**Urinary Trypsin Inhibitor Attenuates Seawater-Induced Acute Lung Injury by Influencing the Activities of Nuclear Factor-κB and Its Related Inflammatory Mediators**

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**Introduction**

Acute lung injury (ALI) is a syndrome of severe dyspnea, hypoxemia, and diffuse pulmonary infiltrates leading to respiratory failure. It is caused by many disorders that induce either a direct or an indirect type of lung injury. Although various causes of ALI result in similar pathologies in the late stage, evidence indicates that the pathophysiology of early ALI may differ according to the type of primary insult [1].

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Respiratory involvement is a very common consequence of submersion accidents. Exposure of the lower respiratory tract to seawater can simulate ALI induced by seawater drowning (SWD-ALI) [2]. Seawater-related respiratory involvement can result from seawater-induced oxidant stress and tissue injury and/or from the local up-regulation of inflammatory mediators. Whatever the stimulus, neutrophil invasion often exacerbates the lung damage caused by the primary insult. The stimulated neutrophils release various molecules, such as pro-inflammatory cytokines, chemokines, reactive oxygen species, and proteases, contributing to the pulmonary inflammatory process that characterizes ALI. Among proteases, neutrophil elastase (NE), the strongest serine protease, has been shown to play a crucial role in neutrophil-mediated endothelial cell injury, thus contributing to vascular injury [3–5]. Nevertheless, few data are available on the role of NE in the course of SWD-ALI.

Increasing evidence suggests that nuclear factor-κB (NF-κB) is a central transcriptional factor in the regulation of inflammatory factors [6] and plays pivotal roles in the initiation and amplification of immune and inflammatory responses [7]. The primary form of NF-κB consists of a heterodimer of p50 and p65, which are localized in the cytoplasm and bind to inhibitory proteins of the IκB family. In response to inflammatory cytokines such as TNF-α, IκB is phosphorylated and degraded, which unMASKS the nuclear localization sequence of NF-κB sub-units, allowing translocation to the nucleus where NF-κB binds to DNA and induces target gene transcription [8].

Binding sites for the transcriptional regulatory factor NF-κB are present in the promoters of TNF-α, and activation of NF-κB is important in modulating its expression [9]. As a cytokine synthesis inhibiting factor, it was suggested [10] that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting NF-κB and the synthesis of TNF-α. In fact, a number of studies were able to demonstrate that IL-10 blocks NF-κB nuclear translocation by inhibiting IKK activity and DNA binding of NF-κB already present in the nucleus. The NF-κB signal transduction pathway is central to the pro- and anti-inflammatory actions in many clinical conditions. Until now, the activities of NF-κB in the seawater-injured lung, to our knowledge, have not been reported.

Urinary trypsin inhibitor (UTI), also referred to as ulinastatin, is a multivalent Kunitz-type serine protease inhibitor that is purified from the fresh urine of healthy men [11]. UTI decreases the elastase release from neutrophils and suppresses the activity of NE [12, 13]. It also stabilizes lysosomal membranes and suppresses the release of lysosomal enzymes [14]. Apart from blocking the protease pathway, UTI has anti-inflammatory properties in vitro [15]. Although some investigators have shown beneficial effects of UTI on several inflammatory animal models such as ischemia-reperfusion injury [16], septic shock [17], hemorrhagic shock [18], and glomerulonephritis [19] in vivo, its protective role in SWD-ALI has not been described yet.

The objective of this study was to explore the role of NF-κB and NE in ALI induced by intratracheal instillation of seawater using rabbits and to evaluate the effect of UTI on SWD-ALI.

Animals and Methods

Animal Preparation

All animal experiments were undertaken with protocols approved by the Naval General Hospital of PLA Committee on Animal Research. Our preliminary data suggested that ALI of fresh water were milder than SWD-ALI [20] and the peak of inflammation appeared between 6 and 12 h with SWD-ALI [21]. We randomly assigned 42 healthy male or female New Zealand white rabbits weighing 2.23 ± 0.22 kg (certificate No. SCXK-jing-2002-0005; Keyu animal feeding center, Beijing, China) to the groups of control (C group), seawater drowning (S group), and UTI treatment (U group). The C group included 3 time subgroups: 0, 3, and 6 h. The other two groups included 2 time subgroups: 3 and 6 h. There were 6 rabbits in each subgroup.

