Acta Biochim Biophys Sin, 2017, 49(10), 907–915
doi: 10.1093/abbs/gmx085
Advance Access Publication Date: 31 August 2017
Original Article

Original Article

High-mobility group box 1 protein is involved in the protective effect of Saquinavir on ventilation-induced lung injury in mice

Xin Wang[1,2,†], Renlingzi Zhang[1,†], Yao Tong[1,†], Xibing Ding[1], Shuqing Jin[1], Xiang Zhao[1], Jiaying Zong[1,2], Zhixia Chen[1], Timothy R. Billiar[3], and Quan Li[1,*]

[1]Department of Anesthesiology, East Hospital, Tongji University School of Medicine, Shanghai 200120, China, [2]Department of Anesthesiology, The First Clinical Medical College of Nanjing Medical University, Nanjing 210029, China, and [3]Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15260, USA

[†]These authors contributed equally to this work.
*Correspondence address. Tel/Fax: +86-21-66307531; E-mail: quanligene@126.com

Received 13 February 2017; Editorial Decision 15 June 2017

Abstract

Saquinavir (SQV) is the first FDA approved HIV protease inhibitor. Previous studies showed that SQV can limit Toll-like receptor-4 (TLR4)-mediated inflammatory pathway and nuclear factor-κB (NF-κB) activation, thereby playing a protective role in many kinds of diseases. High-mobility group box 1 (HMGB1) has been identified as an inflammatory mediator and it might express its toxicity in a short period of time in ventilator-induced lung injury (VILI). In this study, C57BL/6 mice were randomly divided into four groups ($n = 10$): control group and control with SQV group (Con + SQV) were spontaneous breath. HTV group (HTV) received high tidal volume ventilation (HTV) for 4 h. HTV with SQV group (HTV + SQV) were pretreated with 5 mg/kg of SQV for 7 days before HTV. Mice were sacrificed after 4 h of HTV. Lung wet/dry weight (W/D) ratio, alveolar-capillary permeability to Evans blue albumin (EBA), cell counts, total proteins in bronchoalveolar lavage fluid (BALF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) level in BALF and lung tissue, and lung histopathology were examined. Our results showed that HTV caused significant lung injury and NF-κB activation, which was correlated with the increase of TNF-α and IL-6 levels in BALF and plasma. SQV pretreatment significantly attenuated pulmonary inflammatory injury, as well as NF-κB activation. These findings indicate that the protective effect of SQV may be associated with the inhibition of NF-κB activation and HMGB1 expression in mice.

Key words: ventilator-induced lung injury, VILI, Saquinavir, HMGB1, NF-κB

Introduction

Mechanical ventilation (MV) is a widely used life-saving therapy in the management of critically ill patients, such as acute respiratory failure. However, both clinical and experimental studies have shown that it can cause or worsen lung injury, which is recognized as ventilator-induced lung injury (VILI) [1–6]. The exact underlying mechanisms remain unclear, but mechanical stretch and inflammatory process may be the two main aspects that contribute to VILI [7–9]. MV, particularly at large tidal volume, leads to the release of multiple inflammatory cytokines, such as high-mobility group box 1 (HMGB1) protein, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β, resulting in detrimental effects on the lungs. Our previous study demonstrated a critical role of TLR4-MyD88 pathway in the development of VILI in mice, which may possibly act through

mechanisms involving the activation of nuclear factor-κB (NF-κB) and MAPK [10].

Saquinavir (SQV), the pioneer of the HIV protease inhibitors (PIs), can inhibit the biological activity of HIV protease to terminate the replication of the HIV virus. In addition to its antiviral activity, SQV has many other effects. It can be beneficial in some other diseases like cancers, inflammation and immune-mediated diseases [11–13]. Previous studies showed that SQV can inhibit NF-κB activation [14,15], thereby playing a protective role in many kinds of diseases. *In vitro* experiments by Billiar revealed that SQV can inhibit HMGB1-induced TLR4 activation [16], which represents a potential therapy for the acute and chronic inflammation.

HMGB1 is a nonhistone chromatin-associated protein [17] which is actively secreted by macrophages and monocytes, and exhibits proinflammatory activity. HMGB1 has been reported to be increased in the lung epithelial lining fluid of acute lung injury (ALI) patients [18], and it has been identified as a late mediator of endotoxin [19]. More importantly, extracellular HMGB1 can bind to TLR4, then activates NF-κB through MyD88 pathway and stimulates the expressions of inflammatory cytokines, thereby playing an important role in ALI [20,21]. On the other hand, HMGB1 protein levels were also increased in response to MV in both animal and clinical studies [22,23]. A previous study revealed that HMGB1 showed its toxicity in short time of MV in mice [22]. It might be a potent inflammatory mediator in VILI. The high expression of HMGB1 protein caused by cyclic stretch is achieved possibly through p38 MAPK and NF-κB pathways [24].

Therefore, in this study, we hypothesized that SQV pretreatment may inhibit HMGB1 expression following HTV by interfering with the NF-κB signaling pathway, and tested the protective effects of SQV in a murine VILI model.

Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji University. A total of 40 male C57BL/6 mice (weighing 20–25 g, 6–8 weeks old) were included in the experiment. They were obtained from the Laboratory Animal Research Center of Shanghai (Shanghai, China). All animals were handled in compliance with the national guide for care and use of laboratory animals. Mice were randomly assigned to four groups ($n = 10$ per group): spontaneous breath (control); spontaneous breath with SQV pretreatment (Con + SQV); high tidal volume ventilation (HTV); and high tidal volume ventilation with SQV pretreatment (HTV + SQV).

