行政院國家科學委員會專題研究計畫　成果報告

第二型巨噬細胞發炎蛋白在動物模式呼吸器引發肺損傷角色之探討

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-002-223-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 24 日

The Role of Macrophage Inflammatory Protein-2 in the Development of Ventilator-Induced Lung Injury in a Rat Model

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ABSTRACT

Rationale: Mechanical ventilation may be responsible not only for worsening the underlying lung injury, but may also lead to the development of new lung injury, i.e., ventilator-induced lung injury (VILI) as well as systemic inflammatory response syndrome and multiple organ failure. **Objectives:** To study the role of macrophage inflammatory protein-2 (MIP-2), a functional equivalent of interleukin-8 of the chemokine group, in the pathogenesis of VILI, and to investigate the renin-angiotensin system in the involvement of VILI and possible of regulation of MIP-2 expression in VILI. **Methods:** Sprague-Dawley rats were mechanically ventilated for four hours with either low (7 ml/kg) or high (40 ml/kg) tidal volumes. Histologic studies, measurements of the myeloperoxidase activity and nuclear factor-κB activity, quantification of the mRNA as well as protein levels of MIP-2 in the lungs, and assessment of the expression after treatment with captopril, and angiotensin-converting enzyme inhibitor, were performed. **Main Results:** In the high-volume group, lung tissue neutrophil infiltration, activity of nuclear factor -κB, tumor-necrosis factor-α, and macrophage inflammatory protein-2 were significantly increased, and survival was significantly reduced. Treatment with captopril attenuated the lung neutrophil infiltration, tumor-necrosis factor-α, macrophage inflammatory protein-2, and nuclear factor-κB activity in the high-volume group, with significant survival benefit. **Conclusions:** Injurious mechanical ventilation is associated with significantly increased expression of macrophage inflammatory protein-2 in rat lungs, with prominently increased neutrophil infiltration. Treatment with angiotensin-converting enzyme inhibitor can attenuate MIP-2 expression and ventilator-induced lung injury in the animal model.

Key Words: Ventilator-induced lung injury, macrophage-inflammatory protein-2, angiotensin-converting enzyme

INTRODUCTION

Mechanical ventilation (MV) is indispensable in the management of critically ill patients with respiratory failure including the acute respiratory distress syndrome (ARDS) (1-4). MV can subject the lungs to substantial abnormal stretching stress, resulting in structural change, impaired gas exchange, activation of the inflammatory process leading to ventilator-induced lung injury (VILI) with significant risk to the patients (5-7). VILI can also complicate pre-existing lung injury, making the management of critically ill patients even more difficult. Inflammatory cells and mediators may play an important role in the pathogenesis of VILI (8-10).

Macrophage inflammatory protein-2 (MIP-2) belongs to a family of leukocyte chemokines and is considered functional equivalent of human interleukin-8, with the main biologic function of chemoattraction. Increased MIP-2 expression in the lungs may be associated with neutrophil infiltration into the lung parenchyma, therefore the activation of MIP-2 expression is considered an early event that might lead to on overt inflammatory process in the local environment. Despite a number of studies have shown the expression of MIP-2 in acute lung injury/acute respiratory distress syndrome, recently, however, the possible involvement of the renin- angiotensin system (RAS) in the pathogenesis and evolution of inflammatory response has gained substantial interest, and its possible association with the cytokine regulation has remained uncertain. Mechanical stress can activate the inflammatory process via the nuclear factor (NF)-κB pathway (11). Activation of RAS as well as mechanical stress could stimulate production of tumor-necrosis factor (TNF)-α in fibroblasts (12). Angiotensin II may activate the inflammatory process by up-regulation of the synthesis of proinflammatory cytokines and chemokines via the type 1 (AT1) and type 2 (AT2) angiotensin II receptors and the NF-κB pathway (13-15). Systemic infusion of angiotensin II may activate NF-κB in rats (16). Angiotensin II may also induce apoptosis of pulmonary endothelial cells (17) and alveolar epithelial cells (18). The apoptosis could be abrogated by angiotensin receptor antagonists or other angiotensin II blockers (19). Angiotensin II may play also an important role in the fibrotic response to acute lung injury via the action of transforming growth factor-α (20).