Experimental Protocols

The rabbit models of intratracheal instillation of seawater-induced lung injury were prepared using a modification of the method described by Xinmin et al. [2]. Seawater was prepared according to the Standard of the Third Institute of Oceanography of the State Ocean Bureau [22]. The rabbits were anesthetized with ketamine hydrochloride (50 mg/kg i.m.). Additional intramuscular ketamine hydrochloride was provided at 0.25 g/kg/h to maintain anesthesia. A PE-90 catheter was inserted into the right carotid artery to continually monitor the mean arterial pressure, obtain blood samples, and administer fluid. A Y-shape endotracheal tube was inserted through a tracheostomy. Following a 20-min stable period, an instillation tube was gently passed through the tracheal tube until it was advanced to a position approximately 1 cm above the carina. With a 10-ml syringe, 2 ml/kg body weight of seawater was instilled into both lungs within 5 min. After instillation, the rabbits breathed room air via an endotracheal tube and were maintained in the supine position during the monitoring period.

In the S group, seawater-treated animals received arterial injection of 2 ml normal saline (NS) immediately after the instillation of seawater.

In the U group, UTI + seawater-treated animals received arterial injection of UTI (50,000 U/kg) immediately after the instillation of seawater.

In the C group, animals received the same anesthesia and intubation as indicated above and were restrained in a supine position for the same duration, but neither intratracheal instillation nor arterial injection of UTI was performed.
After the treatment, the rabbits were sacrificed by exsanguinations under general anesthesia at 3 h, or the catheters were removed, the vessels ligated, and the cervical incisions closed, whereafter the rabbits returned to their cages. These animals were sacrificed 6 h after instillation in the same way.

Sample Processing

The samples of arterial blood were drawn to measure PaO₂ before (designated as 0 h) and 0.5, 1, 3, and 6 h after instillation of seawater. Two minutes before the end of each time point, arterial blood was drawn once more and centrifuged, and serum was collected to detect total protein (TP) concentrations.

The lung tissue of the sacrificed animals was processed as follows: firstly, the upper and middle right lung lobes were rinsed in NS, weighed immediately (g, wet weight), and then dried at 70°C in a Thelco Lab oven for 3 days and reweighed until the weight became constant (g, dry weight). The change in the ratio of wet weight to dry weight (W/D) was taken as an indicator of lung edema. Secondly, the right lower lung lobe was used for bronchoalveolar lavage (BAL) by instilling 10 ml of sterile saline via a cannula ligated to the bronchus. Approximately 8–9 ml of fluid was retrieved and centrifuged, and the supernatants were used for determining TP concentrations. Thirdly, the left lower lung lobe was cut into two pieces. One portion of the sample was stored at −70°C for later parameter analysis and the other was fixed in 4% paraformaldehyde in PBS for 24 h at room temperature, paraffin embedded, and processed routinely. Sections 4 µm thick were stained with hematoxylin/eosin stain (H&E) for light-microscopic investigations.

Measurement of Inflammatory Mediators

Frozen samples were homogenized in cold NS and aliquoted to measure the inflammatory markers using kits according to manufacturer’s instructions. The MPO assay kit was purchased from Nanjing Jiancheng Bioeng Institute, China. MPO activity, as an index of neutrophil sequestration in the lungs, was expressed as units per gram of protein. Immunoreactive NE, TNF-α, and IL-10 were quantitated with commercially available ELISA kits (Rapidbio Co.). The curves of NE, TNF-α, and IL-10 changes were observed for rabbits in each group, respectively.

NF-κB DNA Binding Activity

To determine the nuclear translocation of NF-κB, a nonradioactive EMSA was performed according to the manufacturer’s protocol. Briefly, nuclear extracts (5 µg) were incubated at room temperature for 20 min with 400 fmol of double-stranded NF-κB oligonucleotide containing the NF-κB binding site (5’-AGT TGA GGG GAC TTT C AGGC-3’) and double end labeled with biotin (Viogene Biotech Co., Ltd). The DNA protein complex was separated from free oligonucleotides on 6.5% native polyacrylamide gels, and the specificity of binding was analyzed by competition with unlabeled oligonucleotides. The NF-κB-positive nuclear extracts came from THP-1 cells stimulated by TNF-α. After transfer, the binding membrane was removed from Whatman papers and transferred to a UV crosslink apparatus for 10 min. Then the membrane was treated as follows. Firstly, it was blocked for 30 min and then incubated with 1:750 diluted streptavidin-HRP in blocking buffer at room temperature for 30 min. It was then equilibrated for 5 min and incubated with chemiluminescence substrate buffer for 3–5 min. Finally, the chemiluminescence imager of nonradioactive EMSA was attained using Viogene’s

