

Original

Effect of Rhubarb on Heat Shock Protein 70 Expression in Lung Tissues from Rats with Acute Lung Injury Induced by Lipopolysaccharide

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Abstract:We investigated the expression of HSP70 in acute lung injury (ALI) induced by endotoxin (lipopolysaccharide: LPS) and the protective mechanisms of rhubarb against ALI. LPS was injected into rats via the internal jugular vein to induce ALI and rhubarb extract was given by intraperitoneal injection. The rats were divided into 4 groups: saline control group; LPS-induced ALI group; ALI treated with prophylactic rhubarb group; and ALI treated with therapeutic rhubarb group. The rats were sacrificed 2 hours after injection of LPS or saline. Physiological and pathological examinations were conducted, including arterial gas analysis, lung coefficient, hematoxylin-eosin staining, and HSP70 expression (immunohistochemistry and Western blotting). In the LPS-induced ALI group, interstitial edema, neutrophil infiltration, plasma exudation in alveoli, and damaged vascular endothelium were observed. HSP70 levels were slightly increased in LPS-treated rats, while HSP70 expression was enhanced significantly and overall lung function was improved in rhubarb-treated groups compared to ALI model rats. These data confirm that rhubarb protects rats from LPS-induced ALI and upregulates HSP70 expression. Thus, the protective effect of rhubarb may be mediated by the HSP70 signaling pathway.

Key words: Acute lung injury, Lipopolysaccharide, Rhubarb, HSP70.

Introduction

Rhubarb is an important traditional Chinese herbal medicine widely used in traditional Chinese medicine for thousands of years, and has recently been developed for the treatment of acute and emergency diseases¹⁾. Studies on the functions of rhubarb in modern medical research both in clinical and basic science settings have revealed that rhubarb has multiple effects including defervescence, antibacterial properties, anti-inflammatory actions, and especially expelling a variety of harmful materials such as endogenous as well as exogenous toxins from the bowel and the body.

Although rhubarb has been applied in traditional Chinese medicine for a long time, the mechanisms of action remain poorly understood. In recent investigations, a model of experimental acute lung injury induced by lipopolysaccharide (LPS) has been established to explore the functions of rhubarb, especially the signal transduction pathways potentially involved.

It is known that rhubarb protects against acute lung injury

induced by LPS, and that rhubarb administration improves respiratory function of the body. Its effect is apparently related to the regulation of the production of nitric oxide as well as phospholipase A₂ and platelet-activating factor activities^{2,3)}.

Heat shock protein 70 (HSP70), the predominant member of a specific family of proteins induced by hyperthermia, is also induced by other perturbing stressors such as hypoxia, hyperosmolarity, glucose deprivation and infection. It has been suggested that HSPs act as molecular chaperones and enhance the viability of cells exposed to environmental perturbations. Therefore, HSP70 can be considered a marker protein in the control and regulation of body environmental perturbation. In this study we examined HSP70 expression in lung tissue of rats with acute lung injury induced by LPS and explored the effect of rhubarb treatment on HSP70 expression in acute lung injury.

Materials and Methods

Experimental animals

Healthy Wistar rats (200-250 g in weight, supplied by the Experimental Animal Center, China Medical University) were divided into 4 groups (15 rats per group) for the experiments described below. All rats in the experiments were anesthetized by

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Table 1. Arterial gas analysis and lung coefficient in various experimental groups

Group	No. of animals	PO ₂ (Kpa)	Lung coefficient
A Saline control	15	16.289±0.501	0.533±0.023
B LPS-induced ALI	15	13.486±0.467*	0.800±0.079*
C Prophylactic rhubarb	15	17.843±0.793 [#]	0.527±0.042 [#]
D Therapeutic rhubarb	15	16.400±0.464	0.633±0.032‡

*: $P < 0.05$ compared with saline-treated rats, #: $P < 0.05$ compared with ALI rats, ‡: $P < 0.05$ compared with prophylactic rhubarb group.

intraperitoneal injection of 20% urethane at 6 ml/kg body weight, and subjected to injection of LPS or saline via the internal jugular vein. Group A (control group) received an injection of 5 ml of saline per kg body weight via the internal jugular vein. Group B group (LPS-induced ALI group) received an injection of LPS (L2880, Sigma USA) at 5 mg/kg body weight via the internal jugular vein. Group C (prophylactic rhubarb group) received an intraperitoneal injection of rhubarb extract (1 ml of extract contains 1 g of original rhubarb, supplied by Laoning Chinese Medical Research Institute) at 10 g/kg body weight, followed 1 hour later by an injection of LPS as above. Group D (therapeutic rhubarb group) received an injection of LPS followed immediately by an intraperitoneal injection of rhubarb extract.