VILI animal model

In brief, animals were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and anesthesia was maintained by supplementing of ketamine and xylazine with 30 mg/kg every 35 min during the experimental period. Mice were placed in the supine position. The trachea was exposed and a 20-gauge, 1-inch-long catheter was inserted, and then connected to a small animal ventilator (Inspira ASV; Harvard Apparatus, Holliston, USA). Mice in HTV group and HTV + SQV group were ventilated at 20 ml/kg (high tidal volume) with 100 breaths/min and no PEEP (positive end expiratory pressure) for 4 h, while mice in the control and Con + SQV groups underwent tracheotomy but breathed spontaneously. Mice with MV were administrated with saline (0.01 ml/g body weight) intraperitoneally every 45 min in order to maintain intravascular volume status. Animals were sacrificed at the end of 4 h MV. Blood samples were collected and centrifuged for 15 min at 500 *g* at 4°C, and serum was collected and stored at −80°C. Lungs were harvested for bronchoalveolar lavage fluid (BALF) preparation, wet/dry weight (W/D) ratios determination, histopathological examination, immunohistochemical analysis, and lung tissue homogenate preparation. The levels of TNF-α, IL-6 and HMGB1 in the serum or BALF were measured by ELISA.

SQV treatment

Animals in the Con + SQV group and HTV + SQV group were treated every 24 h by intraperitoneal injection of a mixture of 5 mg/kg SQV (provided by Dr. T.R.B. of the Department of Surgery, University of Pittsburgh, Pittsburgh, USA) and 1.25 mg/kg ritonavir (provided by Dr. T.R.B.) for 7 days before MV. A small dose of ritonavir (1.25 mg/kg) was used to boost plasma concentrations.

Evans blue albumin permeability measurement

Evans blue albumin (EBA) permeability was used as a marker of pulmonary vascular permeability as previously described [15]. Evans blue (0.5% EB; Sigma-Aldrich, St Louis, USA) was dissolved in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Sigma-Aldrich), and mixed with bovine serum albumin (Sigma-Aldrich) to prepare EBA (4%). After gentle stirring with a magnetic bar, the EBA solution was filtered through a 0.22-μm syringe filter and aliquots were stored at −80°C until use. Each aliquot was used only once for each experiment. To evaluate alveolar-capillary barrier function, EBA (25 mg/kg body weight) was injected into the internal jugular vein 1 h before euthanasia and lung harvesting. Blood samples were obtained from the right ventricle, and the pulmonary vasculature was subsequently infused with 1 ml PBS. The right lung was ligated at the level of the right mainstem bronchus, excised, blotted dry, weighed, and stored in liquid nitrogen until use for EBA analysis.

After freeze/thaw, the lung tissue was homogenized in 2 ml PBS and incubated with 2 ml of formamide (Sigma-Aldrich) for 18 h at 60°C. Formamide extracts were centrifuged at 15,000 *g* for 30 min at 4°C, and the supernatants were collected to measure the lung EBA content using a dual-wavelength (620 nm and 740 nm) spectrophotometric method. Pulmonary EBA absorbance at 620 nm was corrected by a correction factor with EBA absorbance at 740 nm. EBA permeability index was the ratio of the corrected pulmonary tissue EBA absorbance (620 nm) per gram of lung tissue and the corrected serum EBA absorbance (620 nm).

Lung wet/dry weight ratio

At the end of each experiment, the left lung was weighed immediately after removal to get wet weight, and then dried in an oven at 65°C for 48 h to get the dry weight. The lung W/D weight ratio was the ratio of the wet weight to the dry weight.

Lung histological examination and immunohistochemical staining for HMGB1

The effects of ventilation on lung morphology were examined by histological examination. Lungs were fixed in 4% paraformaldehyde phosphate-buffered saline for 24 h. After dehydration, the lungs were embedded in paraffin, and cut into 4-μm sections, and then stained with hematoxylin and eosin (H&E). Immunohistochemistry for HMGB1 was performed using a 1:1000 dilution of the anti-HMGB1 antibody (Abcam, Cambridge, UK) to semi-quantitatively determine changes of the expression of HMGB1 in the nuclei.

Bronchoalveolar lavage

At the end of the experiments, lungs were lavaged 15 times with 1 ml of cold sterile phosphate-buffered saline, and approximately 80% of the BALF was collected. Total cell numbers were counted using a hemocytometer. Total protein concentration was measured with BCA protein assay (Pierce Chemical Co., Rockford, USA). TNF-α and IL-6 levels in the BALF were measured using ELISA kits (R&D, Minneapolis, USA). Serum and BALF levels of HMGB1 were also measured using ELISA kits (Cusabio Biotech, Wuhan, China).

Western blot analysis

Lungs were ground in liquid nitrogen, and the cytoplasmic and nuclear proteins were extracted. Protein concentration was quantitated with BCA protein assay. Western blot analysis for HMGB1 and NF-κB was performed as described previously [25]. Briefly, protein samples were subject to 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes (Thermo Fisher, Waltham, USA). After being blocked with 5% nonfat milk for 1 h, the membrane was incubated with primary antibody against HMGB1 (1:2000; Abcam), p65 (1:1000; Cell Signaling Technology, Beverly, USA), β-actin (1:1500; Abcam), or proliferating cell nuclear antigen (PCNA; 1:1000; Bioworld, St, Louis, USA), and subsequently incubated with HRP (horse radish peroxidase) labeled secondary antibody. Odyssey (LI-COR Biosciences, Lincoln, USA) image analysis system was used to detect the bands.

SYBR green real-time RT-PCR

Total RNA was extracted from lung tissues using the Trizol reagent (Sigma-Aldrich) according to the manufacturer's recommendations. Total RNA was transcribed into cDNA using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China). SYBR green quantitative RT-PCR was prepared to measure the expressions of the target genes. Primers for HMGB1 amplification were purchased from Sangon Biotech (Shanghai, China). The sequences are as follows: HMGB1 forward: 5′-TGGGCAAAGGAGATCCTAAA-3′; HMGB1 reverse: 5′- GCAGACATGGTCTTCCACCT-3′; amplification 160 bp.

Statistical analysis

SPSS 20.0 (SPSS, IBM Corporation, Armonk, USA) was used to analyze data, which are expressed as the mean ± standard deviation (SD). One-way ANOVA was performed to compare statistical differences between groups. $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using the GraphPad Prism 5 program.