In this study we demonstrated that MIP-2 expression can be up-regulated by injurious mechanical ventilation in the rats, and the RAS may play an important role in the pathogenesis of VILI. Treatment with ACEI can attenuate expression of MIP-2 as well as VILI in the animal model. Some of the results of this study have been previously reported in the form of an abstract (21).

METHODS

All experiments were performed after the approval by the Institutional Animal Committee of the National Taiwan University College of Medicine.

Reagents

Captopril, PD123319, lipopolysaccharide from *Escherichia coli* (serotype 055:b5) and all other chemicals, unless indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Losartan, a specific antagonist of type 1 angiotensin receptor (AT1) for angiotensin II, was a gift from Merck (Merck & Co., Inc., Whitehouse Station, NJ). PD123319, a specific antagonist of type 2 angiotensin receptor (AT2) for angiotensin II, was purchased from Sigma. Antibodies to rat tumor-necrosis factor-α, type 1 and type 2 angiotensin II receptors, and nuclear factor-κB p65 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies to rat I-κB and phosphorylated I-κB were purchased from Cell Signaling (Beverly, MA).

Animal Preparation

Male Sprague-Dawley rats weighing 200-250 g were cared, handled and maintained in the animal resource facility of the National Taiwan University College of Medicine in accordance with the Institutional Guidelines. The rats were fed with rat chow and water ad libitum and housed in standard care facilities for ten days before being used for experiments.

The animals were anesthetized by intraperitoneal injection of urethane (1.3 g/kg) before being applied with mechanical ventilation. Tracheostomy was performed, followed by arterial and venous catheterizations. The tracheostomy tube was then connected to a volume-controlled ventilator for small animals (New England Medical Instruments, Inc., Medway, MA) and ventilated according to the study design protocol and the general descriptions of the manufacturer. Immediately before starting mechanical ventilation, the animals were given intravenous pancuronium (4 mg/kg), with further doses added to ensure that there was no spontaneous breathing effort during the course as observed by the investigators.

Mechanical Ventilation Protocol

The animals were generally divided into the following three experimental groups: 1) non-ventilated control group; 2) mechanical ventilation with high tidal volumes (40 ml/kg tidal volume, zero PEEP, 20 breaths/min), with room air; 3) mechanical ventilation with low tidal volumes (7 ml/kg tidal volume, zero PEEP, 100 breaths/min), with room air. For the animals of the control group, only urethane was injected intraperitoneally. For the low-tidal volume and high-tidal volume groups, mechanical ventilation was applied for 4 hours for each animal, and peak airway pressure was monitored throughout the course of mechanical ventilation. Blood samples, 0.1 ml each time, were obtained from the left femoral artery via the cannula immediately before starting ventilation and every 60 minutes after starting ventilation. Arterial blood gas analysis was performed on site immediately after blood sampling using a portable analyzer (i-STAT, Abbott Laboratory, Abbott Park, IL, USA) performed according to the manufacturer's description. The respiratory rates were set at 25 breaths/min for the high volume group, and 80 breaths/min for the low volume group. Intravenous infusion of normal saline was used to maintain a MAP of no less than 70 mmHg, while during the experiments, normal saline, 0.5 ml/hr, would be infused via the femoral vein to prevent dehydration. After four hours of ventilation, animals were given a lethal dose of intraperitoneal pentobarbital for euthanasia. The lungs were then removed en block after the pulmonary vessels were flushed with intracardiac injection of normal saline. The right lung was frozen in liquid nitrogen immediately after removal and stored at -80°C for further analysis, while the left lung was intratracheally flushed and fixed with preservative agent and stored at room temperature for histologic studies. Another group of non-ventilated rats received E. coli LPS (Sigma Chemical, St. Louis, MO) instilled transtracheally at a dose of 0.2 mg/100 g of body weight dissolved in 0.5 mL of phosphate-buffered saline. The LPS solution was dispersed in the trachea just above the level of tracheal bifurcation.