All samples were collected at 2 hours after LPS injection. Blood was collected from the abdominal artery for blood gas analysis. Bilateral lung tissues were sampled for determination of wet and dry weights as well as for immunohistochemistry and Western blotting.

Arterial oxygen analysis and the lung coefficient measurement

All artery blood samples were subjected to blood oxygen analysis using the 238PH/Blood Gas Analyzer (Corning, USA). The lung coefficient of each rat was calculated by the following formula: lung wet weight (g) per body weight (g) × 100.

HE histological staining and immunohistochemistry for HSP70

Lung tissue samples collected from the same location of each rat were fixed in 4% paraformaldehyde. Then the sections were processed using a standard immunohistochemical protocol. Sections were 5 μm in thickness and picked up onto APES-coated slides. Hematoxylin and eosin (HE) staining was performed according to the routine protocol of the pathological laboratory in the Hospital. HSP70 immunostaining was carried out according to the manufacturer’s instructions (Wuhan Boshengde Biological Engineering Company, China). Ten high-power fields (×400) were randomly selected on each slide and the number of HSP70-positive cells and total number of cells in each field were counted, and the HSP70-positive cell percentage was calculated for analysis.

Western Blotting

Protein samples were extracted from homogenized lung tissue

specimens, then separated by SDS-PAGE and transferred onto PVDF membrane for HSP70 probing. Bands corresponding to HSP70 were scanned and the densities of these bands were analyzed with Motic Advanced 3.1 software.

Statistical Analysis

Data are presented as means ± SD and statistical analysis was performed by one-way analysis of variance. The significance of differences between two groups was evaluated by *t* test. The ANOVA procedure was carried out for multiple comparison. *P* values less than 0.05 were considered statistical significant.

Results

Artery gas analysis and lung coefficient measurement

Analysis of arterial gas showed that the PO₂ was significantly lowered in the LPS-induced ALI group compared with the control group. On the other hand, the lung coefficient increased significantly in the ALI group, suggesting lung function was damaged due to LPS treatment. However, in the prophylactic rhubarb group, PO₂ was not affected despite LPS treatment and lung coefficient also was not significantly changed. Thus, administration of rhubarb prophylactically appeared to protect the animals against lung damage caused by LPS. In the therapeutic rhubarb group, administration of rhubarb therapeutically did improve lung damage induced by LPS, but the efficiency was not as good as prophylactic use of rhubarb (Table 1).

Lung histopathological examination

In the lung tissue of LPS-induced ALI, prominent histopathological changes were observed. Spot-like necrosis could be seen with naked eyes and the whole lung appeared hyperemic. In contrast, in both the prophylactic and therapeutic rhubarb groups, only a few necrotic spots could be observed and the lung surface was only very slightly hyperemic. The differences from the ALI group were especially marked in the prophylactic group. HE staining of lung tissue sections from the LPS-induced ALI group revealed interstitial edema, thickening of lung marking, diffuse alveolar hemorrhage and sprinkle bronchial hemorrhage, as well as significant cellular infiltration (erythrocytes and neutrophils). In contrast, in the prophylactic and therapeutic

Table 2. Percentage of HSP70-positive cells in immunostaining and quantitative analysis of HSP70 protein expression by Western blotting

Group	No. of animals	HSP70-positive cells (%)	HSP70 band (Western blot)
A. Saline control	15	0.555±0.142	169.00±7.85
B LPS-induced ALI	15	5.900±1.137*	182.50±5.68*
C. Prophylactic rhubarb	15	22.460±1.868#	214.67±7.20#
D. Therapeutic rhubarb	15	11.283±0.939‡	183.67±5.82‡

*: $P < 0.05$ compared with saline-treated rats, #: $P < 0.05$ compared with ALI rats, ‡: $P < 0.05$ compared with prophylactic rhubarb group.

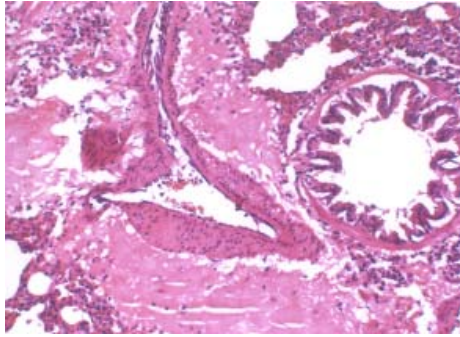


Fig. 1. A representative HE stained section of rat lung tissue in the prophylactic rhubarb group ($\times 400$).

