Alterations of Pulmonary Zinc Homeostasis and Cytokine Production Following Traumatic Brain Injury in Rats

Lin Zhu,1 Wei Yan,1 Meng Qi,1 Ze Lan Hu,2 Ting Jia Lu,2 Min Chen,2 Jin Zhou,2 Chun Hua Hang,1 and Ji Xin Shi1

1Department of Neurosurgery, Jinling Hospital, Nanjing University School of Medicine, Nanjing, Jiangsu Province, China; 2Institute of Neuroscience of Shanghai, Chinese Academy of Science, Shanghai, China

Abstract. Previous studies have shown that labile zinc and inflammatory mediators participate in many pathophysiological processes. The present study investigated the effects of traumatic brain injury (TBI) on the levels of labile zinc and certain proinflammatory factors in rat lung. Male Wistar rats were randomly assigned to 7 groups as follows: normal group, group with sham operation, and TBI groups that were sacrificed respectively at 1, 6, 24, and 72 hr, and on day 7 post-injury. Pulmonary labile zinc, tumor necrosis factor alpha (TNF-α), interleukin (IL)-8, and wet/dry weight ratio were measured at the specified time intervals. TBI caused a gradual increase of pulmonary labile zinc as demonstrated by fluorescence staining with Zinpyr-4 (ZP4). The levels of TNF-α and IL-8 and the lung wet/dry weight ratios were higher in the TBI groups compared to the normal and sham-operated groups (p <0.05). There were highly positive correlations between the intensity of ZP4 fluorescence and the pulmonary levels of TNF-α and IL-8. The results suggest that TBI induces rapid increases of labile zinc and inflammatory mediators in lung, which may participate in the pathogenesis of acute lung injury.

Keywords: brain trauma, lung, zinc, cytokines, TNF-α, IL-8

Introduction

Acute lung injury (ALI) is commonly seen in comatose victims with isolated traumatic brain injury (TBI). The results of some animal studies have shown a relation between TBI and ALI in the absence of underlying cardiac and pulmonary disease [1,2]. This pathologic course may not only influence the lung epithelium itself, but also impair brain oxygenation and enhance the neurogenic injury. A previous study demonstrated pulmonary inflammation in organ donors after fatal brain injury [3]. However, the underlying mechanism for the ALI induced by TBI remains unclear.

Zinc (Zn) is the second most abundant trace element in the human body. It participates in a wide variety of physiological and pathological processes and has important functions as an immunoregulatory agent and growth cofactor [4]. Zn has particular importance in maintaining homeostasis of epithelial tissues that are at the front line of defense, such as pulmonary alveoli [5]. Bao and Knoell [6] reported that zinc acts as a cytoprotector of lung epithelium during inflammatory stress and that cellular depletion of Zn enhances susceptibility to apoptosis. On the other hand, exposure to high concentrations of zinc can cause acute lung injury in humans [7]. It is evident that zinc plays multiple roles and may be involved in the pathogenesis of ALI after TBI. Whether and how TBI may alter pulmonary zinc homeostasis and inflammation have not been elucidated to date.
Using various fluorescent Zn indicators, previous studies have concentrated mainly on the histological changes of the brain that are associated with ischemia, seizures, and trauma [8-10]. In the present study, we evaluate the temporal pattern of pulmonary labile Zn levels and proinflammatory cytokine expression following TBI.

Materials and Methods

Animals. All procedures in animals were approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Male Wistar rats (220 to 250 g) were obtained from the Animal Center of the Chinese Academy of Sciences, Shanghai, China. The rats were housed at 23±1°C in humidity-controlled animal quarters with 12-hr light/dark cycle. Body weight was monitored as an index of nutritional status. Rats had free access to drinking water and food throughout the study.

Experiment protocol. The rats were randomly allocated to 7 groups: (a) sham operation without cortical contusion trauma; (b) normal rats without any operation; and (c, d, e, f, and g) TBI groups, decapitated respectively at 1, 6, 24, 72 hr, or 7 days post-injury (n = 10 rats/group).

After a rat was anesthetized with sodium pentobarbital (50 mg/kg, ip), the head was fixed in a stereotactic device and the rat was allowed to breathe spontaneously. A contusion injury of the right parietal cortex was produced using a modification of the Feeney model [11]. The scalp and temporalis muscles were reflected and a burr hole (3 mm diameter) was drilled through the skull 3.0 mm lateral to the midline and 4.0 mm caudal to the bregma. Traumatic brain injury (TBI) was produced by allowing a steel rod (weight 40 g) with a flat end (diameter 4 mm) to fall from a height of 25 cm onto a piston that rested on the dura. The piston was allowed to compress brain tissue for a maximum of 5 mm.

At timed intervals post-injury, the rats were killed by an overdose of anesthesia and were exsanguinated by cardiac puncture. The lungs were immediately excised and the wet/dry wt ratio was determined. Specimens of lung tissue were frozen in liquid nitrogen prior to determinations of cytokine levels (TNF-α and IL-8) and assessment of labile zinc.

