaline membrane, 3) the proteinaceous debris in the air spaces, and 4) the thickening of alveolar septum. Eight separated fields from each slide were analysed.

Tissue preparation, RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)

At 24 h following TBI, rats (n=6/group) were deeply anesthetized with chloral hydrate (7%, 0.5ml/100g) and perfused intracardially with PBS at 4°C. Lungs were quickly removed and stored in liquid nitrogen until RNA isolation. Total RNA was isolated and reverse transcribed into cDNA by using RNAPrep Pure Tissue Kit (TIANGEN Biotech, Beijing, China) and Quantscript RT Kit (TIANGEN Biotech, Beijing, China), then qRT-PCR was carried out according to the manufacturer's protocol (TIANGEN Biotech, Beijing, China). The relative gene expression was detected by qRT-PCR using the standard $2^{-\Delta\Delta CT}$ method [24]. Each sample was run in triplicate. The mRNA expression was normalized to β -actin. The relative gene expression values were expressed as the relative mRNA expression (fold change) compared to control group, whose expression was set as 1. Primers are: β -actin (sense, CCG TCT TCC CCT CCA TCG T; antisense, ATC GTC CCA GTT GGT TAC AAT GC), TNF- α (sense, TGA TCG GTC CCA ACA AGG A; antisense, TGC TTG GTG GTT TGC TAC GA), IL-6 (sense, GCC CTT CAG GAA CAG CTA TG; antisense, CAG AAT TGC CAT TGC ACA AC), IL-1 β (sense, TGC TGA TGT ACC AGT TGG GG; antisense, CTC CAT GAG CTT TGT ACA AG), IL-10 (sense, CCT GCT CTT ACT GGC TGG AG; antisense, TCT CCC AGG GAA TTC AAA TG) and iNOS (sense, TCT GTG CCT TTG CTC ATG ACA; antisense, TGC TTC GAA CAT CGA ACG TC).

Data evaluation and statistical analysis

After immunostaining, the lung tissues were observed by light microscopy. The counting of CD68⁺ cells was based on previous studies [25]. In detail, CD68⁺ cells were counted in six non-overlapping high-power fields ($\times 400$ magnification) for each section (6 sections were appraised per rat and 2 sections were counted for each rat). The high-power fields were selected to have a maximum of positive cells. Then, CD68⁺ cells were calculated by ImageJ. In each field, only positive cells with DAPI⁺ were counted. The results were shown as the arithmetic means of CD68⁺ cells per high-power fields and SEM.

Unpaired t-tests were performed for paired comparisons (GraphPad Prism 4.0 Software, San Diego, USA). For all statistical analyses, significance levels were set at a P value less than 0.05.

Results

Effects of pHBSP on pulmonary oedema (PE) in TBI-Induced ALI

The wet/dry weight ratio is one of the most significant indexes that reflects PE formation. Lung samples were collected at 8 h and 24 h following TBI, then wet/dry measurement was conducted immediately. As shown in Figure 1A, wet/dry ratios were increased greatly both at 8 h ($p < 0.05$) and 24 h ($p < 0.01$) following TBI, in comparison with sham operation control. Intervention with pHBSP significantly decreased wet/dry ratios both at 8 h ($p < 0.05$) and 24 h ($p < 0.05$) following TBI.

Effects of pHBSP on the concentrations of total BALF protein in TBI-Induced ALI

TBI rats were treated by pHBSP or PBS as described and deeply anaesthetized at 8 h and 24 h for BALF protein analysis. As shown in Figure 1B, BALF proteins were increased at 8 h ($p < 0.05$) and 24 h ($p < 0.01$) following TBI, in comparison with sham operation control.

Fig. 1. Effects of pHBSP on PE and BALF protein in TBI-Induced ALI. pHBSP (60 µg/kg) or equally doses of PBS was therapeutically given to TBI rats (n=6). A: Lung samples were collected at 8 h and 24 h following TBI, then wet/dry ratios in sham, PBS and pHBSP groups were measured. pHBSP treatment decreased lung wet-dry ratios. B: BALF protein of sham, PBS and pHBSP groups were measured. pHBSP treatment significantly decreased TBI-induced increase of BALF protein. Data are mean ± SEM; error bars indicate s.e.m.

Fig. 2. Effects of pHBSP on PE and BALF protein in TBI-Induced ALI. At 8 h and 24 h following TBI, the lungs from the sham operation group (A-B), TBI group (C-D), the pHBSP group (E-F) were subjected to HE staining. A-F: Representative microimages of lung sections from each group are shown, and the arrows indicate inflammatory cell clusters in lung (magnification: 100×). G: The lung injury score decreased with pHBSP intervention (60 µg/kg) after injury. Data are mean ± SEM; error bars indicate s.e.m. Scale bars are 100 µm.