Course and Survival of Rats after Mechanical Ventilation

To evaluate the possible prolonged effect of injurious mechanical ventilation, additional groups of rats received mechanical ventilation for 4 hours with endotracheal intubation without tracheostomy. The skin and subcutaneous tissue were dissected after intubation and the trachea was then exposed and ligated to maintain the endotracheal tube in proper position without air leak during mechanical ventilation. After completion of the 4-hour ventilation, the endotracheal tube was removed and the cervical wound was closed. The animals were then fed and cared as described above. To assess the lung injury after mechanical ventilation were stopped, rats were sacrificed 1, 2, and 3 days after the MV, respectively, and their lungs were then removed for further studies (n=3 for each group). To assess the long-term survival of rats after stopping mechanical ventilation, the rats were then observed closely until 7 days or death (n=10 for groups without captopril, and n=15 for captopril pre-treated group).

Treatment with Captopril, Losartan or PD123319

Additional groups of rats were randomly assigned to receive captopril before receiving mechanical ventilation, or to be treated with either losartan or PD123319 during mechanical ventilation. For animals to receive captopril, 500 mg/l of captopril was added in the drinking water since for three days before the initiation of mechanical ventilation. For the losartan- or PD123319-treated groups, either losartan (10 mg/kg) or PD123319 (10 mg/kg) were intravenously administered via a pump simultaneously during the 4-hour course of mechanical ventilation. All pre-medicated animals will be subjected to experiments according to the protocol described above for different ventilatory strategies.

Histologic Studies

The lungs were removed immediately after the animals were killed and fixed with a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacolate buffer, pH 7.4 for more than 24 hours, and were then dehydrated with graded alcohol and embedded in paraffin at 52°C. Sections were prepared and stained with hematoxylin and eosin for histologic evaluation. Each lung section was assigned blindly and scored for lung injury by a board-certified pathologist using previously published criteria (E1, E2): 1, rare or occasional inflammatory cells scattered through the lung; 3, abundant inflammatory cells scattered through the lung; 2, inflammatory cells between levels of 1 and 3. The total inflammation score for each animal will be calculated as mean of the scores for five lung sections.

Myeloperoxidase Assay

Myeloperoxidase in the lung parenchyma was used as a marker enzyme for neutrophil infiltration into the lung (E3, E4). The lungs were washed with saline, weighed, and immediately homogenized in potassium phosphate buffer (10 mM, pH 7.4) containing 1.0 mM ethylenediaminetetraacetic acid. The homogenate was centrifuged at 10,000 *g* at 4°C for 20 min. The pellet was resuspended in potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% (vol/vol) hexadecyltrimethyl-ammonium bromide (Sigma, Saint Louis, MO). The pellet was re-homogenized and soniated and then centrifuged at 40,000 *g* at 4°C for 15 min. The supernatant (0.1 ml) was added to 2.9 ml of potassium phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml *O*-dianisidine hydrochloride (Sigma, Saint Louis, MO) and 0.0005% (wt/vol) hydrogen peroxide. Absorbance of the solution was measured over 3 min at 460 nm. MPO activity was expressed as international units per gram of dry tissue. One unit of enzyme activity was defined as the amount of peroxidase that produced an absorbance change of 1.0 optical density unit/min at 25°C.

Tissue RNA Extraction

Total cellular RNA from rat lung recovered 4 h after treatment was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Briefly, the lung tissue was homogenized with liquid nitrogen, and 1 ml of TRIzol was added. One ml of the mixture was obtained and 200 μl of chloroform was added. The mixture was centrifuged 13000 *g* at 4°C for 15 min. The supernatant was obtained and mixed with 0.5 ml isopropanol, and then centrifuged at 13000 *g* at 4°C for 15 min. The supernatant was discarded and the sample was washed with 1 ml of 75% ethanol. The ethanol was then discarded and the pellet was dissolved in 20 μl of DEPC-H_2O. The purity and integrity of the RNA samples were assessed by OD_{260}/OD_{280} spectrophotometric measurements and by agarose gel (1% agarose-formaldehyde gel containing 20 mM morpholino sulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0).

