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Original Article

TLR4 signaling is activated in ventilator-induced diaphragm dysfunction in rats

Pei Liu[1], Hongmei Zhang[2], Ke Hu[1], Hongmei Zheng[3]

[1]*Division of Respiratory Disease, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, P. R. C.;* [2]*Department of Respiratory Disease, Taihe Hospital, Hubei University of Medicine, Hubei, P. R. C.;* [3]*Department of Skill Training Center, Taihe Hospital, Hubei University of Medicine, Hubei, P. R. C.*

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Abstract: Inflammation is involved in ventilator-induced diaphragm dysfunction. Toll-like receptor 4 (TLR4) is an important inflammatory factor, but it remains unclear whether TLR4 contributes to ventilator-induced diaphragm dysfunction. This study aimed to investigate the role of TLR4 signaling in ventilator-induced diaphragm dysfunction. Total 30 adult male SD rats were randomly divided into control group, low tidal volume and high tidal volume group (n = 10). Control group received tracheotomy and endotracheal intubation but no mechanical ventilation; low tidal volume group and high tidal volume group received tracheotomy, mechanical ventilation after intubation, and then received tidal volume 6 ml/kg and 20 ml/kg, respectively. Ventilation rate was 60 beats/min, inspiratory to expiratory ratio was 1:3, FiO_2 was 21%, ventilation was 24 h. Diaphragmatic muscle contraction, tumor necrosis factor (TNF-α) and TLR4 expression, and malondialdehyde (MDA) and superoxide dismutase (SOD) contents in the diaphragm tissues were detected. TLR4 and TNF-α expression in diaphragm tissues of high tidal volume group were significantly increased and diaphragm muscle contraction was significantly decreased, compared to other groups. In conclusion, high tidal volume ventilation may activate TLR4 signaling and cause pathological changes in diaphragm tissues. TLR4 is a promising target for the prevention and treatment of ventilator-associated diaphragmatic injury.

Keywords: Ventilator-induced diaphragm dysfunction, TLR4 signal, TNF-α, malondialdehyde, superoxide dismutase

Introduction

Mechanical ventilation has been increasingly used in the clinic for the treatment of various causes of respiratory failure, but improper use of mechanical ventilation will lead to ventilator-associated diaphragmatic injury [1]. Toll-like receptors are transmembrane receptor mainly expressed in innate immune cells and function as pathogen pattern recognition receptors [2]. Recent studies have shown that TLR4 plays important role in early inflammation of acute lung injury and thus is involved in acute lung injury occurrence and development [3, 4]. However, no study has reported the role of TLR4 in ventilator-associated diaphragmatic injury. Therefore, this study attempts to investigate the role of TLR4 signaling in mechanical ventilation associated diaphragmatic injury.

Materials and methods

Animals and groups

Thirty specific-pathogen-free (SPF) adult male SD rats (weight 250-300 g) were provided by the Animal Experimental Center of Hubei Medical College, and randomly divided into control group, low tidal volume group and high tidal volume group (n = 10 for each). Control group, SD rats underwent intraperitoneal anesthetizing using 10% chloral hydrate (5 ml/kg body weight), and underwent tracheotomy, intubation and the right carotid artery intubation after successful anesthesia, without mechanical ventilation. Low tidal volume group and high tidal volume group, rats underwent intraperitoneal anesthetizing using 10% chloral hydrate (5 ml/kg body weight), and underwent tracheotomy,

intubation and the right carotid artery intubation after successful anesthesia, then underwent mechanical ventilation using the animal ventilator with the tidal volume of 6 ml/kg body weight and 20 ml/kg body weight in low tidal volume group and high tidal volume group, respectively. The mechanical ventilation frequency was 60 beats/min, inspiratory to expiratory ratio was 1:3, FiO_2 was 21%, and ventilation lasted 24 h [5]. At the end of ventilation, all rats were sacrificed by air embolism and right diaphragm specimens were taken for pathological and immunohistochemically analysis, left diaphragm specimens were taken to detect diaphragmatic contractility. Animal experiments were approved by animal ethics committee at the Renmin Hospital of Wuhan University.

Pathological examination

Paraffin sections were stained with HE, pathological changes of the diaphragm was observed by a pathologist under light microscope.

Immunohistochemical analysis

Lung tissues were fixed in paraformaldehyde and paraffin embedded, and then cut into 4 μm thick serial sections. The sections were washed with phosphate buffered saline (PBS) three times and then blocked by incubation with 2% goat serum in PBS containing 0.3% Triton X-100 (PBS-X) for 1 h at room temperature. Next the sections were incubated with anti-rat TLR4 or anti-TNF-α primary antibody (Boster, Wuhan, China) at 4°C overnight, and then incubated with secondary antibody and DAB chromogen. The section was counterstained with Hematoxylin and eosin and observed under optical microscope. The staining was analyzed by Image-Pro 6.0 software.