Intervention with pHBSP significantly decreased total BALF protein both at 8 h ($p < 0.05$) and 24 h ($p < 0.05$) following TBI.

Effects of pHBSP on histological changes in lungs of TBI rats

TBI rats were treated by pHBSP or PBS as described and sacrificed at 8 h and 24 h for histological analysis. HE staining was carried out to show cell infiltration, PE, and alveolar collapse in the lung tissues and compared with the sham operation group. As shown in Figure 2, the lung sections of sham operated rats showed a normal alveolar morphology both at 8 h and 24 h following TBI (Fig. 2A-B), however, the lung sections of TBI group showed obvious PE, alveolar wall thickening, and increased exudates as well as inflammatory cell infiltration in the alveolar spaces (Fig. 2C-D), especially at 24 h following TBI (Fig. 2D). The pHBSP intervention significantly amended TBI-induced histopathologic changes (Fig. 2E-F).

In addition, lung injury scores were calculated. Lung injury scores in the pHBSP-treated group were significantly lower than the scores of the TBI group (Fig. 2G), both at 8 h ($p < 0.05$) and 24 h ($p < 0.01$) following TBI.

Effects of pHBSP on macrophage accumulation in the lungs of TBI rats

At 24 h following TBI, lungs in each group were collected to detect the accumulation of macrophages by immunofluorescence staining. The accumulation of CD68⁺ macrophages was significantly increased at 24 h post-TBI (Fig. 3D-2F), compared with sham operation control ($p < 0.01$, Fig. 3A-2C). The pHBSP treatment significantly decreased the accumulation

Fig. 3. Effects of pHBSP on macrophages accumulation in lung of TBI rats. 24 h following TBI, the lungs from the sham operation group (A-C, K-M), TBI group (D-F, N-Q), the pHBSP group (G-I, R-T) were subjected to CD68 immunofluorescence staining. Representative microimages of lung sections from each group are shown, and the arrows indicate macrophages clusters (magnification: 100×). J: Numbers of CD68⁺ cells were counted in six nonoverlapping high-power fields (× 400 magnification) for each section. n=6 per group. Data are mean ± SEM; error bars indicate s.e.m. Results are presented as arithmetic means of positive cells per high-power field and SEM. Scale bars are 100 μm for (A-P) and (R-T) and 50 μm for Q.

Fig. 4. Effects of pHBSP on the expression of iNOS and inflammatory cytokines in lungs of TBI rats. At 24 h following TBI, lungs in each group were taken for qRT-PCR analysis. The levels of iNOS, TNF-α, IL-6 and IL-1β mRNA expression were decreased following pHBSP treatment, while IL-10 mRNA expression were increased following pHBSP treatment. n=6 per group. Data are mean ± SEM; error bars indicate s.e.m.

of CD68⁺ macrophages in the lung ($p < 0.01$, Fig. 2G-2J). Interestingly, compared with the sham operation group, a drastic increase in CD68⁺ macrophage clusters was observed at 24 h post-TBI (Fig. 2K-2Q); we inferred that these macrophages were perivascular macrophages or bronchial macrophages. As expected, the administration of pHBSP significantly decreased the accumulation of CD68⁺ macrophage clusters (Fig. 2R-2T).

Beneficial effects of pHBSP on the expression of iNOS, proinflammatory cytokines and anti-inflammatory cytokines in the lungs of TBI rats

At 24 h following TBI, lungs in each group were collected to detect the mRNA expression of inflammatory cytokines by qRT-PCR analyses. As shown in Figure 4, the mRNA expression of iNOS and proinflammatory cytokines (TNF-α, IL-6 and IL-1β) were significantly increased at 24 h post-TBI, compared with sham operation control ($p < 0.01$). The pHBSP treatment significantly reduced TNF-α ($p < 0.05$), IL-6 ($p < 0.01$), IL-1β ($p < 0.05$) and iNOS ($p < 0.05$) mRNA expression levels in the lungs of TBI rats. We next assessed the mRNA expression of IL-10 in the lungs of TBI rats. pHBSP treatment significantly increased IL-10 mRNA expression levels in the lungs of TBI rats ($p < 0.01$).

