Tanshinone IIA Attenuates Seawater Aspiration-Induced Lung Injury by Inhibiting Macrophage Migration Inhibitory Factor

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Inflammation takes responsibility for the seawater aspiration-induced lung injury. Tanshinone IIA (TIIA) can protect lipopolysaccharide-induced lung injury in mice through the inhibition of inflammation, but it is not reported whether TIIA have a protective effect on lung injury induced by seawater aspiration. Macrophage migration inhibitory factor (MIF) plays an important role in acute lung injury. In this study, we observed the effect of TIIA on the seawater aspiration-induced lung injury and the role of MIF in it. Seawater was aspirated into trachea of rats to make the lung injury model. TIIA was administered to investigate its beneficial effect on seawater-induced acute lung injury. The results showed that seawater aspiration led to hypoxemia, pulmonary edema, neutrophil infiltration, and lung histopathologic changes, with the elevated MIF expression in the lung tissues and plasma. However, these changes were attenuated by TIIA. In macrophage cells we also demonstrated that TIIA could inhibit MIF expression, nuclear factor κ B (NF- κ B) activity and release of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) induced by seawater. Besides, pretreatment with (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1), the MIF antagonist, elevated NF- κ B and cytokines induced by seawater were also reduced markedly. Furthermore, rMIF treatment alone increased the phosphorylation level of NF- κ B and release of cytokines, which was almost abolished by TIIA. Taken together, our results suggested that TIIA exert a protective effect on the seawater aspiration-induced lung injury partly through downregulation of MIF and the subsequent NF- κ B activity, as well as expression of IL-6 and TNF- α .

Key words seawater aspiration; lung injury; tanshinone IIA; macrophage migration inhibitory factor; nuclear factor κ B; cytokine

In drowning victims, acute respiratory failure and hypoxia are very common,¹⁾ and hypoxia-induced organ damage due to pulmonary edema caused by aspiration of water has been recognized as a major cause of death in near-drowning victims.^{2–4)} But the exact mechanisms remain unclear. Previous clinical studies have shown that white blood cell and neutrophils apparently increased in most acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) drowning patients' plasma with bilateral diffuse or localized alveolar infiltrates on chest X-ray obtained shortly after arrival.⁵⁾ In seawater aspiration-induced ALI rabbits model, the infiltration of inflammatory cells are increased in alveolar spaces with evidence of increasing of myeloperoxidase (MPO) activity and tumor necrosis factor- α (TNF- α) concentration in lung tissue.⁶⁾ These results may provide a potential mechanism that inflammation could partly explain the lung injury following seawater aspiration.

Danshen, a traditional medical ingredient herbal drug deriving from dried roots of *Salvia miltiorrhiza* BUNGE, has been widely used in the treatment of acute or chronic diseases as circulatory, cerebrovascular disorders.⁷⁾ Tanshinone IIA (TIIA), one of the major active components of Danshen, has been reported to protect against lipopolysaccharide (LPS)-induced lung injury in mice.⁸⁾ It is also shown that TIIA has an anti-inflammatory effect through the inhibition of nuclear factor- κ B (NF- κ B) activity⁹⁾ and the expression of inflammatory mediators such as nitric oxide, TNF- α , interleukin-1 (IL-1) and interleukin-6 (IL-6) in macrophage cells.¹⁰⁾ Whether TIIA plays a protective effect on lung injury following seawater aspiration is unknown.

Macrophage migration inhibitory factor (MIF) is recognized as a proinflammatory cytokine which derived from many cell types including macrophages and T lymphocytes.¹¹⁾ It is reported that serum MIF levels are significantly increased in ALI patients or animal model compared to healthy controls, as revealed MIF is a putative biomarker in ALI.¹²⁾ Additionally, after activating NF- κ B, MIF can induce the production of subsequent cytokines.¹³⁾ The selective inhibition of inflammatory cytokine activities may remain an important goal for the effective treatment of acute lung injury.