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

One microgram RNA was subjected to first-strand cDNA synthesis in a 20-μl reaction mixture containing avian myelo blastosis virus reverse transcriptase (10 U), 2 μl of dNTP mixture (2.5 mM concentrations of each dNTP), 0.5 μl of RNAase inhibitor, 1 μl of oligo(dT)12–18 primers (10 μM), and reaction buffer as supplied with the enzyme (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, and 10 mM DDT). The samples were incubated in a MJ Research PTC-200 thermal cycler (MJ Research) at 42°C for 60 min followed by enzyme denaturation step at 94°C for 2 min. The reverse transcription mixture was stored at -20°C for use in PCR. All reagents were obtained from Promega (Southampton, U.K.).

Four microlitters of reverse transcribed products were used as the template for RT-PCR. The PCR amplification was performed using r*Taq* DNA polymerase (Takara, Shiga, JP) under the following conditions: an initial denaturation at 95°C for 5 min, amplification was conducted through 27-30 cycles of denaturation at 94°C for 30s, annealing at 55°C (GAPDH) or 60°C (for all other transcripts) for 30 s and extension at 72°C for 45 s, final extension was at 72°C for 7 min. Gene-specific oligonucleotide primers of TNF-α, MIP-2 and GAPDH were described by Haddad et al. The PCR products were electrophoresed by 2% agarose gel and stained with ethidium bromide. Bands of each target transcript were visualized by ultraviolet transillumination and captured using a digital camera. ODs for each band were quantified by image analysis software AlphaImager System (Alpha Innotech Corporation). The level of gene expression of each transcript was normalized to that of the housekeeping gene GAPDH.

Real-time Reverse Transcription-Polymerase Chain Reaction

Oligonucleotide primers and for rat angiotensinogen, angiotensin-converting enzyme, type 1 and type 2 angiotensin II receptors were designed from the GenBank databases (NM_012544 and NM_134432) (Table E1) using Primer Express (PE Applied Biosystems, Inc). Real-time RT-PCR was performed in an ABI PRISM 7500 Sequence Detector (PE Applied Biosystems) according to the instructtions from the manufacturer. In brief, reverse transcription was carried out in a total volume of 25 μl that contained the following components: 5 μl RT sample (250 ng), 12.5 μl of QIAGEN SYBR Green Master Mix (QIAGEN, Valencia, CA), 1 μl of each forward and reverse primer (5 μM) and 5.5μl H_2O. Thermocycling conditions consisted of 1 cycle at 50°C for 2 min and 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. RT quantities were determined by comparing the cycle threshold of each sample to those of RT standard curves. The mRNA values of these RAS components were normalized to the content of GAPDH RNA in each sample and expressed as fold increase compared with control group.

Measurement of Plasma Angiotensin II Level

Rat plasma was centrifuged using Microcon YM-3 filter (Millipore, Bedford, MA) according to the manufacturer's instruction to obtain proteins less than 3 kD. The eluate was evaporated under vacuum rotation using a Speed Vac concentrator (Savant Instruments). An Angiotensin II Radioimmunoassay kit (Peninsula Laboratories, San Carlos, CA) was used to determine serum angiotensin II level. Briefly, samples were reconstituted with RIA buffer and incubated with 50 μl of tracer (^{125}I-ANG II, 4,000 cpm) and 50 μl of angiotensin II antiserum for 20 h at 4°C. The goat anti-rabbit IgG serum was added and incubated for 90 min in room temperature. Free radioactive angiotensin II (pellet) was counted in a gamma counter (1470 Wizard, Wallac ADL). Standards ranged from 1 to 250 pg/tube and were compared with the World Health Organization angiotensin II reference standard. The detection limit was 1 pg of angiotensin II per tube.