Results

SQV attenuates vascular leak of VILI model

Lung W/D weight ratio (**Fig. 1A**), EBA permeability (**Fig. 1B**), protein concentrations in BALF (**Fig. 1C**), and total cells in BALF

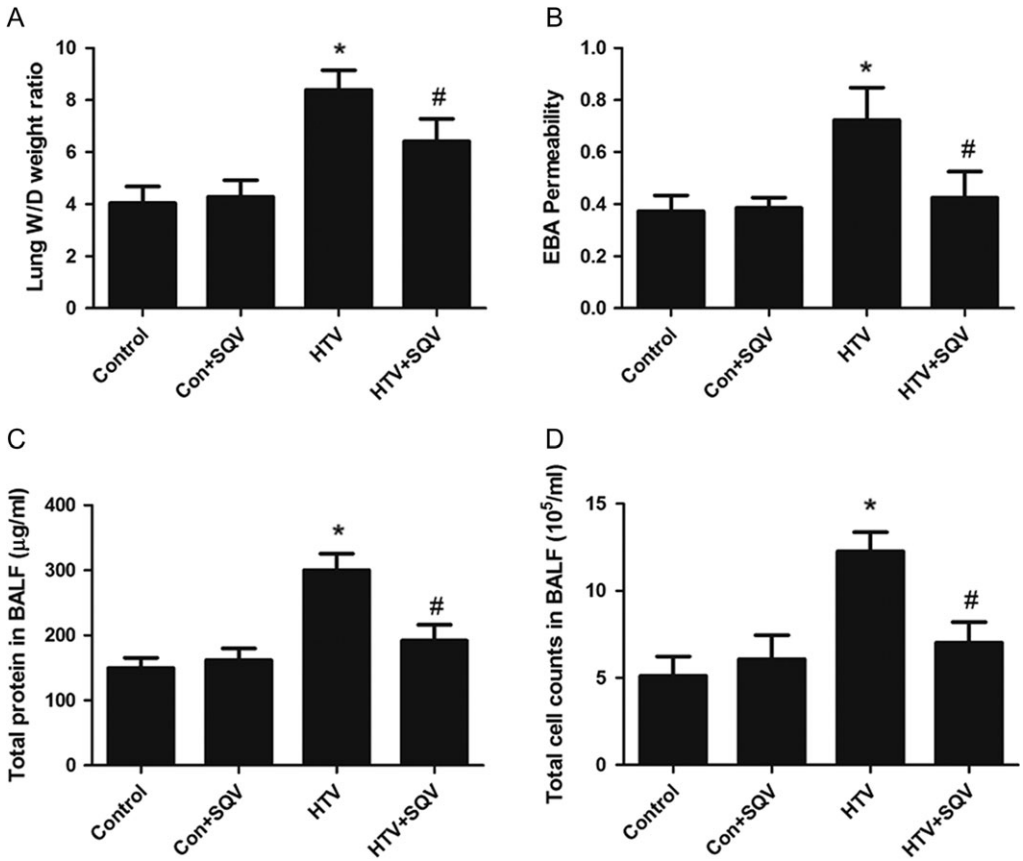

Figure 1. SQV attenuates vascular leak of VILI model Mice in the HTV group and control group were pretreated with SQV or vehicle. Lung wet/dry weight ratio (A), EBA permeability (B), protein concentrations in BALF (C), and total cell counts in BALF (D) were measured from four groups of mice, i.e. control, Con + SQV, HTV, and HTV + SQV. SQV pretreatment significantly attenuates vascular leak caused by HTV. Data are expressed as the mean ± SD ($n = 10$ mice per group). $^{*}P < 0.05$ vs. control group mice; $^{\#}P < 0.05$ vs. HTV group mice.

(**Fig. 1D**) in HTV group were significantly increased compared with control and Con + SQV groups ($P < 0.05$). In contrast to HTV injury alone, pretreatment with SQV significantly prevented these increases ($P < 0.05$). The effects of SQV on lung injury were confirmed by assessing histological changes. Representative H&E stain images from lung tissues of the four groups are shown in **Fig. 2**. A significant increase in the infiltration of inflammatory cells, welling of parenchyma as well as alveoli, and pulmonary edema was observed in the lung from HTV group compared with the lungs from control and Con + SQV groups. In the HTV + SQV group, the extent of interstitial edema and inflammatory cell infiltration were significantly attenuated.

SQV decreases inflammation cytokine secretion in VILI model

To examine the effect of SQV on cytokine levels both in BALF and serum, IL-6 and TNF-α levels were measured in each group. As shown in **Fig. 3**, low levels of IL-6 and TNF-α were detected in control group and Con + SQV group. They were enhanced significantly after 4 h of HTV ($P < 0.05$). However, pretreatment with SQV prevented the increase of IL-6 and TNF-α expressions following HTV ($P < 0.05$).

SQV inhibits NF-κB activation in VILI model

In order to investigate whether HTV would leads to NF-κB activation and whether SQV affects such change, the nuclear translocation of NF-κB was examined by western blot analysis. As shown in **Fig. 4**, NF-κB factor p65, the main subunit of NF-κB, was strongly translocated from cytoplasm to the nucleus by 4 h of HTV challenge. Pretreatment with SQV significantly prevented cytoplasmic NF-κB-p65 from translocation to the nucleus. These results show that SQV administration can reduce NF-κB activation compared with the HTV group ($P < 0.05$).

SQV reduces the expression of HMGB1 protein in the lung of VILI model

To determine whether SQV administration influences HMGB1 protein expression following HTV, western blot analysis was performed using lung tissues from mice in each group. There was no significant difference between control group and Con + SQV group. As shown in **Fig. 5**, compared with the control and Con + SQV groups, HTV group showed significantly elevated level of HMGB1 protein ($P < 0.05$). By contrast, HMGB1 level in the HTV + SQV group was significantly lower than that in the HTV group ($P < 0.05$), which indicated that SQV pretreatment could inhibit the high expression of HMGB1 protein caused by cyclic stretch, which is likely correlated with p38 MAPK and NF-κB pathways following 4 h of HTV. On the other hand, real-time RT-PCR was used to analyze mRNA expression of HMGB1. HTV enhanced HMGB1 mRNA expression, but SQV pretreatment significantly reduced its expression ($P < 0.05$).