In the present study, we reported that TIIA treatment significantly attenuated seawater aspiration-induced lung injury. It was probably associated with downregulation of MIF and the subsequent inhibition of NF- κ B activity as well as expression of IL-6 and TNF- α .

MATERIALS AND METHODS

Chemicals Tanshinone IIA (sulfonate, purity is 99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICBP, Beijing, China). The kits for determination of myeloperoxidase activity were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Anti-phospho-NF- κ B p65 and anti- β -actin monoclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Anti-MIF monoclonal antibody was gifted from Dr. Chuan-Min Hu, the Third Military Medical University, Chong Qing, China. Enzyme-linked immunosorbent assay (ELISA) kits of MIF, TNF- α and IL-6 were purchased from R&D Systems (Min-

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neapolis, MN, U.S.A.). Recombinant mouse MIF was purchased from R&D Systems (Minneapolis, MN, U.S.A.). (*S,R*)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) was obtained from Calbiochem (San Diego, CA, U.S.A.).

Model and Grouping Male Sprague-Dawley (SD) rats, which weighed 250–300 g, were obtained from the animal center (Fourth Military Medical University, Xi'an, P.R. China), and all animal experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

The seawater aspiration model was prepared according to the previous report.⁶⁾ The rats were anesthetized with 20% urethane (4 ml/kg) and maintained in the supine position during experiments. A “Y” shape endotracheal tube was inserted through a tracheostomy. Within 3 min 4 ml/kg body weight of seawater was aspirated into trachea. Seawater (osmolality 1300 mmol/l, pH 8.2, specific weight 1.05, NaCl 26.518 g/l, MgSO₄ 3.305 g/l, MgCl₂ 2.447 g/l, CaCl₂ 1.141 g/l, KCl 0.725 g/l, NaHCO₃ 0.202 g/l, NaBr 0.083 g/l) was prepared according to the major composition of the East China Sea provided by Chinese Ocean Bureau. In the control group normal saline was given intraperitoneally in the same manner. TIHA (10 mg/kg) was administered intraperitoneally 30 min after seawater aspiration. In another group TIHA was given intraperitoneally without seawater aspiration. The animals were killed at 4 h after seawater aspiration.

PaO₂ Study In the separated groups, a PE-50 catheter was inserted in the right carotid artery to obtain blood samples. Arterial blood gas analysis was performed at 0 min, 1 h, 2 h, 4 h, 6 h and 8 h after seawater aspiration respectively.

Lung Wet/Dry Weight Ratio To quantify the magnitude of pulmonary edema, we evaluated the wet to dry weight ratios at different time points (0 min, 1 h, 4 h, 8 h) after seawater aspiration. Tissue samples were weighed immediately after removal (wet weight) and then subjected to desiccation in an oven at 80 °C until a stable dry weight was achieved after 72 h. The ratio of the wet/dry weight was then calculated.

MPO Assay The accumulation of neutrophils in the lung tissue was assessed by measuring the MPO activity according to the manufacturer's instructions. Briefly, the lung tissue samples obtained at different time points (0 min, 1 h, 4 h, 8 h) after seawater aspiration were homogenized in cool normal saline (lung tissue to normal saline 1 : 10). MPO activity was measured with a spectrophotometer at 460 nm.

Histological Study After the lungs were removed aseptically 4 h after seawater aspiration, the lung tissues were fixed in 4% paraformaldehyde for at least 72 h. The tissues were embedded in paraffin and cut into 5 μ m sections. Hematoxylin-eosin stains were performed using standard protocol.

Western Blot Assay The lung tissues and cultured cells were prepared to extract proteins with lysis buffer (50 mmol/l NaCl, 50 mmol/l NaF, 50 mmol/l sodium pyrophosphate, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 5 mmol/l ethyleneglycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mmol/l Na₃VO₄, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 10 mmol/l *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.4, along with 0.1% Triton X-100, 10 mg/l leupeptin, and 5% apro-

tinin). After protein quantitation using a bicinchoninic acid (BCA) assay, 50 μ g protein was boiled in loading buffer, electrophoretically separated using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to nitrocellulose membranes, and probed with anti-MIF primary monoclonal antibody (1 μ g/ml), polyclonal antibody against phospho-NF- κ B p65 (1 : 1000) overnight at 4 °C. The secondary antibody was incubated and the relative content of target proteins was detected by chemiluminescence.