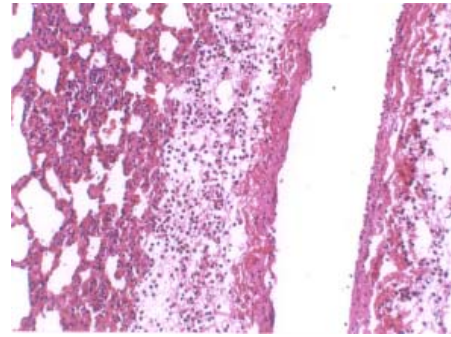


Fig. 2. A representative HE stained section of rat lung tissue in the therapeutic rhubarb group ($\times 400$).

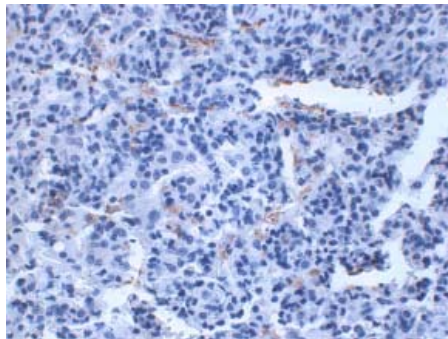


Fig. 3. Immunohistochemical staining for heat shock protein 70 in lung tissue section of a rat in the LPS-induced ALI group ($\times 400$).

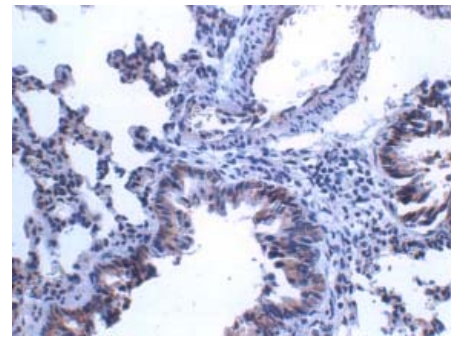


Fig. 4. Immunohistochemical staining for heat shock protein 70 in lung tissue section of a rat in the prophylactic rhubarb group ($\times 400$).

rhubarb groups, little exudation, decreased infiltration of neutrophils and erythrocytes, and thinner lung markings were observed compared with the LPS-induced ALI group (Fig. 1). In the therapeutic rhubarb group, however, interstitial vascular exudation was present and peripheral inflammatory cell infiltration also was observed (Fig. 2).

Immunohistochemistry of HSP70

In order to investigate the HSP70 distribution patterns in normal lung tissue and LPS-induced ALI, HSP70-specific immunostaining was applied to these specimens. In the control rat lung tissue, weak immunoreactivity for HSP70 was observed in the bronchial and bronchiolar epithelial cells as well as alveolar cells, while macrophages showed slight and sprinkle positive staining, but the pulmonary interstitial cells and vascular endothelium were

almost negative for HSP 70 antibody. In comparison with normal controls, the HSP70-specific immunoreactions were stronger in LPS-treated rat lung tissue (Fig. 3). The bronchial and bronchiolar epithelia were stained deeply. Neutrophils and macrophages were sprinkle stained. The HSP70-positive cells in alveolar epithelium increased slightly. The percentage of positive cells was higher than the normal control group with a significant statistical difference (Table 2). In the prophylactic rhubarb group, bronchial and bronchiolar epithelial cells as well as vascular endothelial cells showed strongly positive staining, some were stained a dark brown color. The surrounding smooth muscle cells expressed HSP70 slightly (Fig. 4). The number of positive alveolar epithelial cells, mononuclear phagocytes, lymphocytes and neutrophils increased markedly. The percentage of positive cells increased to as high as 22.5% (Table 2). In the therapeutic rhubarb group, the

bronchial and bronchiolar epithelial cells as well as vascular endothelial cells were positively stained while sprinkled positive lymphocytes and neutrophils were observed, but the percentage of positive cells was only 11.3% (Table 2).

Western Blotting

HSP70 expression in all four groups was evaluated by Western blotting. The amount of 70-kDa protein from every sample was quantitatively analyzed and the data are summarized in Table 2. HSP70 expression was increased in the LPS-treated ALI group, prophylactic rhubarb group and therapeutic rhubarb group compared to control rats, with the highest expression observed in the prophylactic rhubarb group. HSP70 level in the therapeutic rhubarb group was not different from that observed in the LPS-induced ALI group. The higher HSP70 expression in the prophylactic rhubarb group compared with the therapeutic rhubarb group suggests that the timing of drug administration is the key point.