Effect of SQV on lung tissue levels of HMGB1

Immunohistochemistry was also used to assess the expression of HMGB1 in the lung tissues (**Fig. 6**). Lungs in control and Con + SQV groups showed sparsely distributed inflammatory cells, and they were

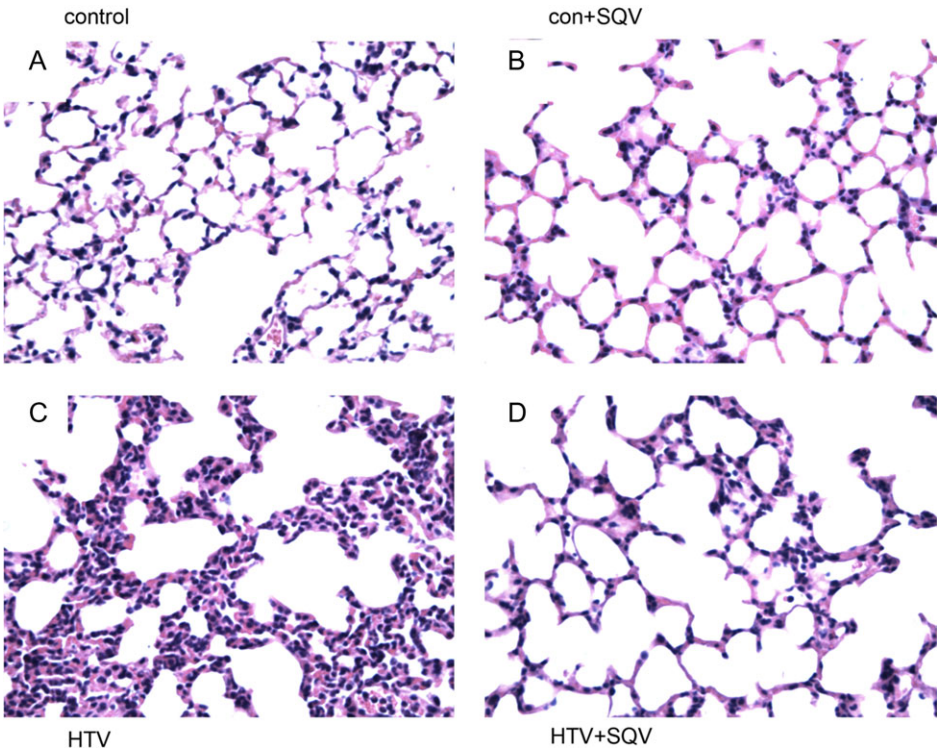

Figure 2. SQV restores normal morphology in VILI model Mice in the HTV group and control group were pretreated with SQV. Representative images from each group are shown (original magnification ×400). Lung sections from control (A) and Con + SQV (B) groups showed normal alveolar structures; HTV group (C) showed a marked increase in inflammatory cells infiltration and septal thickening, as well as pulmonary edema; whereas SQV pretreatment ameliorated these pathological changes (D).

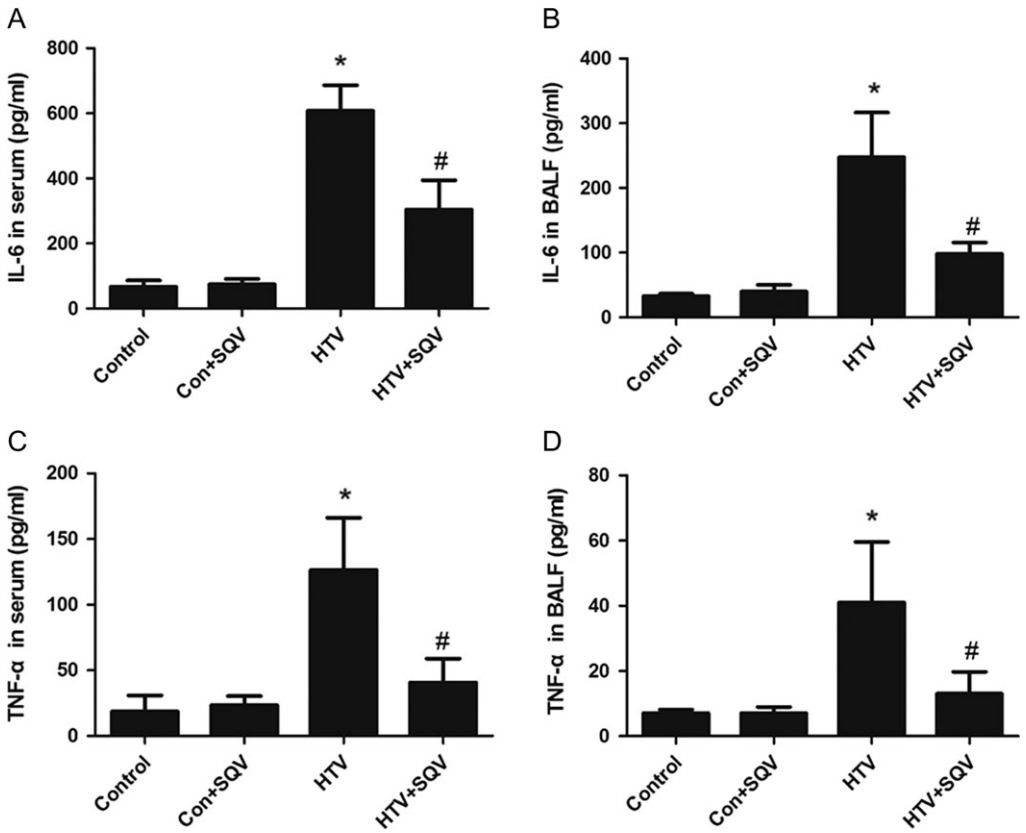

Figure 3. SQV decreases inflammation cytokines secretion in VILI model Mice in the HTV group and control group were pretreated with SQV or vehicle. ELISA was performed to determine the protein levels of IL-6 (A,B) and TNF-α (C,D) both in BALF and in serum. SQV pretreatment can significantly attenuated the IL-6 and TNF-α levels following HTV. Data are expressed as the mean ± SD ($n = 10$ mice per group). $^{*}P < 0.05$ vs. control group mice; $^{\#}P < 0.05$ vs. HTV group mice.

mostly not immunoreactive for HMGB1. Lungs exposed to HTV showed increased number of macrophages and neutrophils. Some of these inflammatory cells that had migrated into the alveolar spaces were strongly immunoreactive for HMGB1 (stained in brown). Only a few epithelial cells were also immunoreactive for HMGB1. However, specific staining of HMGB1 protein was significantly reduced in lung slices from HTV + SQV mice compared with 4 h HTV-treated mice.