Detection of oxidative stress

Malondialdehyde (MDA) content in the diaphragm was detected by using thiobarbituric acid (TBA) kit (Jiancheng Bioengineering Institute, Nanjing, China) and Superoxide dismutase (SOD) activity was detected by using Xanthine oxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's manuals.

Diaphragm muscle contraction force detection

After the animals were sacrificed, the left rib diaphragm was taken comprising a central tendon and ribs, and connected to 95% O_2 and 5% CO_2 gas mixture Ringer bath (pH 7.4) at 37°C. The diaphragm was cut into 5 mm × 15 mm muscle strips. Muscle strips were fixed to the bottom end of the bath, central tendon end was connected to a standardized and calibrated tension transducer, which was connected to a polygraph recorder. The muscle strips were placed between two platinum electrodes, a stimulator was used to give 0.5 ms single square wave pulse stimulation (stimulation parameters preload 2 g, time constant DC, high frequency filter 30 Hz, gain 10 g), each stimulus had an interval of 2 min. Initial intensity of the stimulus was 0.1 mA, the stimulus intensity was gradually increased with increments of 0.05 mA, when the contractile force of muscle contraction was not increased we obtained the maximum contraction force. Diaphragm muscle contraction force = maximum contraction force/muscle strip cross-sectional area, and muscle cross-sectional area = weight of the bar (g)/[muscle strip length × muscle density (1.056 g/cm^3)] [6].

Statistical analysis

Data were expressed as x ± s and analyzed by SPSS13.0 software. The differences between groups were compared by using one-way analysis of variance. $P<0.05$ was considered as significant difference.

Results

Diaphragmatic biopsy in each group

Diaphragmatic structure in the control group was normal (**Figure 1A**). In the diaphragm in low tidal volume group, mild edema and a small amount of inflammatory cell infiltration were observed (**Figure 1B**). In the diaphragm in high tidal volume group, obvious edema and some inflammatory cell infiltration were observed (**Figure 1C**).

TLR4 and TNF-α expression in the diaphragm in each group

We performed immunohistochemically staining to detect TLR4 and TNF-α expression in diaphragm tissues. The results showed that TLR4 expression was very weak in control group, strong in low tidal volume group, and very strong in high tidal volume group (**Figure 2**).

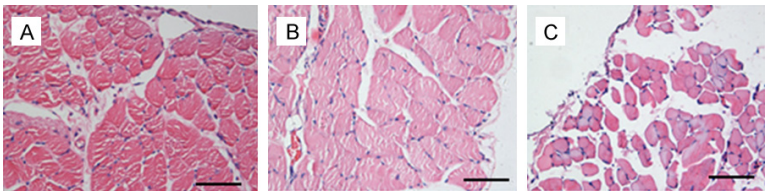

Figure 1. Histopathology of diaphragm in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Shown were representative images of HE staining. Scale bar: 100 μm.

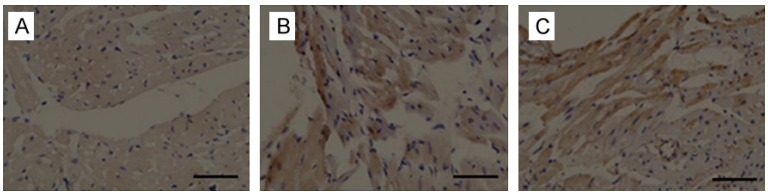

Figure 2. TLR4 expression in the diaphragm in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Scale bar: 100 μm.

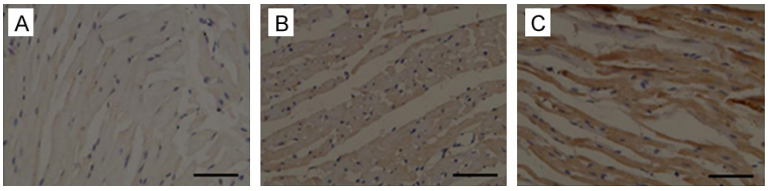

Figure 3. TNF-α expression in the diaphragm in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Scale bar: 100 μm.

Table 1. TRL4 and TNF-α expression in diaphragm in each group

Group	No.	Relative TRL4 level	Relative TNF-α level
Control	10	11.3±1.73	13.1±1.48
Low tidal volume	10	43.57±5.27*	35.7±4.69*
High tidal volume	10	165.2±18.65*,#	197.4±11.2*,#

*P<0.05 compared to control group, #P<0.05 compared to low tidal volume group.

Similarly, TNF-α expression was very weak in control group, strong in low tidal volume group, and very strong in high tidal volume group (**Figure 3**). Quantitative analysis showed that both TLR4 and TNF-α expression levels were significantly higher in low tidal volume group and high tidal volume group than in control group. Furthermore, both TLR4 and TNF-α expression levels in the diaphragm in high tidal volume group were significantly higher than in low tidal volume group control (**Table 1**). In addition, TLR4 expression level was positively correlated with TNF-α expression level, with a correlation coefficient of 0.904.