Zinpyr-4 (ZP4) staining and counting of ZP α-positive cells. ZP4 fluorescence was assessed using 10 µm sections, as previously described [13,14]. Zinpyr-4 was freshly diluted in PBS to a final concentration of 25 µM, immediately pipetted onto tissue sections, and incubated in the dark for 60 sec at room temperature. After the sections were briefly rinsed in PBS, images were collected with a compound fluorescence microscope (Zeiss Universal; excitation wave-length, 420 to 490 nm; dichroic beam splitter, 500 nm; barrier, 550 nm long-pass). The images were captured by a CCD camera (Retiga 1300R) and quantified with a PC work-station. Five high-power (x200) fields in each section were evaluated and the mean number of cells that showed ZP4-positive fluorescence in the 5 fields was used as the index of labile zinc in the sample.

Enzyme-linked immunosorbent assay (ELISA). Pulmonary levels of inflammatory mediators were quantified using specific ELISA kits for rats according to the manufacturers’ instructions (TNF-α kit from Diacclone Research, France; IL-8 kit from Biosource Europe SA, Nivelles, Belgium) and a previous study [15]. Values were expressed as pg/mg protein.

Statistical analysis. All data are presented as mean ± SD. One-way ANOVA with Tukey’s post test was performed using Graphpad Prism (version 4.0) for Windows, (Graphpad Software, San Diego California, CA, USA). Statistical significance was assumed for p values <0.05.

Results

The wet/dry weight ratio, which represents the percentage of tissue water, is an index of tissue microvascular permeability. TBI significantly increased the wet/dry weight ratio in the lungs at 24 hr post-injury (Fig. 1). Compared to the normal group or the sham-operation group, the lung wet/dry weight ratio began to increase at 6 hr, was highest at 24 hr (p <0.05), and remained slightly elevated at 7 days post-injury.

Lung wet/dry weight ratio. The lung wet/dry weight ratio was measured as previously described [12]. Briefly, immediately after the lung tissue samples were taken, excess fluid was blotted from specimens and wet weights were measured. Dry weights were measured after the specimens were dried to constant weight at 80°C for 72 hr.

Zinpyr-4 (ZP4) staining and counting of ZP α-positive cells. ZP4 fluorescence was assessed using 10 µm sections, as previously described [13,14]. Zinpyr-4 was freshly diluted in PBS to a final concentration of 25 µM, immediately pipetted onto tissue sections, and incubated in the dark for 60 sec at room temperature. After the sections were briefly rinsed in PBS, images were collected with a compound fluorescence microscope (Zeiss Universal; excitation wave-length, 420 to 490 nm; dichroic beam splitter, 500 nm; barrier, 550 nm long-pass). The images were captured by a CCD camera (Retiga 1300R) and quantified with a PC work-station. Five high-power (x200) fields in each section were evaluated and the mean number of cells that showed ZP4-positive fluorescence in the 5 fields was used as the index of labile zinc in the sample.
TBI increased the abundance and brightness of pulmonary ZP4 fluorescent spots. In the normal group, labile zinc could be detected faintly, showing a fuzzy pattern by ZP4 staining (Fig. 2A). The number and intensity of fluorescent signals in the normal and the sham-operation group were similar. ZP4 fluorescence intensity in the lung started to increase at 6 hr after TBI. The intensity of ZP4 fluorescence was generally maximal at 72 hr after TBI (Fig. 2B) and remained elevated for 7 days post-injury. The fluorescence was concentrated mainly in the alveolar epithelium and interstitial spaces, which agrees with a previous study [14]. Quantification of the ZP4-labeled cells showed that TBI significantly increased pulmonary labile Zn accumulation, with the maximum increase at 72 hr post-injury (p <0.001) (Fig. 3).

TBI increased TNF-α and IL-8 levels in lung. The mean TNF-α level was almost the same in the sham-operated and normal groups of rats (Fig. 4A). Compared to the normal group, the pulmonary TNF-α level was induced at 6 hr after TBI (4.39 ± 1.55 ng/mg protein), was maximal at 24 hr post-injury (11.19 ± 2.16 ng/mg protein), remained elevated at 72 hr post-injury (9.99 ± 1.79 ng/mg protein), and declined at 7 days post-injury (5.94 ± 2.54 ng/mg protein), but were still higher than the levels of the control group. There was positive correlation between the abundance of ZP4 fluorescence and pulmonary TNF-α concentrations (r = 0.82, p < 0.05).

There was no significant difference in the mean IL-8 concentrations of lung tissue from the normal group of rats compared to the sham-operated group (Fig. 4B). After TBI, IL-8 concentrations in the lung tissue were increased from 1 hr to 7 days, compared to the control group. IL-8 concentration was maximal at 24 hr post-injury. The pulmonary IL-8 levels after TBI were significantly correlated with the abundance of ZP4 fluorescence (r = 0.78, p < 0.05).
Discussion

The most important finding of this study is that TBI can induce significant up-regulation of tissue microvascular permeability, labile zinc, TNF-α, and IL-8 in the lung. Labile zinc was significantly increased from 24 hr to 7 days post-injury, with the maximum at 72 hr. Moreover, pulmonary capillary permeability, TNF-α, and IL-8 were increased at 6 hr, highest at 24 hr, and remained elevated on day 7 post-injury. There was positive correlation between the expression of labile zinc and the pulmonary levels of proinflammatory cytokines, TNF-α and IL-8, suggesting that labile zinc may be a marker for ALI induced by TBI.