Cytokine ELISA Blood was centrifuged (2500 *g*, 4 °C), and the plasma was collected. Lung tissue homogenate was also prepared. Levels of MIF, IL-6 and TNF- α were determined by using commercially available ELISA kits according to the manufacturer's instructions.

Effect of TIHA on MIF Expression Induced by Seawater in Cells We used rat alveolar macrophage cell line NR8383 in the following cells experiments. Cells were seeded into flat-bottom 24-wells plates and were then stimulated with 25% seawater (seawater: Ham's F12 medium = 1 : 4) in the presence or absence of TIHA (0–20 μ g/ml) for 4 h. Supernatant was collected and used for determination of MIF expression by ELISA.

Effect of TIHA on MIF and Cytokines Expression Induced by Seawater at Different Time Cells were administered with 25% seawater, and some cells were in combination with TIHA. The expression of MIF, IL-6 and TNF- α in the cells supernatant was determined by ELISA at 0 h, 1 h, 4 h and 8 h.

Effect of TIHA on NF- κ B and Cytokines Expression Induced by Seawater We investigated the effect of TIHA on NF- κ B and cytokines expression in cells incubated with seawater, as well as the role of MIF in it. Cells were stimulated with 25% seawater in the presence or absence of TIHA (20 μ g/ml) for 4 h. In inhibitor treatment group, cells were pretreated with MIF inhibitor ISO-1 (50 μ M) for 30 min before seawater treatment. Supernatant was collected to test MIF, IL-6 and TNF- α expression by ELISA. Then cell protein was extracted to determine the level of phospho-NF- κ B p65 by Western blot.

Effect of TIHA on NF- κ B and Cytokines Expression Induced by MIF We also examined whether TIHA alone could inhibit MIF-induced NF- κ B and cytokines expression. Cells were incubated with rMIF (250 ng/ml) in the presence or absence of TIHA (20 μ g/ml) for 4 h. IL-6 and TNF- α in supernatants were determined by ELISA. The level of phospho-NF- κ B p65 in cells was tested by Western blot.

Statistical Analysis Values were expressed as mean $\pm$ S.D. Statistical analysis was carried out using one-way ANOVA, followed by LSD test for *post hoc* multiple comparisons. A significant difference was accepted at $p < 0.05$.

RESULTS

PaO₂ According to our previous report,⁸⁾ we used 10 mg/kg TIHA in the animal experiments. As Fig. 1 shown, seawater aspiration caused a markedly decrease in the PaO₂, but it was partly improved by TIHA treatment.

Effect of TIHA on Lung Edema To observe the lung edema, we measured the lung wet/dry weight ratio at 0, 1, 4

Fig. 1. Effect of Tanshinone IIA Treatment on Arterial Oxygen Tension

Seawater aspiration caused a markedly decrease in the PaO_2 , which could be partly increased by TIIA treatment. $n=6$. ** $p<0.01$ vs. time-match value in the control group, # $p<0.05$, ## $p<0.01$ vs. time-match value in the seawater group.

Fig. 2. Effect of TIIA on the Lung Edema of Rats Following Seawater Aspiration

At each time point after seawater administration, treatment of TIIA decreased lung wet/dry ratios markedly. $n=6$. ** $p<0.01$ vs. control group; # $p<0.05$ vs. seawater group; ## $p<0.01$ vs. seawater group.

and 8 h after seawater aspiration (Fig. 2). Wet/dry weight ratio was markedly increased in the seawater aspirated rats ($n=6$, $p<0.01$). Treatment with TIIA markedly decreased the lung tissue edema formation at 4 h point.

