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TRPA1 ion channels in vagal afferent nerves contribute to ventilator-induced lung injury in a rat model

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Abstract. In order to investigate the effects of transient receptor potential channel A1 (TRPA1)-mediated neurogenic inflammatory reaction on the process of ventilator-induced lung injury (VILI). A rat VILI model was created, and the TRPA1 selective antagonist, HC-030031, was used to investigate the role of TRPA1 in the process of VILI. 50 rats were randomly divided into five groups: vehicle group, low tidal volume group, high tidal volume group, low tidal volume group with TRPA1 inhibitor, high tidal volume group with TRPA1 inhibitor. Biochemical index of lung injury in each group were determined, including the W/D ratio, total protein, count of WBC, content of MDA, activities of MPO and SOD, content of IL-8, TNF-α and substance P. Results showed that TRPA1 inhibitor could significantly reduce the inflammatory response and generation of reactive oxygen species, improve SOD activity and inhibit the production of inflammatory factors in lung tissues. TRPA1 was expressed in vagal nerve afferents, and the TRPA1 antagonist significantly inhibited the expression of substance P, indicating the involvement of TRPA1 in neurogenic inflammation. In conclusion, TRPA1 might be involved in the pathophysiological process of VILI by inducing the neurogenic inflammation, and TRPA1 inhibitor could inhibit inflammatory response of VILI.

Key words: Ventilator-induced lung injury — TRPA1 — Acute lung injury — Neurogenic inflammation

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; IL-8, interleukin-8; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha; TRPA1, transient receptor potential channel A1; VILI, ventilator-induced lung injury.

Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS), manifesting as non-cardiogenic pulmonary edema, respiratory distress and hypoxemia, could be resulted from lung injury. Mechanical ventilation is an important lifesaving technique, applied in clinical intervention for patients with severe symptoms. However, patients with mechanical ventilation are also at the risk of ventilator-induced lung injury (VILI) (Gattinoni et al. 2005). VILI may increase various complications and can affect the treatment outcomes. The investigation on the cellular and molecular mechanisms of VILI is necessary. Various mechanisms are involved in VILI such as capillary leak, apoptosis, fibrin deposition, inflammatory cytokines, oxidative stress, disrupted angiogenesis and neutrophil infiltration (Lionetti et al. 2005; Ngiam and Kavanagh 2012). The pathogenesis of VILI partly relates to over distension and/or cyclic collapse of distal airways and alveoli. Lung neutrophilic inflammation is involved in the pathogenesis of VILI by integrating the regulation of airway smooth muscle tone into the control of ventilation (Perez Fontan 2002; Liu et al. 2012). The neuropeptide substance P manifests its biological functions through ligation of G protein-coupled receptor, such as neurokinin-1 (NK-1) which revealed a preponderance of proinflammatory properties resulting from ligand activation. It demonstrated that neurogenic component could contribute to multiple forms of inflammation and injury. The release of

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bioactive constituents including substance P from sensory neurons could result from the activation of the transient receptor potential vanilloid 1 (TRPV1) (Dib et al. 2009). The TRPV1 channel is a member of transient receptor potential (TRP) family which comprises a diverse group of cation channels that regulate a variety of intracellular signaling pathways (Ho et al. 2012).

There are 28 mammalian TRPs, divided into 6 subfamilies based on homology: canonical (TRPC1-7), vanilloid (TRPV1-6), melastatin (TRPM1-8), ankyrin (TRPA1), polycystin (TRPP1-3), and mucolipin (TRPML1-3). Recently it was reported that the TRP family participated in the process of VILI, such as TRPV4, a subtype of TRP family, initiating an acute calcium-dependent permeability incensement during VILI in isolated mouse lungs (Hamanaka et al. 2007). TRPA1, another subtype of TRP family, has been implicated as a mechanosensor (Nassenstein et al. 2008). It can be activated by a large number of inhale irritants or irritant compounds including cold air, mustard oil, acrolein, formalin, 4-hydroxynonenal and reactive oxygen species (Brierley et al. 2011). Recent studies suggested that activation of TRPA1 on the vagal sensory afferents could lead to central reflexes, including dyspnea, changes in breathing pattern, and cough (Nassenstein et al. 2008). Thus, we assume that the TRPA1 might be involved in the pathophysiology of VILI. The present study was designed to observe the effect of TRPA1-mediated neurogenic inflammatory reaction on the development of VILI.