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**Fig. 1.** Effects of UTI on the changes in the oxygenation index (PaO₂/FiO₂). PaO₂/FiO₂ = PaO₂/0.21. The values of PaO₂/FiO₂ were significantly decreased in the S and U groups. At 6 h after UTI treatment, PaO₂/FiO₂ was significantly upregulated. Change significance indicators to *p < 0.05, **p < 0.01 versus time-matched values in the C group; ∗p < 0.05 versus time-matched values in the S group.

CoolImager and the specific band intensities were quantified using ScionImage software.

Histopathology

Lung injury severity (LIS) was semiquantitatively scored by visual inspection of H&E-stained lung sections as previously described [21]. Lung injury was scored based on: (1) alveolar capillary congestion, (2) hemorrhage, (3) infiltration or aggregation of neutrophils in the air space or the vessel wall, (4) the thickness of the alveolar wall/hyaline membrane formation, and (5) bronchial epithelia disruption and slough. Each component was graded on a scale of 0 (minimal damage) to 3 or 4 (maximal damage). The degree of lung injury was assessed by the sum of the scores of items from 0 to 18 in 5 random high-power fields (×100 magnification) and by 2 investigators blinded to treatment assignment. The average sum of each field score was compared among groups.

Statistical Analysis

Data were presented as means ± SD for each experimental group and statistically analyzed using the following tests for multiple comparisons: two-way repeated measures analysis of variance (ANOVA) followed by Dunnett’s test for multiple observations, Student’s unpaired t test for single observations, and Wilcoxon’s U test for histological data. p < 0.05 was considered statistically significant.

**Results**

**Ratios of PaO₂/FiO₂**

Dynamic observation of arterial PaO₂/FiO₂ ratios was performed in three groups (fig. 1). The ratio of PaO₂/FiO₂ was relatively stable in rabbits of the C group. However,
the ratio of PaO₂/FiO₂ dropped sharply to 203.76 mm of mercury 0.5 h following aspiration and remained below 300 mm of mercury until 6 h on average in the S groups. The ratios of PaO₂/FiO₂ were significantly lower in the rabbits of the U and S groups compared to rabbits of the C group (p < 0.05 or 0.01). There was no significant difference in the ratios of PaO₂/FiO₂ between the U and S groups 0.5–3 h after instillation. At 6 h, the values of PaO₂/FiO₂ were significantly higher in the U groups than in the S groups (p < 0.01).

Pulmonary Endothelial Permeability

We measured the W/D ratio and lung permeability index (LPI = BALF TP/serum TP) to assess extravascular lung water and albumin leakage into the pulmonary interstitium (fig. 2). Seawater treatment significantly increased the W/D ratio and LPI compared with the C groups (p < 0.01). The values of W/D and LPI were higher in the U groups than in the C groups but lower than in the S groups (p < 0.05).

SWD-ALI Increased the Activities or Contents of NE, MPO, TNF-α, and IL-10, Which Could Be Alleviated by UTI Treatment, except for IL-10

The activities or contents of NE, MPO, TNF-α, and IL-10 were significantly upregulated in rabbits of the S group compared to rabbits of the C group (p < 0.05 or <0.01) (fig. 3, 4). UTI treatment significantly decreased the values of NE, MPO, and TNF-α and increased the value of IL-10 in the rabbits with SWD-ALI.

Inhibition of NF-κB DNA Binding Activity by UTI

The activity of increased translocated NF-κB was evaluated by κB DNA motif binding assay for the NF-κB in the isolated nuclear fraction. As illustrated in figure 5a, a shifted band of NF-κB DNA complex was evidently observed in nuclear extracts from lung tissues 3 and 6 h after seawater instillation (lane 2–3); this was barely or not visible in the rabbits of the C and U groups (lanes 1, 4–5). The assay of cold competition was performed to confirm that the signal was specific for the putative NF-κB DNA complex with positive controls. Quantization of activated NF-κB DNA complexes by densitometry analysis (fig. 5b) confirmed the significantly higher amount in the rabbits of S groups (3 h: 107.82 ± 9.16; 6 h: 125.34 ± 7.7; p < 0.01 vs. C and U) compared to the other groups (C: 42.65 ± 10.1; U group at 3 h: 47.94 ± 2.74; U group at 6 h: 32.22 ± 7.21; fig. 5b).