SQV decreases the level of HMGB1 in BALF but not in serum of VILI model

The effect of SQV on HMGB1 levels both in BALF and in serum was examined. As shown in **Fig. 7A**, low level of HMGB1 was detected in control group and Con + SQV group in BALF. They were enhanced significantly after 4 h of HTV in BALF ($P < 0.05$). However, pretreatment with SQV prevented the increase in HMGB1 following HTV in BALF ($P < 0.05$). But no significant difference was found in serum (**Fig. 7B**).

Discussion

HIV PIs have been successfully used for the treatment of HIV infection. Previous studies have shown that these inhibitors had additional effects on the immune system. HIV PIs directly affect several pathways involved in invasion, inflammation, angiogenesis, tumor-cell proliferation and survival in HIV-free models [26]. Pati *et al.* [27] showed that HIV PIs may inhibit the activation and proliferation of endothelial cells, and inhibit the production of inflammatory cytokines and chemokines including IL-6, IL-8, and TNF-α. Our previous screening of 5546 pharmacologically active compounds and clinically used drugs identified SQV and nelfinavir as potent inhibitors of HMGB1-induced TNF-α production [28]. Our recent study discovered that SQV can suppress the interaction between the TLR4-MyD88 receptor complex and cathepsin V induced by HMGB1 [16]. A study based on murine CLP-induced sepsis revealed that HIV PIs therapy was associated with improved survival, reduced lymphocyte apoptosis, as well as lower plasma cytokines compared with CLP only [12]. HIV PIs are beneficial in some other diseases like cancer, inflammation, and immune-mediated diseases [11,13].

The activation of NF-κB is centrally regulated by the proteasome. The main subunit p65 of NF-κB is sequestered in the cytoplasm as inactive forms by binding to the inhibiting protein IκBα. After IκBα degradation by the ubiquintin-proteasome pathway, p65 is translocated to the nucleus to initiate transcription activity [29]. Previous studies have proved that NF-κB plays critical roles in the regulation of proinflammatory genes [30,31]. It has been found that mechanical stretch can activate IκBα degradation and p65 translocation, which induces the secretion of IL-8 [32]. In addition, NF-κB *cis*-elements have been identified in the promoter region of the *HMGB1* gene [30]. *In vitro* experiment has shown that high expression of HMGB1 is induced by cyclic stretch partly through the signal pathway NF-κB [18]. Ding *et al.* [24] showed that NF-κB inhibitor pretreatment blocked mechanical stretch-induced expression of HMGB1 following lipopolysaccharides (LPS) challenge.

Proteasome and NF-κB are key mediators of inflammation. Previous studies showed that HIV PIs including SQV have a significant anti-proteasome activity [14,15], which both SQV and RTV interfere with the catalytic proteases of proteases, thus inhibit 26S proteasome activity, thereby preventing IκB degradation and blocking NF-κB activation. Rosanna *et al.* [28] focused on the beneficial effect of SQV on idiopathic nephritic syndrome. The rationale was to down-regulate the activity of proteasome and the unclear translocation of NF-κB [3,4] and they observed that SQV significantly blunted the NF-κB up-regulation. Inhibitors of proteasome or of NF-κB activation have an anti-inflammatory activity [13]. In TLR4 cellular responses, 26S proteasome is required for NF-κB activation. In our recent study, we also assessed the degradation of IRAK1 and phosphorylation of IRAK4, which is the upstream of NF-κB activation [16]. SQV suppresses the IRAK1 and IRAK4 activation, indicating that SQV can act as an inhibitor of NF-κB. HMGB1 may activate TLR4 signaling pathway. On the other hand, NF-κB may regulate proinflammatory cytokines including HMGB1. We have just reported that SQV can block TLR4/MyD88-NF-κB signaling, so we hypothesize here that SQV pretreatment may inhibit HMGB1 expression following HTV by interfering with NF-κB signaling. Our findings showed that SQV showed positive results in reducing inflammation caused by HTV, which was in accordance with previous studies [3,4,12,27,29,33]. NF-κB nuclear translocation was significantly down-regulated, as well as the expression of HMGB1 protein.

HMGB1 has been identified as a molecule of danger-associated molecular patterns (DAMPs) in ALI [34]. It may be released passively in the setting of cell necrosis or actively from macrophages/monocytes activated by LPS or hypoxia. Although Wang *et al.* [19] suggested that HMGB1 was a late mediator of endotoxin lethality in mice, previous studies and our current findings supported that HMGB1 may be an early mediator following VILI [22,24]. Eileen *et al.* [22] reported that MV with a large V_T (30 ml/kg) for 4 h increased HMGB1 concentration in BALF, IL-8, and TNF-α levels in both BALF and plasma. Administration of HMGB1 by intratracheal instillation caused an acute, diffuse

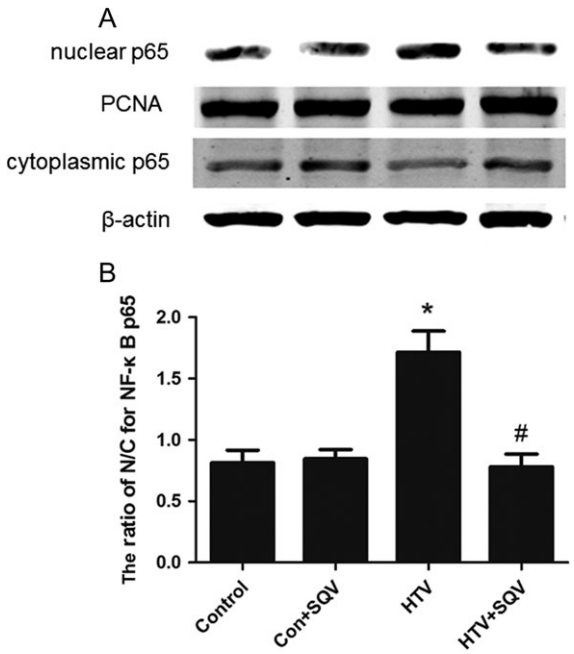

Figure 4. SQV inhibits NF-κB activation in VILI model Mice in the HTV group and control group were treated with SQV or vehicle. Lung tissues were used for the analysis of p65 subunit of NF-κB both in the nucleus and in the cytoplasm by western blot analysis (A). The ratio of densitometric quantifications of p65 in the nucleus and in the cytoplasm of mice from the control, Control + SQV, HTV, and HTV + SQV group. (B). Data are expressed as the mean ± SD (n = 10 mice per group). *P < 0.05 vs. control group mice; #P < 0.05 vs. HTV group mice.