Materials and Methods

Materials and animal experiments

HC-030031, a TRPA1 selective antagonist, was purchased from Merck (Germany). The enzyme-linked immunosorbent assay (ELISA) kits for myeloperoxidase (MPO) and substance P were purchased from JianCheng Biotechnology Company (Nanjing, China). The ELISA kits for tumor necrosis factor (TNF)-α, interleukin (IL)-8, malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from Beyotime Company (Shanghai, China). DNAse I, dNTP, TRIzol reagent, oligodT primers were purchased from Invitrogen (USA). Pentobarbital, coomassie brilliant blue, bovine serum albumin (BSA), agarose and all other chemicals were of analytical grade and purchased from Sigma (USA).

All animal experiments were implemented according to the internationally accredited guidelines with the approval of the Animal Care and Use Committee of Shandong University. Experiments were performed on 50 male Sprague-Dawley rats, 280–320 g, 8 weeks of age. The animals were housed under pathogen-free conditions (temperature of 19–21°C, relative humidity of 50–60%) and acclimated for one week prior to the experiment.

Preparation of the VILI model in rats

50 Sprague-Dawley rats were randomly divided into the following experimental groups (10 rats in each group): group A: rats were unventilated; group B: rats were ventilated with 7 ml/kg tidal volume room air for 4 hours; group C: rats were ventilated with 40 ml/ kg tidal volume room air for 4 hours; group D: rats were pre-drug 100 mg/kg of HC-030031 in 2 ml of vehicle (NS: DMSO = 95: 5, dosed *i.p.*), and then were ventilated with 7 ml/kg tidal volume room air for 4 hours; group E: rats were also pre-drug 100 mg/kg of HC-030031, and then were ventilated with 40 ml/kg tidal volume room air for 4 hours.

The rat VILI model was prepared as previously described (Ricard et al. 2001; Karzai et al. 2005). Rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg). A tracheostomy was performed and then the animal was ventilated with a rodent volume ventilator (ALC-V8A, China). Rats were placed in a supine position on a warming device. The anterior soft tissue of neck was dissected to expose the trachea and the tracheotomy was performed under sterile condition. A 3.5 mm inner diameter angio catheter was inserted into the trachea and sutured in place. The intratracheal angio catheter was connected to the ventilator. Peak pressure and respiration rate were regulated to the level when the targeted tidal volume reached 7 or 40 ml/kg. Heart rate, blood pressure and temperature were monitored continuously throughout the experiments. Then the animal was sacrificed by CO_2 asphyxiation post experiments. The thorax was opened, and blood was sampled by cardiac puncture. The samples of pulmonary tissue and lung lavage fluid were collected. The jugular/nodose ganglia which were near lungs were dissected and cleared of adhering connective tissue. The lung homogenate was obtained from each group. Total protein, wet/dry (W/D) ratios, MDA, activities of SOD and MPO, count of white blood cells (WBC) in the lung tissue or lavage fluid were measured.

Measurement of W/D ratios and count of WBC

In order to detect the inflammation level of lung induced by mechanical ventilation, the W/D ratios of lung, contents of WBC were detected in lung tissue or lavage fluid. The right lung leaf of the rats from different groups were isolated, and weighed as the wet weight, then placed in the oven, 70°C, dried to the constant weight as the dry weight, and calculated the ratio of the lung W/D. The count of WBC in the lung tissue and lavage fluid was measured by cell counting method.

Assay of total protein, MDA and SOD activities

Cytological studies indicated that ventilation could significantly activate the lung epithelial cells to generate a large number of intracellular reactive oxygen species (ROS). In

addition to direct damage effects on cells, a large number of ROS were also involved in the inflammatory response and increased the expression of a variety of pro-inflammatory cytokine (Vlahakis and Hubmayr 2005). MDA was one of the major metabolite of ROS-induced lipid peroxidation reactions, and reflected the level of oxidative stress. SOD was the strongest oxygen free radical scavenging enzymes in the lung which could protect the lung from injury. In this study, the contents of MDA and the activity of SOD in lung tissues were detected. The lung tissues obtained from each group were accurately weighed and then homogenized at 4°C. The total protein was determined with Coomassie brilliant blue method. Chemical colorimetric assay of MDA content and SOD activity was conducted with the ELISA kits.

Determination of MPO, TNF-α and IL-8

MPO, an important peroxidase enzyme, was significantly related to the release of neutrophil cells. The activity of MPO could reflect the neutrophil invasion level of lung tissue. It was reported that high tidal volume ventilation led to the biological lung injury which manifested as a lot of inflammatory factors hoarding in the lung, such as cytokine IL-1, IL-8, TNF-a and nuclear factor-κB (NF-κB) (Imanaka et al. 2001; Uhlig 2002; Contreras et al. 2012). The activity of MPO and the level of TNF-α and IL-8 in lung tissues from each group were determined. The lung homogenate obtained from each group were processed for measuring activity of MPO with the ELISA kit. Cytokine TNF-α and IL-8 concentrations in lung homogenate were also measured with commercial ELISA kits according to manufacturer instructions.