Effect of TIIA on Neutrophil Infiltration in the Lung Tissues Next, we further performed MPO activity assay which indicated the neutrophil influx in the lung (Fig. 3). MPO activity was significantly increased in the seawater-aspirated rats compared with that of control ($n=6$, $p<0.01$). The increased MPO activity was reduced by treatment with TIIA following seawater aspiration at different time point ($p<0.01$).

Histology Histological analysis was carried out to evaluate the protective effect of TIIA on the seawater aspiration-induced lung injury at different time point after seawater aspiration (data not shown). As the data showed that the best protective effect of TIIA on the seawater aspiration-induced lung injury was at 4 h after seawater aspiration. There were normal lung tissue structure and clean pulmonary alveoli in

Fig. 3. Effect of TIIA on MPO Activity in Rats Following Seawater Aspiration

MPO activity at each time point reflects the neutrophil infiltration in the lung. Data are mean $\pm$ S.D., $n=6$. ** $p<0.01$ vs. control group; # $p<0.05$ vs. seawater group; ## $p<0.01$ vs. seawater group.

Fig. 4. Microscopic Findings of Lung Tissues Stained with Hematoxylin-Eosin

(A) Control group; (B) seawater group: hemorrhage, edema, thickened alveolar septum, formation of hyaline membranes and the infiltration of inflammatory cells in alveolar spaces were observed in lung tissue of seawater-aspirated rats; (C) seawater + TIIA group: lung injury was improved by TIIA treatment; (D) TIIA group: no difference compared with control group.

the control and TIIA group (Figs. 4A,D). Seawater aspiration caused acute alveolar damage and inflammation which was represented by thickening of alveolar septum, distortion of normal lung architecture, the appearance and sequestration of inflammatory cells, and hemorrhagic congestion in the terminal bronchus and alveolar spaces (Fig. 4B). TIIA treatment significantly attenuated lung injury induced by seawater (Fig. 4C).

MIF Expression in Rats As Fig. 5A shown, in the plasma of seawater-aspirated rats, MIF levels were higher than those of normal rats ($n=6$, $p<0.01$). Western blot results also showed that seawater treatment caused an obvious

increase of MIF in lung tissues (Fig. 5B), and there were no significant differences between control and TIHA groups.

Effects of TIHA on MIF Expression Induced by Seawater We further investigated the mechanism of protective effect of TIHA on lung injury induced by seawater *in vitro*. As shown in Fig. 6, seawater increased the MIF expression in cells ($n=5$, $p<0.01$), while administration of TIHA attenuated MIF expression concentration-dependently ($p<0.01$).

Fig. 5. MIF Expression in Rats Following Seawater Aspiration

(A) MIF level was elevated in the plasma of rats subjected to seawater determined by ELISA. (B) Western blot assay of MIF protein in lung tissue following seawater aspiration. The ratios between MIF protein and β -actin of 6 independent experiments were obtained by density scanning of the film using software. $n=6$. ** $p<0.01$ vs. control group; ## $p<0.01$ vs. seawater group.

Effect of TIHA on MIF and Cytokines Expression Induced by Seawater at Different Time We also examine the effect of TIHA on the expression of MIF, IL-6 and TNF- α induced by the seawater at different time. As shown in Fig. 7, after treated with seawater, MIF content in the supernatant was significantly increased at 1 h with maximal at 4 h and then decreased ($p<0.01$), and TIHA markedly inhibited the expression of MIF at 4 h and 8 h following seawater treatment ($p<0.01$). Meanwhile, there was no difference of IL-6 expression induced by seawater between 0 h and 1 h. The elevation of IL-6 level was observed at 4 h and found to be decreased at 8 h ($p<0.01$). The change of TNF- α expression was similar to IL-6 level. The increased expression of IL-6 and TNF- α at 4 h and 8 h following seawater treatment were also downregulated by TIHA treatment ($p<0.01$).