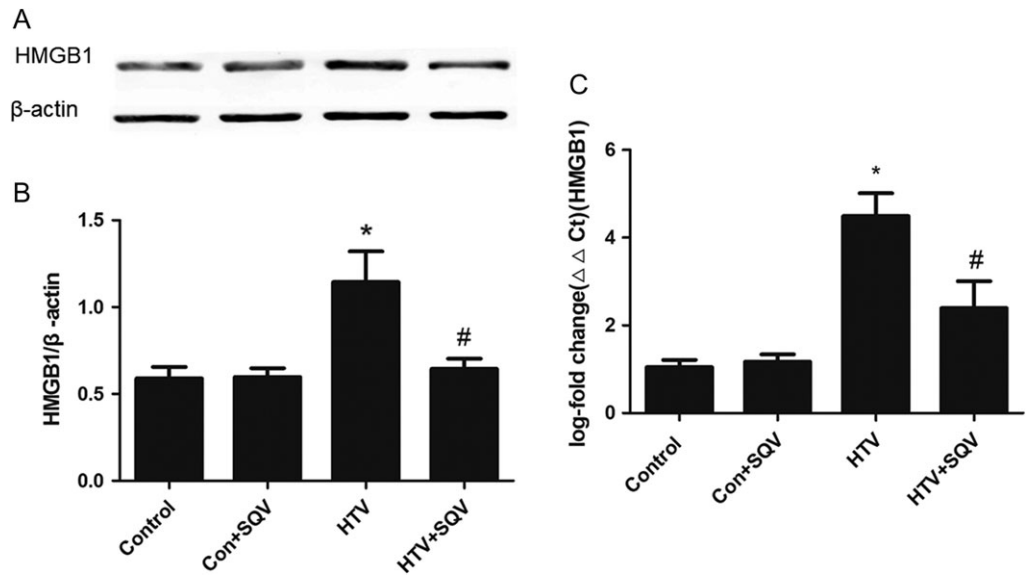

Figure 5. SQV reduces the expression of HMGB1 protein and mRNA expressions in the lung of VILI model Mice in the HTV group and control group were treated with SQV or vehicle. (A) HMGB1 was examined by western blot analysis. (B) Densitometric quantifications of HMGB1 in mice from control, Control + SQV, HTV, and HTV + SQV groups. SQV administrated 7 days before HTV significantly reduced the high expression of HMGB1 protein caused by HTV. (C) Real-time RT-PCR was performed with total RNA. The expression of HMGB1 mRNA increased significantly following HTV compared with the control and Con + SQV groups. Pretreatment with SQV significantly reduced HMGB1 mRNA expression in lung tissue after VILI. Data are expressed as the mean ± SD (n = 10 mice per group). *P < 0.05 vs. control group mice; #P < 0.05 vs. HTV group mice.

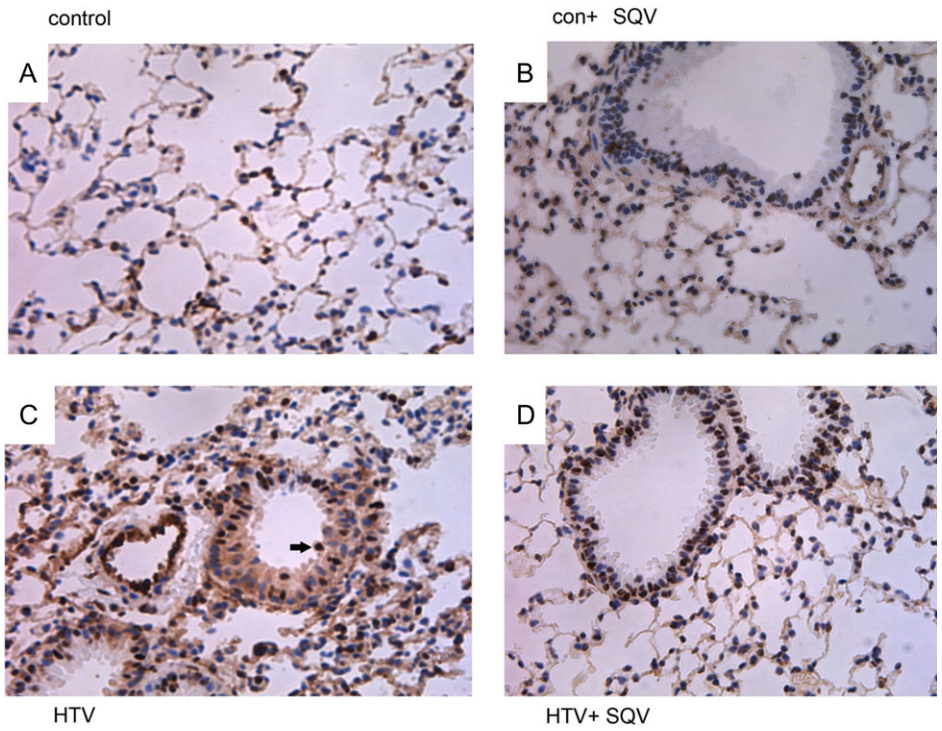

Figure 6. Immunohistochemical staining of lung sections for HMGB1 in VILI model Representative images from each group are shown (original magnification ×400). Lung sections from control (A) and Con + SQV (B) groups showed no immunoreaction. (C) In HTV group, mononuclear cells and inflammatory cells infiltrated in the lungs injured by HTV were immunoreactive for HMGB1 (stained in brown). (D) There was only a fraction of immunoreactive inflammatory cells in HTV + SQV group.