Determination of substance P in vagal nervous ganglia

It was reported that TRPA1 involved in the process of neurogenic injury (Wei et al. 2010). However, the neurogenic injury was often induced by substance P released from sensory nerves (O'Connor et al. 2004). The expression level of substance P was determined in the vagal nerve afferents. Isolated ganglia were subject to homogenize and ultrasonication, and then centrifuged for 10 min (2,000 r/min, 4°C). The supernatants were collected to determine the content of substance P using an ELISA kit.

RT-PCR and PCR

The mRNA for TRPA1 expression and β-actin were determined semi-quantitatively by RT-PCR (Table 1). Total RNA from the vagal nervous ganglia was extracted with TRIzol reagent according to the manufacturer's protocol. The content of RNA was determined by measuring the absorbance at 260 nm, and the integrity was verified by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing agarose gel.

RNA (2 μg) was reverse transcribed into cDNA using oligodT primer and the AMV reverse transcriptase (Promega Corp, USA). PCR was performed using a Gene Amp PCR System 9700 thermo cycler (PerkinElmer, USA). Cycling parameters included 40 cycles of denaturation at 94°C for 30 s, followed by annealing (60°C for 30 s) and extension (72°C for 1 min), ending with a final extension period of 72°C for 10 min. Products were then visualized in ethidium bromide-stained 1.0% agarose gels.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software package, and all the results were presented as the mean ± standard deviation (SD). Statistical significances among groups were then tested using a one-way analysis of variance (ANOVA). Differences between groups were further confirmed by Student's *t*-test and considered to be statistically significant if $p < 0.05$.

Results

Development of VILI model and effects of TRPA1 inhibitor

Table 2 showed that compared with the unventilated group (group A), the W/D rate, WBC counts and MPO activity in lung tissue were significantly increased when ventilated, especially on the high tidal volume ventilation group (group C) ($p < 0.01$). It showed that the lung injury was caused by mechanical ventilation. The lung injury on the normal ventilation group (group B) was relatively less than the high tidal volume ventilation group (group C). When pretreatment with TRPA1 inhibitor, HC-030031, the W/D rate of the hy-

Table 1. Sequence of primers used for analysis of murine TRPA1 receptor transcripts

Gene	Primer	Sequence (5'-to 3')	GenBank	Product length
β-actin	Forward	CTGGTCGTCGACAACGGCTCC	NM_007393	238 bp
	Reverse	GCCAGATCTTCTCCATG		
TRPA1	Forward	GGAGCAGACATCAACAGCAC	AY231177	393 bp
	Reverse	GCAGGGGCGACTTCTTATC		

TRPA1, transient receptor potential channel A1.

Table 2. Effects of TRPA1 inhibitor pretreatment on various indicators of VILI (n = 10)

Groups	W/D	WBC ($\times 10^4$)	Total protein (g/l)	MPO (U/g)	MDA (nmol/mg protein)	SOD (U/mg protein)
A	4.26 ± 0.57	21.40 ± 2.90	0.36 ± 0.05	0.32 ± 0.04	1.23 ± 0.21	66.84 ± 8.93
B	5.78 ± 0.53^{+}	127.60 ± 25.10*	1.89 ± 0.09*	3.68 ± 0.65*	2.45 ± 0.49*	53.78 ± 8.13
C	8.12 ± 0.95*	228.40 ± 35.70*	6.96 ± 1.07*	7.98 ± 1.03*	4.27 ± 0.63*	36.14 ± 4.03*
D	5.21 ± 0.49	125.90 ± 26.30	1.66 ± 0.08	3.41 ± 0.54	1.89 ± 0.36	56.99 ± 7.95
E	6.48 ± 0.67$^{\#}$	167.80 ± 23.70$^{\#}$	4.29 ± 0.68$^{\#}$	6.07 ± 0.73$^{\#}$	3.08 ± 0.55$^{\#}$	48.67 ± 6.14$^{\#}$

* $p < 0.01$, $^{+}p < 0.05$compared with the group A (vehicle group). $^{\#}$ $p < 0.05$, compared with the injury groups (group C) without pretreatment with TRPA1 antagonist. MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; TRPA1, transient receptor potential channel A1; VILI, ventilator induced lung injury;WBC, white blood cells; W/D, ratio of wet/dry total protein.

per-ventilation group was significantly reduced, as well as the content of WBC in lung tissues ($p < 0.05$, group E compared with group C). However, there was no significant protective effect on the low tidal volume ventilation group treated with HC-030031. Moreover, HC-030031 could reduce the release of MPO, and alleviate the VILI.