Oxidative stress parameters and diaphragmatic contractility in each group

As shown in **Table 2**, MDA content in the diaphragm in low tidal volume group and high tidal volume group was significantly higher than in control group, and MDA content was higher in high tidal volume group than in low tidal volume group. SOD content in the diaphragm and diaphragm contraction in low tidal volume group and high tidal volume group decreased significantly compared to the control group, and SOD content and diaphragm contraction were significantly lower in high tidal volume group than in low tidal volume group (**Figure 4**).

Discussion

Mechanical ventilation is an important method for the treatment of patients with severe respiratory failure [7]. However, 20% to 50% patients treated with mechanical ventilation have difficulty to get rid of ventilator, and recent data suggest that ventilator-induced diaphragm dysfunction may be an important reason [8, 9]. In this study, we showed that after mechanical ventilation diaphragmatic contractility in rats decreased significantly, especially in rats with high tidal volume ventilation. TNF-α expression in diaphragm in high tidal volume group increased significantly, and we observed diaphragm edema and inflammatory cell infiltration, which indicated that inflammatory response can play an important role in ventilator-induced diaphragm dysfunction.

Toll-like receptors are frequently expressed in the surface of innate immune cells where they recognize pathogens to activate innate immune system to produce pro-inflammatory cytokines [10]. Toll-like receptors are distributed in vari-

Table 2. Oxidative stress in diaphragm and diaphragmatic contractility in each group

Group	No.	MDA value (nmol/mgprot)	SOD activity (U/mgprot)	contractility (N/cm^2)*10^{-3}
Control	10	6.1±0.72	114.7±8.52	995.26±71.43
Low tidal volume	10	8.4±0.67*	95.3±7.34*	708.34±59.98*
High tidal volume	10	10.6±0.83*,#	54.8±8.75*,#	214.73±38.83*,#

*P<0.05 compared to control group, #P<0.05 compared to low tidal volume group.

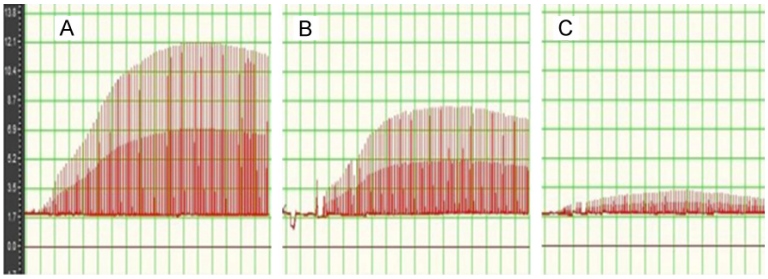

Figure 4. Diaphragmatic contractility in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group.

ous organs and play important role in recognizing different pathogens [11]. Lipopolysaccharide (LPS) from bacteria is an important structure recognized by TLR4 and it induces the activation of TLR4 signal pathway [12]. In acute lung injury caused by bacterial infection, TLR4 initiates early inflammation and contributes to the occurrence and development of acute lung injury [13].

In this study we showed that TLR4 expression in diaphragm of high tidal volume group was significantly higher than that of control group and low tidal volume group, and there was a positive correlation between TRL4 and TNF-α expression. The mechanism may be that the binding of TLR4 and ligand promotes the activation of NF-kB, which then activates the transcription of inflammatory cytokines such as TNF-α and IL-6 [14, 15]. These inflammatory cytokines may further promote the release of TLR4, thus forming a vicious cycle for progressive inflammation [16]. Furthermore, we found that MDA content increased significantly while SOD activity decreased significantly in high tidal volume group, indicating oxidative stress in diaphragm. And it was reported that active oxygen radicals can activate TLR4 signal to mediate inflammatory responses [17]. Therefore, we speculate that oxidative stress can lead to the activation of TLR4 mediated inflammation, thereby aggravating diaphragm injury. TLR4 signal may play an important role in the initiation of ventilator-induced diaphragm dysfunction and subsequent maintenance of inflammation.

In summary, we found that during high tidal volume ventilation TRL4 expression in the diaphragm was significantly increased, accompanied by pathological and biochemical changes in the diaphragm. TLR4 signal may play an important role in the initiation and subsequent maintenance of ventilator-induced diaphragm dysfunction inflammation.

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Disclosure of conflict of interest

None.

Address correspondence to: Ke Hu, Division of Respiratory Disease, Renmin Hospital of Wuhan University, 99 Zhangzhidong Road, Wuhan 430060, Hubei Province, P. R. C. E-mail: huke-rmhospital@163.com

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