Extraction of Cytosolic and Nuclear Protein from Rat Lung Tissue

The rat lung was homogenized and washed by cold phosphate buffer saline. For extraction of cytosolic and nuclear proteins, 200 μl 0f cold buffer A (10mM HEPES-KOH, 1.5mM $MgCl_2$, 10mM KCl) containing protease inhibitor 10 μl/l buffer (Sigma Protease Inhibitor, Sigma-Aldrich), was added and placed on ice for 10 minutes. The mixture was centrifuged at 8000 *g* for 1 minute at 4°C.

The supernatant was collected as cytosolic protein, and the remnant was resuspended with cold Buffer C (20 mM HEPES, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, and 25% glycerol), and placed on ice for 10 min, then centrifuged at 13000 *g* for 2 min at 4°C. The supernatant was collected as nuclear protein.

Western blot analysis for NF-κB and I-κB of the rat lung tissue

For analysis of NF-κB p65 and I-κB, 20 μg of cytosolic or nuclear protein were electrophoresed on 10 % SDS-PAGE gel and transferred onto a polyvinylidine difluoride (PVDF) membrane (Amersham, Arlington Heights, IL). Membranes were blocked for I h in 5 % non-fat dried milk in TBST (0.24 % (w/v) Tris, 0.8 % (w/v) NaCl, 0.05 % (v/v) Tween 20, pH 7.6) and incubated with either monoclonal anti-rat NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-rat IκB antibody (Cell Signaling, Beverly, MA) for 1 h. After washing three times in TBST, the blots were incubated with a peroxidase-conjugated-goat anti-(rabbit IgG) for 1 h and washed extensively three times in TBST for 10 min. Immunocomplexes were detected with the ECL reagent (Amersham). Analysis of the immunoreactive bands was performed with AlphaImager 2200 System (Alpha Innotech Corporation).

Emzyme-linked Immunosorbent Assay (ELISA) for Macrophage-inflammatory Protein-2 (MIP-2)

For the tissue MIP-2 assays, 0.5 g of frozen rat lung were added to 2 ml of cold-acid-ethanol (93% ethanol, 2% concentrated HCl). 85 μg/ml phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin A, and homogenized 1min with a polytron homogenizer. The samples were extracted overnight. MIP-2 was then measured using a rat MIP-2 ELISA kit (Biosource International, Camarillo, CA). Each sample was run in duplicate according to the manufacturer's instructions.

Data Presentation and Statistics

All data are shown as mean $\pm$ SD. Comparisons between groups were performed by paired t tests and one-way analysis of variance, using SPSS 10 software (SPSS, Inc, Chicago, IL). For survival analysis, the Kaplan-Meier method was performed, with the Log-Rank test to compare different groups of treatment. A p value of < 0.05 was considered significant.

RESULTS

Histological changes and neutrophil infiltration of rat lungs undergoing mechanical ventilation and the effect of captopril

We first examined the histological changes of rat lungs exposed to a four-hour course of mechanical ventilation. The histological findings of low-volume ventilation group were comparable with control group, whereas high-volume ventilation resulted in a mild form of lung injury, which could be attenuated by pretreatment with captopril at a dosage of 500 mg/l in drinking water for three days (Fig. 1A). The pathologic lung injury scores were compatible with the histologic findings, showing that high-volume ventilation increased lung injury, which could be attenuated by captopril (Figure 1B). We also measured the myeloperoxidase activity in rat lungs to assess the degree of neutrophil infiltration. Figure 1C shows that high-volume ventilation increased the neutrophil infiltration in rat lungs, while pretreatment with captopril could attenuate the neutrophil infiltration.

Expression of proinflammatory cytokines in rat lungs undergoing mechanical ventilation and the effect of captopril

We next evaluated the expressions of proinflammatory cytokines in the rat lungs. Figure 2 shows the chronologic expression pattern of TNF-α mRNA in the rat lungs during and after 4 hours of high-volume ventilation. The mRNA expression of TNF-α was gradually increased up to 4 hours, and decreased gradually after cessation of mechanical ventilation (Figure 2A). We compared the expression of TNF-α and MIP-2 in the lungs of control, low-volume and high-volume groups, and evaluated the effect of prophylactic treatment of captopril. At 4 hours, high-volume ventilation induced significantly higher mRNA expression of TNF-α and MIP-2 as compared to low-volume and control groups. The increased expressions by high-volume ventilation could be attenuated by prophylactic captopril.