Effect of TIHA on NF- κ B and Cytokines Expression Induced by Seawater Next, we investigated the effect of TIHA on expression of MIF, activated NF- κ B and cytokines after seawater treatment. The results showed that MIF expression was elevated as well as activated phospho-NF- κ B p65 and induced IL-6 and TNF- α after incubated with seawater ($n=6$, $p<0.01$) (Fig. 8). Pretreated with ISO-1, upregulated phospho-NF- κ B p65, IL-6 and TNF- α by seawater were reduced significantly ($n=6$, $p<0.01$), while MIF expression was not affected. Consistent with ISO-1, TIHA also attenuated MIF, IL-6 and TNF- α levels, as well as the phos-

Fig. 6. Effect of TIHA on MIF Expression Induced by Seawater in Cells

Seawater increased the MIF expression, which were attenuated by TIHA concentration-dependently. $n=5$. ** $p<0.01$ vs. control group. ## $p<0.01$ vs. seawater group.

Fig. 7. Effect of TIHA on Cytokines Expression Induced by Seawater at Different Time

Expression of MIF (A), IL-6 (B) and TNF- α (C) in the cell supernatants were tested by ELISA. $n=6$. ** $p<0.01$ vs. control group. ## $p<0.01$ vs. seawater group.

Fig. 8. Effect of TIHA on MIF, NF- κ B and Cytokines Expression Induced by Seawater

Levels of MIF (A), IL-6 (C) and TNF- α (D) in the cell supernatants were tested by ELISA. Phospho-NF- κ B p65 was examined by Western blot (B). ISO-1 (50 μ M) was pretreated 30 min before seawater treatment. $n=6$. ** $p<0.01$ vs. control group. ## $p<0.01$ vs. seawater group.

Fig. 9. Effect of TIHA on MIF-Induced Cytokines Expression

Phospho-NF- κ B p65 was tested by Western blot (A). Concentrations of IL-6 (B) and TNF- α (C) in the cell supernatants were determined by ELISA. $n=6$. ** $p<0.01$ vs. control group. ## $p<0.01$ vs. rMIF.

pho-NF- κ B p65 expression following seawater treatment. TIHA alone has no effect on cytokines and NF- κ B expression. It was suggested that TIHA could inhibit the expression of MIF, NF- κ B activity and inflammatory mediators. Meanwhile, the upregulation of NF- κ B activity and levels of IL-6 and TNF- α induced by seawater treatment was mediated by MIF.

Effect of TIHA on NF- κ B and Cytokines Expression Induced by MIF We finally examined whether TIHA pre-

vented cytokines expression through inhibition of MIF directly and subsequent activated phospho-NF- κ B p65 (Fig. 9). Administration of rMIF increased the levels of phospho-NF- κ B p65, IL-6 and TNF- α ($n=6$, $p<0.01$), while TIHA significantly inhibited the expression of phospho-NF- κ B p65 and cytokines induced by rMIF ($n=6$, $p<0.01$). This data indicated that TIHA inhibit the NF- κ B activity and release of IL-6 and TNF- α by downregulation of MIF.

DISCUSSION

In the present study, we found that TIHA treatment improved seawater aspiration-induced hypoxia and lung injury. Our finding also suggested that MIF inhibition play an important role in attenuation of seawater aspiration induced-lung injury by TIHA.

In the seawater drowning rabbit model, the level of MPO and TNF- α is significantly increased with the massive inflammatory cells infiltration and serious alveolar edema in lung tissue.⁶⁾ In the current study, we reported the similar finding that pulmonary edema reflected by wet-to-dry ratios and neutrophil cells infiltration determined by MPO assay were increased following seawater aspiration. It was reported that TIHA exerted a protective effect on LPS-induced lung injury *in vivo*.¹⁴⁾ TIHA also played an anti-inflammatory role by inhibition of NO synthesis and inflammatory cytokine (IL-1 β , IL-6 and TNF- α) expression in macrophages.¹⁵⁾ These results suggest that anti-inflammation effect of TIHA contribute to the tissue protection. In our study, administration of TIHA markedly attenuated seawater aspiration-induced lung edema, neutrophil infiltration and histopathologic change. It was indicated that TIHA may play a protective effect on the lung injury induced by seawater aspiration.