Discussion

In our ALI model, the Wistar rats presented typical ALI symptoms 2 hours after jugular injection of LPS. Several investigations have indicated that LPS directly causes damage of alveoli epithelial cells and vascular endothelial cells, thereby inducing the infiltration of blood cells such as neutrophils and cellular proteins into the alveolar cavity. The lung injury finally causes rapid increases in the number of neutrophils and amount of total protein, as well as the ratio of wet weight to dry weight of the lung. All these histological changes are in accord with ALI characteristics^{4,5}. On the other hand, LPS able promotes activation and expression of various biological activators, thereby enhancing lung injury and worsening the clinical situation.

Heat shock proteins (HSP family) are highly conserved stress-response proteins that play a central role in cellular repair and adaptation to stress. Among them, HSP70 is an important member of stress-induced proteins, and its expression is upregulated when cell or body is placed under stressful conditions⁶. The physiological functions of HSP70 have been widely studied. It has been shown that under conditions of cellular stress, overexpression of HSP70 can protect cells from apoptosis induced by c-Jun N-terminal kinase (JNK) activity, because HSP72 interacts with components of the JNK signaling pathway, thereby suppressing JNK-induced cell death⁷. On the other hand HSP is also able to inhibit cellular apoptosis through protection of the cell skeleton.

Koh et al²) reported that in rats exposed to mild heat for 18 hours prior to intravenous injection of LPS, HSP was induced and the HSP overexpression subsequently prevented lung injury, suppressed lung damage, and diminished the death rate. The investigation of Wischemyer et al⁸) indicated that pretreatment of human peripheral blood mononuclear cells with glutamine

enhanced HSP72 production and attenuated proinflammatory cytokine (such as TNF- α) release after LPS treatment of the cell culture.

In the transplant experiment of Hiratsuka et al⁹), the mean arterial oxygenation 24 hours after reperfusion was superior in donor lungs that underwent adenovirus-mediated HSP70 gene transfer compared with control groups, while wet to dry weight ratio and myeloperoxidase activity were significantly lower in the transfected group than in the control. These results indicate that HSP70 overexpression induced by transfection of adenovirus-HSP70 plasmid decreases subsequent ischemia-reperfusion injury in rat lung isografts. Increasing evidences have demonstrated that HSP70 functions as a biological indicator and activator in early lung injury.

In the present study, we demonstrated that rhubarb has the ability to induce HSP70 overexpression and subsequently protects ALI induced by LPS in rats. In the group given rhubarb prophylactically, HSP70 expression levels increased compared with ALI control in both Western blotting and immunohistochemical studies. Meanwhile, regarding the pathophysiological indicators, the prophylactic group showed a higher arterial PO₂ and lower lung coefficient, as well as minimal pathological changes in lung tissues. In the group given rhubarb therapeutically, although the physiological status was somewhat improved in comparison with the ALI group (such as an increase in arterial PO₂ and a decrease in lung coefficient), pathological examination and measurement of HSP70 expression demonstrate lower efficacy in this group than in the prophylactic group.

In recent years, evidences from our laboratory and other investigators have revealed that rhubarb confers protection against LPS-induced ALI in rats. The administration of rhubarb decreases pulmonary tissue fluid leakage and inflammatory cell infiltration, and markedly reduces vascular permeability, thereby improving pulmonary respiratory dynamics. Nevertheless, little is known concerning the mechanism of actions of rhubarb in ALI. Li et al³) reported that rhubarb suppressed the ALI-induced upregulation of nitric oxide generation and inducible nitric oxide synthase activity, thus providing protection and minimizing lung damage of ALI.

Our present data indicate that rhubarb confers protection against LPS-induced ALI in rats. Functionally, rhubarb decreases pulmonary vascular and alveolar permeability, thereby reducing fluid leaking and inflammatory cell infiltration and improving lung respiratory dynamics. While the mechanism of rhubarb-mediated protection against ALI in rat is poorly understood, one investigator³) has implied that the mechanism may involve the nitric oxidation signaling pathway because rhubarb treatment nitric oxide generation and inducible nitric oxide synthase activity, thus diminishing the damage in ALI induced by LPS.

Our work suggests that the heat shock protein family, especially HSP70, is related to rhubarb-mediated actions, as

rhubarb increased HSP70 levels in bronchial and bronchiolar epithelial cells and vascular endothelial cells when administered both prophylactically and therapeutically. It should be emphasized that only in the prophylactic group did rhubarb-induced HSP70 upregulation provide protection against ALI; whereas in the therapeutic group, although HSP70 expression appeared to increase although not significantly, lung improvement was not observed. These results suggest that HSP70 inhibits the early steps in LPS-induced lung injury. This result is very valuable in determining the appropriate timing of rhubarb administration.

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