Comparison of Histopathology

The lungs of rabbits in the S and U groups were not collapsed like those of rabbits in the C group.
Fig. 3. Effects of UTI on MPO (a) and NE (b). The values of MPO and NE were higher in U groups than in C groups but lower in U groups than in S groups. S3h = S group at 3 h; S6h = S group at 6 h; U3h = U group at 3 h; U6h = U group at 6 h. * p < 0.05; ** p < 0.01 versus time-matched values in the S group.

Fig. 4. Effects of UTI on TNF-α (a) and IL-10 (b) of lung tissue. The values of TNF-α and IL-10 were increased by seawater. UTI treatment downregulated the level of TNF-α and upregulated the level of IL-10. S3h = S group at 3 h; S6h = S group at 6 h; U3h = U group at 3 h; U6h = U group at 6 h. ** p < 0.01 versus time-matched values in the S group.

Fig. 5. Shifted band and amount of NF-κB DNA complexes in different groups. a Shifted band of NF-κB DNA complexes. Lane 1: 0 h or C group, lane 2: S group at 3 h, lane 3: S group at 6 h, lane 4: U group at 3 h, lane 5: U group at 6 h, lane 6: cold competition assay, lane 7: positive control. b Amount of NF-κB DNA complexes. The activity of NF-κB in S groups was significantly higher than in C and U groups. S3h = S group at 3 h; S6h = S group at 6 h; U3h = U group at 3 h; U6h = U group at 6 h. ** p < 0.01 versus 0 h or the C group, ∆ p < 0.01 versus the S group at matched time.
of rabbits were heavy, boggy, and water logged in the S groups and they were significantly bigger than those in the U group. Light microscopy analyses of representative sections showed an increment in alveolar septal thickening, tissue cellularity, alveolar collapse, edema, and other signs 3 h after the induction of the lesion. The extent of lung cellularity and other pathological changes increased in a time-dependent manner in the S group during our experiment (data not shown). In the U groups, these changes were less pronounced (fig. 6).

Semiquantitative lung injury scores (LIS) were comparably and significantly increased in the U and S groups at 3 and 6 h compared with the C groups (p < 0.01) (fig. 3). Histological injury scores were significantly lower in the U group compared to the S groups (p < 0.01). LIS had a similar varying trend with NF-κB activity, neutrophil infiltration, and inflammatory mediators.

Discussion

Drowning is the process of experiencing respiratory impairment from submersion/immersion in liquid [23]. According to the World Health Organization (WHO), drowning represents a major cause of accidental deaths, with the majority of drowning victims being younger than 20 years of age [24]. For a correlation analysis of the morphological alterations and inflammatory pathways involved in this process, the experimental model of spontaneous breath was used for the ALI study, which allowed investigation of the pure lung effects of SWD-ALI. Our previous data strongly suggest that the lung injury induced by seawater drowning is more severe [20]. The animals suffered from a longer duration of low PaO2/FiO2 (less than 300 mm of mercury). Seawater instillation also yielded rapidly more pronounced inflammatory responses, which induced morphological changes. ALI comprises neutrophilic inflammation, interstitial edema, and alveolar hemorrhage. This enhancement of ALI is concomitant with the elevated lung injury parameters, including...
the W/D ratio, LPI, and LIS, and the increased expression of inflammatory mediators at levels of proteins and NF-κB DNA binding activity in lung tissue.