Effects of TRPA1 inhibitor on activity of MDA and SOD

Compared with the vehicle group, the content of MDA was significantly increased and the activity of SOD was reduced in the ventilation groups (Table 2). Moreover, with the increasing of the tidal volume of ventilation, this trend was more apparent. However, the HC-030031 pretreatment could inhibit the MDA response and increase the SOD activity, which can improve the balance of oxidant/antioxidant capacity in lung tissue.

Effects of TRPA1 inhibitor on the expression of cytokine TNF-α and IL-8

Figure 1 showed that the contents of TNF-α and IL-8 were significantly increased in the tidal volume ventilation groups compared with the vehicle group ($p < 0.01$). The TRPA1 inhibitor pretreatment could inhibit the response of inflammation ($p < 0.05$, group E compared with group C).

Effects of TRPA1 inhibitor on the expression of substance P in vagal nerve afferents

As shown in Figure 2, the substance P level in the tidal volume ventilation groups was significantly higher than that of the vehicle group ($p < 0.01$). It was also found that when the rats were treated with high tidal volume ventila-

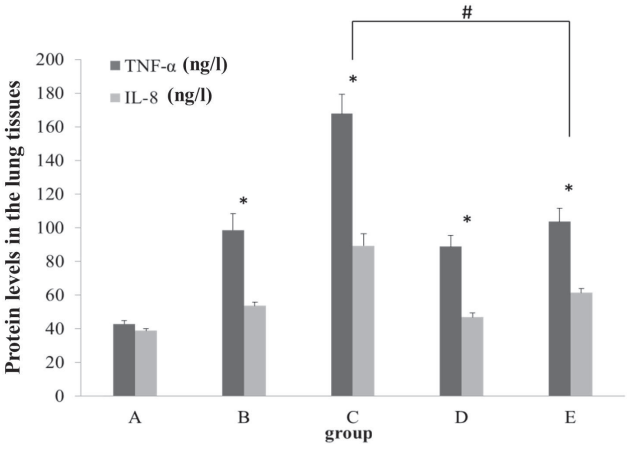

Figure 1. The content of cytokine TNF-α and IL-8 (ng/L) in the lung tissues. Group A, vehicle group; group B, low tidal volume ventilation group; group C, high tidal volume ventilation group; group D, low tidal volume ventilation+TRPA1 inhibitor; group E, high tidal volume ventilation+TRPA1 inhibitor. * $p < 0.01$, compared with the group A (vehicle group); $^{\#}$ $p < 0.05$, group C compared with group E.

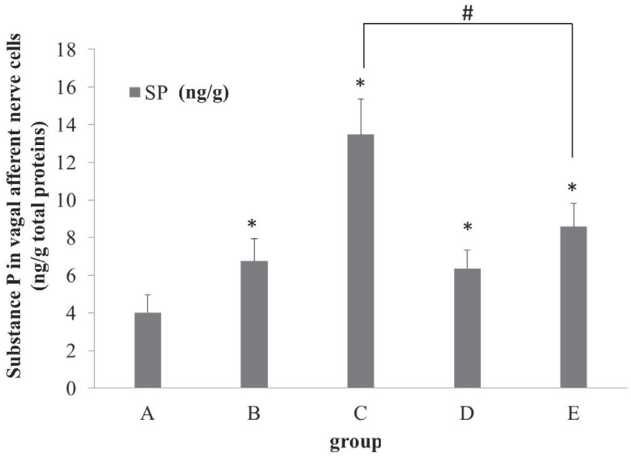

Figure 2. The content of substance P (ng/g total proteins) in vagal afferent nerves. Group A, vehicle group; group B, low tidal volume ventilation group; group C, high tidal volume ventilation group; group D, low tidal volume ventilation+TRPA1 inhibitor; group E, high tidal volume ventilation+TRPA1 inhibitor.* $p < 0.01$, compared with the group A (vehicle group); $^{\#}$ $p < 0.05$, group C compared with group E.

tion, the TRPA1 inhibitor could inhibit the expression of substance P in vagal nerve afferent ($p < 0.05$, group E compared with group C). It could be hypothesized that TRPA1 involved in the ventilator-induced lung injury through affecting the afferent vagal nerve and induced the neurogenic injury which aggravated the degree of lung injury.