MIF, as a critical proinflammatory cytokine, contributes to the lung injury. It is reported that MIF has been identified enhanced expression in alveolar capillary endothelium in lung tissues from ARDS patients with infiltrating macrophages.¹⁶⁾ Additionally, treatment with anti-MIF antibody effectively reduces the LPS-induced acute lung injury.¹⁷⁾ In bleomycin-induced lung injury in rats, treatment with the anti-MIF antibody significantly improves the mortality and histological changes, with the reduction of inflammatory cells and TNF- α in the alveolar space.¹⁸⁾ In the current study, we found that seawater aspiration caused obvious increase of MIF expression in blood and lung tissues of rats, which was significantly prevented by TIHA treatment. We further investigated the mechanism of protective effect of TIHA on lung injury induced by seawater. *In vitro*, TIHA significantly attenuated MIF expression induced by seawater concentration-dependently. The results suggested that TIHA could protect the seawater aspiration-induced lung injury by negative modulation of MIF.

It is known that inflammatory mediators contribute to the lung injury following seawater aspiration. In the freshwater aspiration canis, the NF- κ B activity and TNF- α expression were increased.¹⁹⁾ After activation of NF- κ B, the production of interleukin (IL)-8, TNF- α and interleukin (IL)-1 β were also upregulated in macrophage cells.²⁰⁾ Our study also confirmed that NF- κ B activity, IL-6 and TNF- α expression were elevated by seawater treatment *in vitro*. Furthermore, several studies reported that MIF regulated the inflammatory re-

sponse through modulation of NF- κ B. In thrombin-treated endothelial cells induced MIF secretion can upregulate NF- κ B activation.²¹⁾ Compared with wild-type cells, MIF-deficient macrophages are hyporesponsive to lipopolysaccharide and Gram-negative bacteria through the reduction NF- κ B activity.²²⁾ By antagonizing hydrocortisone MIF results in increased nuclear NF- κ B DNA binding in LPS-stimulated human peripheral blood mononuclear cells.²³⁾ Moreover, MIF can elevate the neutrophil accumulation by upregulation of IL-6 and TNF- α .²⁴⁾ Therefore, we investigated whether MIF could stimulate NF- κ B and induce IL-6 and TNF- α expression in seawater induced injury. In the present study, we found that seawater exposure induced the elevation of MIF expression as well as phospho-NF- κ B p65, IL-6 and TNF- α *in vitro*. The increased MIF expression induced by seawater seemed earlier than IL-6 and TNF- α . After treatment with MIF antagonist ISO-1, elevated phospho-NF- κ B p65, IL-6 and TNF- α levels induced by seawater were reduced markedly. It was suggested that the upregulation of NF- κ B activity and release of TNF- α and IL-6 induced by seawater should be mediated by MIF. We also found that TIIA inhibited the expression of MIF and activation of NF- κ B, as well as IL-6 and TNF- α release following seawater treatment *in vitro*, indicating that both seawater and MIF could activate NF- κ B, but TIIA inhibited the NF- κ B activity and expression of inflammatory mediators. Lastly, we demonstrated whether TIIA exerted a downregulation of inflammatory mediators through inhibition of MIF directly. *In vitro*, administration of rMIF increased the NF- κ B activity and release of IL-6 and TNF- α , which was almost abolished by TIIA. The results indicated that MIF play a central role in TIIA-mediated the inhibition of inflammatory reaction induced by seawater.

CONCLUSION

Our study demonstrated that TIIA exerted a protective effect on the lung injury following seawater aspiration, partly through downregulation of MIF and the subsequent inhibition of NF- κ B activity as well as inflammatory mediators. These observations also suggested that application of TIIA may be a potential effective treatment for seawater drowning.

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