We used ZP4, a second-generation member of the Zinpyr family of Zn-selective sensors. ZP4 is more specific and sensitive for detecting the free or loosely bound (labile) pools of intracellular Zn than the quinoline-based reagents such as TSQ, Zinquin, or TFLZn, which have been used for staining ionic Zn in biological samples [16-18]. Using the Zn fluorophore Zinquin, Truong-Tran and Ruffin et al [14] detected a low content of labile Zn in cryostat sections of alveolar epithelium of normal sheep and pigs [14]. Our results in the normal and sham-operated groups of rats are consistent with their observations.

There have been no previous studies of Zn redistribution in pulmonary alveoli after TBI. To date, the pathological role of increased chelatable zinc has been mainly examined in the central nervous system and pancreas in relation to various acute or chronic diseases [19,23,24]. For example, Zn in pancreatic β-cells, involved in the formation of insoluble insulin hexamer in secretory granules [20], is co-secreted with insulin after stimulation with secretagogues [21]. Excessive secretion of zinc has been linked to the death of the same cells in a model of type 1 diabetes [22,23]. Similar phenomena may occur in certain central neurons [24]. Chelatable zinc in the brain is involved in modulating synaptic activity at excitatory and inhibitory synapses and is believed to be a key factor leading to cell death during episodes of excessive Zn release. Increased lung epithelium death, in vitro and in vivo, after treatment with Zn has been reported previously, although these findings were inconclusive [7,25].

In this experiment, we demonstrated for the first time that TBI can induce a rapid and persistent increase of labile zinc in the lung, suggesting that increased labile zinc may play an important role in the pathogenesis of ALI. However, the mechanisms underlying the activation of pulmonary labile zinc following TBI remain unclear. We speculate that the increased labile zinc might be accumulated from several sources, including Zn mobilization and redistribution from metallothioneins or mitochondria [26] and from the three types of granulocytes that contain high levels of zinc in their cationic protein [27,28].

Fig. 4. Lung cytokine levels for TNF-α (A) and IL-8 (B) in normal, sham operation, and TBI groups at specified intervals post-injury. (Mean ± SD; 5 rats/group; *p <0.01 vs normal group; **p <0.001 vs normal group.)
In general, cytokines are not stored intracellularly and their secretion depends on new protein synthesis. As a consequence, the release of cytokines in response to inflammatory stimuli, such as trauma, sepsis, or hemorrhagic shock, is predominantly regulated by transcription of cytokine genes. Yu-Mee and William et al [25] have demonstrated that zinc can induce IL-8 expression through MAPK and AP-1 activation in human airway epithelial cells [25]. MAPK and AP-1, which play central roles in regulating gene transcription involved in cell proliferation and differentiation, apoptosis, inflammation, and the immune response, are important transducers of extracellular stimuli to the nucleus. Since transcriptional regulation is critical for the production of many cytokines, zinc may play a key role in regulating cytokine-mediated inflammation. The present study shows that pulmonary labile zinc, TNF-α, and IL-8 are up-regulated as early as 6 hr following TBI and that there are positive correlations among them, suggesting that Zn may modulate the expression of these proinflammatory cytokines.

Excessive cytokine-mediated inflammation is likely to be important in the pathogenesis of various diseases, including alterations of intestinal mucosa structure, the sepsis syndrome, and the adult respiratory distress syndrome. Several studies have evaluated the role of Zn during inflammatory reactions in the lung [6,29-31]. However, the role of zinc in the production of inflammatory mediators, including TNF-α and IL-8, in ALI after TBI has not been fully delineated. Many proinflammatory cytokines have cytotoxic effects that induce apoptosis of alveolar epithelium, resulting in destruction of intercellular tight junctions and increased permeability [32,33]. TNF-α, regarded as the most important proinflammatory cytokine, is considered a major initiator of inflammation and is released early after an inflammatory stimulus [33]. IL-8 is an important proinflammatory cytokine in the human lung and is induced in human airway epithelial cells exposed to Zn [25]. Elevated levels of IL-8 are associated with several pathophysiological states in the lung, including bronchoconstriction, edema, and neutrophilia [32,33]. The present study indicates that TBI can increase microvascular permeability in the lungs. We suspect that the pulmonary inflammatory response may be mainly mediated by Zn and that proinflammatory cytokines may contribute to acute lung injury following TBI.

In summary, TBI can induce significant up-regulation of labile zinc and increased levels of inflammatory mediators in lung. Highly positive correlation exists between the pulmonary concentration of labile Zn and the proinflammatory cytokines, TNF-α and IL-8. Inflammatory responses that are mediated by up-regulation of zinc and the proinflammatory cytokines may be involved in the pathogenesis of acute lung injury following TBI.

Acknowledgements

The authors gratefully acknowledge the assistance of Professors Yu-Qiang Ding and Zhi-Qi Xiong.

References