Discussion

This study showed the protective effects of pHBSP on TBI-caused ALI, which demonstrated for the first time that pHBSP suppressed pulmonary inflammation, mainly

by inhibiting the activation of inflammatory macrophages and the expression of pro-inflammatory cytokines in TBI rats.

pHBSP, an EPO-derived short peptide, was designed for specific binding to the non-erythropoietic EPOR-CD131 heterocomplex, instead of the homodimer EPO receptor (EPOR₂), so pHBSP retains the anti-inflammatory functions and not the erythropoietic function of EPO. The anti-inflammatory function of pHBSP has been widely evaluated in various disease models, such as EAN, obesity and neuropathic pain [22, 26, 27]. It was also reported that pHBSP improved rat TBI [18]. Unfortunately, in both clinical and experimental studies, severe TBI severely damages pulmonary function, thus decreasing the outcomes of TBI survivors. In the current study, we proved that pHBSP has protective effects on TBI-caused ALI, indicating that pHBSP could be a hopeful therapeutic candidate for TBI-caused ALI.

Growing evidence has identified a crucial contribution of inflammation to TBI-caused ALI. Following TBI, inflammatory cytokine expression was elevated in the lung such as TNF- α , IL-6, IL-1 β and IL-10. The proinflammatory cytokine TNF- α is regarded as one of the key mediators of inflammation and could be quickly secreted during an inflammatory response. IL-6 is also a proinflammatory cytokine with a function of aggravating uncontrolled inflammation, and IL-1 β is involved in a variety of cellular activities [28, 29]. Consistent with these cytokines, iNOS can be synthesized by NF κ B, a protein transcription factor for pro-inflammatory molecules such as TNF- α . It is reported that iNOS is involved in many inflammatory diseases such as EAN and TBI [30]. In ALI, these inflammatory mediators have cytotoxic effects, such as inducing apoptosis of multiple cells including alveolar epithelial cells, increasing capillary permeability and damaging intercellular tight junctions, further resulting in increasing extravasation of vascular fluid, inflammatory cells and more inflammatory mediators [31]. In addition, Jin and colleagues demonstrated that disruption of TNF- α , IL-6 and IL-1 signalling pathways protected mice from TBI-induced ALI [28]. Also, reducing the expression of iNOS can protect mice from ALI [32], demonstrating that these inflammatory mediators are essential to TBI-induced ALI. Conversely, the anti-inflammatory cytokine IL-10 is reported to protect mice from the inflammation process in TBI-induced ALI [33]. In the current study, we discovered that pHBSP ameliorated TBI-induced decrease of PaO₂, and pHBSP significantly decreased TBI-induced up-regulation of TNF- α , IL-6, IL-1 β and iNOS mRNA expression in the lung, while up-regulated the mRNA expression level of IL-10 in the lung. These findings suggested that pHBSP exerts its lung-protection function mainly by inflammation suppression in rats with TBI-caused ALI.

Macrophages play a central role in inflammation involved in TBI-caused ALI. TBI results in the infiltration of a large number of macrophages in the lung in the initial hyperinflammatory phase, during which macrophages secrete many proinflammatory mediators such as TNF- α , IL-6, and IL-1 β , thus aggravating ALI. Lynne C et al. reported that blocking CD11d, a protein that is located on macrophages, decreased the infiltration of macrophages in the lung, resulting in reduced pulmonary inflammation that further led to ameliorated lung damage [10], pointing to the key role of macrophages in the progression of TBI-caused ALI. Our study revealed that pHBSP reduced the intensity of macrophages in the lung following TBI. Since TNF- α , IL-6, IL-1 β are macrophage-derived cytokines, and previous studies demonstrated that pHBSP suppressed the expression of TNF- α , IL-6, IL-1 β whereas increased the expression of anti-inflammatory cytokine IL-10 in RAW264.7 macrophages [22], it can be deduced that pHBSP exerts its anti-inflammation and lung-protective function following TBI presumably by inhibiting the activation of macrophages.

Conclusions

In summary, we demonstrated that pHBSP significantly inhibited pulmonary inflammation in TBI rats, mainly through reducing iNOS and proinflammatory cytokine (TNF- α , IL-6, and IL-1 β , among other cytokines) expression whereas increasing anti-

inflammatory cytokine IL-10 expression, and reducing the infiltration of macrophages in the lung. Therefore, pHBSP may be a potent candidate for the treatment of TBI patients.

Abbreviations

TBI (traumatic brain injury); ALI (acute lung injury); EPO (erythropoietin); pHBSP (pyroglutamate helix B surface peptide); iNOS (inducible nitric oxide synthase); TNF- α (tumor necrosis factor α); TGF- β (transforming growth factor β); IL-1 β (interleukin 1 β); EAN (experimental autoimmune neuritis); PE (pulmonary edema); BALF (bronchoalveolar lavage fluid).

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Disclosure Statement

The authors report no conflicts of interest.

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