It has been reported that lung tissue inflammation in ALI/ARDS is characterized by early sequestration of neutrophils in the pulmonary microvasculature, followed by adhesion and migration [25–27]. A number of mediators, including lipid mediators, cytokines, free radicals, and proteases, especially the products from neutrophils, contribute to the severe dysfunction of the lungs and to the injury of resident epithelial and endothelial cells. Current studies have also shown a critical role of activated neutrophils during the onset and progression of SWD-ALI. Our preliminary data revealed that seawater caused rapidly notable neutrophil infiltration and elevation of MPO and NE in lung tissue, which were maximized 6 h after reperfusion. The intracellular content of MPO was constant in neutrophils, the MPO concentration correlated with the neutrophil count [28], and the elevated tissue MPO content can be regarded as a marker of neutrophil accumulation in the injured lung [29]. NE can exert the most injurious effects on many kinds of substrates (elastin, type I–IV collagen, fibronectin, laminin, and proteoglycans) among the proteases produced by neutrophils, and it is a critical mediator of tissue injury for ALI [30]. UTI is recognized to be degenerated from pre-α-/inter-α-trypsin inhibitors induced by NE during inflammation [31]. As one of the physiological trypsin inhibitors, UTI reportedly inhibits inflammatory proteases, such as trypsin, chymotrypsin, NE, plasmin, and so on [32]. Based on the multivalent nature of protease inhibition, UTI appears to prevent organ injury by inhibiting the activity of these proteases [33, 34]. Our data showed that administration of UTI significantly suppressed the excessive production of NE caused by seawater. Simultaneously, the lung injury was decreased since the treated rabbits showed significantly lower W/D ratios, LPI, and LIS compared to untreated rabbits. The histopathological findings revealed an apparent difference between U group animals and S group ones. The extent of lung cellularity, alveolar capillary congestion, and other pathological signs was observably ameliorated by UTI. The decline of MPO activity was consistent with the lessening of neutrophil accumulation in the injured lung. As a result, the lung functions of gas exchange of UTI-treated rabbits were better than those in hosts without treatment. At 6 h after administration, UTI attenuated the severity of hypoxia.

NF-κB activation is an integral part of the pathological response that leads to the development of organ dysfunction/injury. Studies using an LPS model of septic shock have consistently demonstrated that blocking the NF-κB pathway improves the outcome of septic shock. Survival rates could be improved from 0–20% (LPS alone) to 80–100% (LPS plus NF-κB inhibitors) [7]. As an NF-κB-dependent proinflammatory cytokine, TNF-α participates in the acute-phase response to the injury by promoting endothelial cell permeability, enhancing neutrophil recruitment to the area of injury, and inducing further cytokine secretion. Positive feedback between NF-κB and TNF-α may also enhance the inflammatory response. IL-10 is an endogenous immunomodulatory and anti-inflammatory cytokine. For many years, IL-10 has been known to inhibit proinflammatory cytokine production [35], improve bacteria-induced lung injury [36], and induce survival in animals following endotoxemia [37]. In our study, the elevation of NF-κB activity in lung tissues obtained from rabbits suffering from seawater instillation as compared with control subjects indicated a dysregulated activation of this transcriptional system in ALI induced by seawater. The elevation of this nuclear factor was consistent with the severity of lung injury. Accordingly, the concentration of TNF-α was also upregulated significantly. These data are consistent with MPO and NE as described above. IL-10 experienced a change as did TNF-α. However, the predominance of the inflammatory response results in an imbalance between the two sets of counter mechanisms, leading to the development of SWD-ALI.

Yamaguchi et al. [38] reported that UTI did not affect NF-κB activation in rat liver ischemia/reperfusion since it did not prevent degradation of phosphorylated IkBa. On the contrary, the inhibiting effect of UTI on NF-κB activation was significant in the study. The indistinct difference indicated that downregulation of NF-κB activation by UTI may have other pathways [39, 40]. Some molecules were verified to be able to protect mice from lethal endotoxemia [37] or to improve survival in severe sepsis patients [41] by inhibiting NF-κB activation (e.g. one of the anti-inflammatory cytokines, IL-10) [42]. The nuclear translocation of the classic NF-κB p65/p50 heterodimer can be blocked by IL-10 [10]. As observed in the study, the significant promotion of IL-10 by UTI could be a contributing factor leading to the inhibition of NF-κB activation. Another mechanism of the inhibition of NF-κB activation may be through downregulation of TNF-α expression and weakening of the positive feedback mechanism of NF-κB activation.
Conclusion

Seawater instillation can trigger a cascade of responses, including a primary insult to the lung parenchyma and an acute inflammatory response accompanied by an increase in anti-inflammatory levels. The ideal therapeutic agent of ALI treatment should downregulate an overactive innate immune response and preserve or upregulate adaptive immunity. The best strategy is to dampen NF-κB activity to reduce proinflammatory cytokine production and release with adequate antibiotherapy to compensate for the impaired adaptive immunity functions [43]. UTI ameliorates SWD-ALI, at least in part, via upregulation of IL-10 by inhibiting the activity of NF-κB, decreasing the accumulation of neutrophils, and suppressing the production of TNF-α, aside from its antiprotease activity. These results provide direct molecular evidence for the ‘rescue’ therapeutic utility of UTI against SWD-ALI.

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References