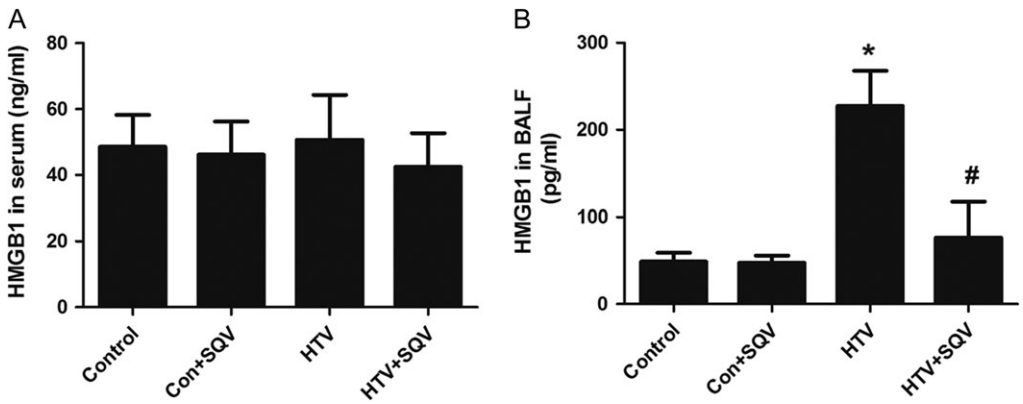

Figure 7. SQV decreases the level of HMGB1 in BALF but not in serum of VILI model Mice in the HTV group and control group were pretreated with SQV or vehicle. ELISA was performed to determine the levels of HMGB1 protein both in BALF (A) and in serum (B). SQV pretreatment can significantly attenuated the HMGB1 levels following HTV in BALF, but no significant difference was found in serum. Data are expressed as the mean ± SD (n = 10 mice per group). $^{*}P < 0.05$ vs. control group mice; $^{\#}P < 0.05$ vs. HTV group mice.

inflammatory response [18,19]. Importantly, VILI was mitigated by anti-HMGB1 antibody [20].

To determine whether SQV pretreatment directly affects the expression of HMGB1, mice received HTV at 0, 2, 4, 6 days post-treatment. SQV administered 4 and 6 days prior to HTV significantly reduced the high expressions of HMGB1 protein and mRNA caused by HTV, and it was more obvious for the 6 days (**Supplementary Figs. 1** and **2**). So in our study, we treated mice with SQV 7 days prior to HTV. Nevertheless, several limitations should be considered when interpreting our investigation. Firstly, we evaluated the time course of the expression of HMGB1 but the time of the SQV administration may not be optimal for the development of inflammation in VILI model. Secondly, we did not perform SQV administration after receiving HTV, which may be more similar to clinically relevant conditions. Thirdly, our further studies will focus on the role of HMGB1 in VILI by using its inhibitor and antagonist. In addition, we need to show the time evaluation of inflammation and activation of NF-κB after MV in further studies.

We chose the V_T of 20 ml/kg in this study, because a previous study showed that MV with a V_T of 20 mg/kg for 4 h caused evident

VILI in mice with significant increase in W/D ratio, pulmonary permeability and inflammation [10]. It was reported that MV with a V_T of 20 mg/kg developed an increase of IL-8 concentration in BALF and an infiltration of neutrophils [35], and elevated CD14 protein expression in alveolar macrophages [36]. We observed that TNF-α and IL-6 levels were significantly increased both in BALF and in plasma, and HMGB1 protein levels were obviously elevated after 4 h of HTV, which were in accordance with lung injury induced by HTV. HMGB1 was significantly increased in BALF after 4 h of HTV and pretreatment with SQV prevented the increase in HMGB1 following HTV in BALF, but there was no significant difference in serum. These results suggest that HTV induces the recruitment and activation of inflammatory cells, leading to alveolar epithelial and endothelial injury, as well as increased alveolar permeability with interstitial edema. We also confirmed that HTV can activate NF-κB pathway and may regulate HMGB1 expression in the lung. In contrast, pretreatment with SQV attenuates pulmonary inflammation and decreases pulmonary vascular permeability, thereby inhibiting NF-κB signaling in the lung.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Acknowledgements

We would like to thank T.R.B. from Department of Surgery, University of Pittsburgh, for valuable advice and supplying the drugs Saquinavir and Ritonavir. We would also like to thank Weifan Xiao, Yue Zhang, and Hui Zeng from Shanghai Tenth People's Hospital, Tongji University School of Medicine for their technical assistance.

Funding

The laboratory equipments of this work were supported by the grants from the National Natural Science Foundation of China (Nos. 81571927 and 81270135). The materials used in this work was funded by the Science and Technology Commission of Shanghai Municipality (No. 13411951700).