TRPA1 was expressed in vagal afferent nerves

In order to address the hypothesis that TRPA1 involved in neurogenic lung injury, the determination of the expression of TRPA1 in vagal nerves was necessary. Herein, we used RT-PCR to semi-quantitatively determine the expression of mRNA of TRPA1. As shown in Figure 3, the TRPA1 was expressed in the vagal nervous ganglia, and there was no significant difference among these groups. This result suggested that volume ventilation could not influence the expression of TRPA1, and it induced the lung injury probably by affecting the activity of TRPA1.

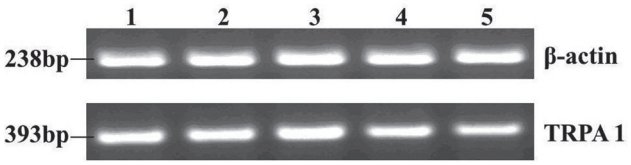

Figure 3. TRPA1 mRNA expression in vagal nerve afferents innervating the airways using RT-PCR method. Lane 1, vehicle group; lane 2, low tidal volume ventilation group; lane 3, high tidal volume ventilation group; lane 4, low tidal volume ventilation+TRPA1 inhibitor; lane 5, high tidal volume ventilation+TRPA1 inhibitor.

Discussion

Full understanding the mechanisms mediating VILI may help to permit potential strategies directed at preventing VILI and reducing the incidence of VILI-induced multiple organ failure (Shimabukuro et al. 2003; Lionetti et al. 2005). The present study investigated the effects of TRPA1-mediated neurogenic inflammatory reaction on the process of VILI. A rat VILI model was created and a TRPA1 selective antagonis was used. Biochemical index of lung injury including the W/D ratio, count of WBC, content of MDA, activities of MPO and SOD, content of IL-8, TNF-α and substance P were tested.

The TRP family was considered to be one of the most important ionotropic receptors which participate in the development of inflammation especially in neurogenic inflammation (Toth et al 2011; Andreev et al. 2012). TRPV4 has been been shown to increase the acute calcium-dependent permeability during VILI. Thus, we hypothesis that neurogenic inflammatory responses to the proinflammatory, participating in the process of VILI through the TRP channel receptors. TRPA1 is a non-selective calcium permeable cation channel, expressed on the vagal afferent nerves, and is a therapeutic target for neurogenic inflammation (Pertovaara et al. 2011). TRPA1 mainly functions as a mechanosensor, so the noxious mechanical stimulation can activate TRPA1, such as the tidal volume ventilation (Nassenstein et al. 2008).

In this study, a selective TRPA1 antagonist was used to observe the effect of TRPA1 on the pathophysiological process of VILI. Previous study has indicated that orally administered small molecule TRPA1 receptor antagonist can effectively inhibit mechanical hypersensitivity in rodent models of inflammatory and neuropathic pain (Eid et al. 2008). In the present study the dose of HC-030031 used was 100 mg/ml, and it showed good specificity and ability to block the direct activation of TRPA1 (data not shown). The TRPA1 antagonist could significantly improve the SOD activity, and reduce the generation of ROS and the inflammatory response in lung tissues. Moreover, the TRPA1 antagonist extremely inhibited the production of inflammatory factors such as IL-8 and TNF-α in lung tissues especially in the high tidal volume ventilation groups. These results indicated that TRPA1 may involve in the pathophysiological process of VILI, and TRPA1 antagonist could help to improve the inflammatory response.

Substance P has proinflammatory effects in immune and epithelial cells and participates in inflammatory diseases of the respiratory systems. It could induce neuropeptide release from sensory nerve, such as allergen, histamine, and leukotrienes, and induce the neurogenic inflammation in lung tissues (O'Connor et al. 2004). Our study indicated that the TRPA1 was expressed in vagal nerve afferents innervating the airways. The TRPA1 antagonist significantly reduced the expression of substance P, indicating that TRPA1 was involved in neurogenic inflammation. In the rat VILI model, both low and high may be able to activate the TRPA1. Activation of TRPA1 in vagal afferent nerves in the early inflammatory process of VILI would contribute to induce the neurogenic inflammation, resulting into activation of downstream inflammatory response, and finally induction of lung injury.

The present study on the effect of TRPA1 on neurogenic inflammation helps to further understand the mechanisms of VILI, moreover, the TRPA1 antagonist may be effective for the VILI treatment. This study is a valuable contribution for the development of potential therapeutic methods for VILI. However, further work such as the *ex vivo* studies is needed to understand the detailed mechanisms in the future work.

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