References

1. Saddy F, Oliveira GP, Garcia CS, Nardelli LM, Rzezinski AF, Ornellas DS, Morales MM, *et al*. Assisted ventilation modes reduce the expression of lung inflammatory and fibrogenic mediators in a model of mild acute lung injury. *Intensive Care Med* 2010, 36: 1417–1426.
2. Kuipers MT, van der Poll T, Schultz MJ, Wieland CW. Bench-to-bedside review: damage-associated molecular patterns in the onset of ventilator-induced lung injury. *Crit Care* 2011, 15: 235.
3. Moriondo A, Pelosi P, Passi A, Viola M, Marcozzi C, Severgnini P, Ottani V, *et al*. Proteoglycan fragmentation and respiratory mechanics in mechanically ventilated healthy rats. *J Appl Physiol* 2007, 103: 747–756.
4. Brower RG, Matthay MA, Morris A, Schoenfeld D, Thompson BT, Wheeler A. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. *N Engl J Med* 2000, 342: 1301–1308.
5. Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, Wolthuis EK, Choi G, *et al*. Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. *Anesthesiology* 2012, 116: 1104–1115.
6. Gattinoni L, Protti A, Caironi P, Carlesso E. Ventilator-induced lung injury: the anatomical and physiological framework. *Crit Care Med* 2010, 38: S539–S548.
7. Ma SF. Bioinformatic identification of novel early stress response genes in rodent models of lung injury. *Am J Physiol Lung Cell Mol Physiol* 2005, 289: L468–L477.
8. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, *et al*. Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial. *JAMA* 1999, 282: 54–61.
9. Gharib SA, Liles WC, Klaff LS, Altemeier WA. Noninjurious mechanical ventilation activates a proinflammatory transcriptional program in the lung. *Physiol Genomics* 2009, 37: 239–248.
10. Li H, Su X, Yan X, Wasserloos K, Chao W, Kaynar AM, Liu Z, *et al*. Toll-like receptor 4-myeloid differentiation factor 88 signaling contributes to ventilator-induced lung injury in mice. *Anesthesiology* 2010, 113: 619–629.
11. Gilquin J, Viard JP, Jubault V, Sert C, Kazatchkine MD. Delayed occurrence of Graves' disease after immune restoration with HAART. Highly active antiretroviral therapy. *Lancet* 1998, 352: 1907–1908.
12. Weaver JG, Rouse MS, Steckelberg JM, Badley AD. Improved survival in experimental sepsis with an orally administered inhibitor of apoptosis. *FASEB J* 2004, 18: 1185–1191.
13. Hosseini H, Andre P, Lefevre N, Viala L, Walzer T, Peschanski M, Lotteau V. Protection against experimental autoimmune encephalomyelitis by a proteasome modulator. *J Neuroimmunol* 2001, 118: 233–244.
14. Piccinini M, Rinaudo MT, Chiapello N, Ricotti E, Baldovino S, Mostert M, Tovo PA. The human 26S proteasome is a target of antiretroviral agents. *AIDS* 2002, 16: 693–700.
15. Piccinini M, Rinaudo MT, Anselmino A, Buccinna B, Ramondetti C, Dematteis A, Ricotti E, *et al*. The HIV protease inhibitors nelfinavir and saquinavir, but not a variety of HIV reverse transcriptase inhibitors, adversely affect human proteasome function. *Antivir Ther* 2005, 10: 215–223.
16. Pribis JP, AI-Abed Y, Yang H, Gero D, Xu H, Montenegro MF, Bauer EM, *et al*. The HIV protease inhibitor saquinavir inhibits HMGB1 driven inflammation by targeting the interaction of cathepsin V with TLR4/MyD88. *Mol Med* 2015, 21: 749–757.
17. Prasad S, Thakur MK. Age-dependent effects of sodium butyrate and hydrocortisone on acetylation of high mobility group proteins of rat liver. *Biochem Int* 1988, 16: 375–382.
18. Ueno H, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, Kobayashi A, *et al*. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004, 170: 1310–1316.
19. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, *et al*. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999, 285: 248–251.
20. Gribar SC, Richardson WM, Sodhi CP, Hackam DJ. No longer an innocent bystander: epithelial toll-like receptor signaling in the development of mucosal inflammation. *Mol Med* 2008, 14: 645–659.
21. Perros F, Lambrecht BN, Hammad H. TLR4 signalling in pulmonary stromal cells is critical for inflammation and immunity in the airways. *Respir Res* 2011, 12: 125.
22. Ogawa EN, Ishizaka A, Tasaka S, Koh H, Ueno H, Amaya F, Ebina M, *et al*. Contribution of high-mobility group box-1 to the development of ventilator-induced lung injury. *Am J Respir Crit Care Med* 2006, 174: 400–407.
23. van Zoelen MA, Ishizaka A, Wolthuls EK, Choi G, van der Poll T, Schultz MJ. Pulmonary levels of high-mobility group box 1 during mechanical ventilation and ventilator-associated pneumonia. *Shock* 2008, 29: 441–445.
24. Ding N, Wang F, Xiao H, Xu L, She S. Mechanical ventilation enhances HMGB1 expression in an LPS-induced lung injury model. *PLoS One* 2013, 8: e74633.
25. Morris SM, Kepka-Lenhart D, Chen LC. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am J Physiol* 1998, 275: E740–E747.

26. Monini P, Sgadari C, Toschi E, Barillari G, Ensoli B. Antitumour effects of antiretroviral therapy. *Nat Rev Cancer* 2004, 4: 861–875.
27. Pati S, Pelser CB, Dufraine J, Bryant JL, Reitz MJ, Weichold FF. Antitumorigenic effects of HIV protease inhibitor ritonavir: inhibition of Kaposi sarcoma. *Blood* 2002, 99: 3771–3779.
28. Gero D, Szoleczky P, Modis K, Pribis JP, Al-Abed Y, Yang H, Chevan S, *et al*. Identification of pharmacological modulators of HMGB1-induced inflammatory response by cell-based screening. *PLoS One* 2013, 8: e65994.
29. Ruland J. Return to homeostasis: downregulation of NF-kappaB responses. *Nat Immunol* 2011, 12: 709–714.
30. Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, Bianchi ME. Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF-κB activation. *J Cell Biol* 2007, 179: 33–40.
31. Klune JR, Billiar TR, Tsung A. HMGB1 preconditioning: therapeutic application for a danger signal? *J Leukoc Biol* 2007, 83: 558–563.
32. Ning Q, Wang X. Role of Rel A and IkappaB of nuclear factor kB in the release of interleukin-8 by cyclic mechanical strain in human alveolar type II epithelial cells A549. *Respirology* 2007, 12: 792–798.
33. Coppo R, Camilla R, Porcellini MG, Peruzzi L, Gianoglio B, Amore A, Dapra V, *et al*. Saquinavir in steroid-dependent and -resistant nephrotic syndrome: a pilot study. *Nephrol Dial Transplant* 2012, 27: 1902–1910.
34. Tolle LB, Standiford TJ. Danger-associated molecular patterns (DAMPs) in acute lung injury. *J Pathol* 2013, 229: 145–156.
35. Kotani M, Kotani T, Ishizaka A, Fujishima S, Koh H, Tasaka S, Sawafuji M, *et al*. Neutrophil depletion attenuates interleukin-8 production in mild-overstretch ventilated normal rabbit lung. *Crit Care Med* 2004, 32: 514–519.
36. Moriyama K. Enhancement of the endotoxin recognition pathway by ventilation with a large tidal volume in rabbits. *Am J Physiol Lung Cell Mol Physiol* 2004, 286: L1114–L1121.