Activation of the Nuclear Factor-κB in Rat Lung Tissue by Mechanical Ventilation and the Effect of Captopril Pretreatment

To evaluate whether injurious mechanical ventilation can activate the nuclear factor-κB (NF-κB), an important transcription factor for various proinflammatory cytokines, we then assessed the amounts of cytosolic and nuclear NF-κB in rat lung tissues by Western blotting. Figure 3 shows the representing findings of rats treated with positive control lipopolysaccharide (LPS), low tidal volume (LV) or high tidal volumes (HV) ventilations. Compared with the normal control and low-volume groups, cytosolic NF-κB of the high-volume as well as the LPS-treated groups were markedly decreased, with a simultaneous distinct increase in their nuclear component amounts (Figure 3A), suggesting an obvious nuclear translocation of this factor induced by injurious MV and LPS. The translocation if NF-κB in the high-volume group could be partially inhibited by the pretreatment with captopril. The cytosolic I-κB in rat lung tissue was also decreased by high-volume MV, with simultaneous increase of phosphorylated I-κB (Figure 3B), suggesting an increased activity of the NF-κB activity pathway.

Expression of the components of the renin-angiotensin system in rat lungs undergoing mechanical ventilation

To elucidate the possible involvement of the renin-angiotensin system in the rat lungs in ventilator-induced proinflammatory response, we then examined the expression of the RAS components in the rat lungs. Figure 4 shows that high-volume ventilation increased the expressions of the mRNA of angiotensinogen, AT1 and AT2 receptors ($p < 0.01$) (Fig 4A), as well as the blood level of angiotensin II ($p < 0.05$) (Fig 4B). The increase of angiotensin II could be attenuated by pretreatment with captopril ($p < 0.05$).

Effects on receptor blockade of the RAS on the expression of proinflammatory cytokines

To elucidate the possible involvement of the AT1 or AT2 receptor pathway, or both, in the induction of inflammation in the rat lungs, we then assessed the effect of AT1 and AT2 receptor blockades on the induction of expression of MIP-2 by high-volume ventilation. Figure 5 shows that the increased expression of MIP-2 mRNA (Fig 5A & B) and protein (Fig 5 C & D) by high-volume ventilation could be attenuated either by the AT1 antagonist losartan or the AT2 antagonist PD123319. Neither of the blockades could completely attenuate the expression of MIP-2 in the rat lungs, suggesting that both receptors may play an important role in high-volume ventilation-induced lung inflammation.

Survival after Mechanical Ventilation

To assess the long-term effects of this temporary injurious ventilation, we then studied the survival of rats by observing their post-ventilation course in different groups. Figure 6 shows that rats receiving 4 hours of high-volume ventilation had a significantly worse survival as compared with those submitted to low-volume ventilation ($p = 0.002$) or control group. The unfavorable outcome in the high-volume MV group tended to be improved by a prophylactic treatment with oral captopril (50 mg/kg) ($p = 0.062$).

DISCUSSION

Mechanical ventilation is indispensable in the treatment of critically ill patients with respiratory failure. The resulting lung distension may activate the inflammatory process associated with VILI and significant risk to patients. The molecular pathogenesis of activation of inflammation in VILI and the potential role for specific anti-inflammatory therapeutic agents remained uncertain.

In this study we reported two novel findings. First, RAS may play an important pathogenic in the inflammatory process, most significantly the expression of macrophage inflammatory protein-2 (MIP-2) and VILI. Lung tissue distension by high-volume ventilation resulted in up-regulation of the RAS, AT1 and AT2 angiotensin II receptors, activation of NF-κB, over- expression of TNF-α, MIP-2 proinflammatory cytokines, and neutrophil infiltration into the lungs. Second, treatment by ACEI can attenuate VILI and suppress TNF-α, MIP-2, NF-κB and angiotensin II over-expression, with significant survival benefit in the animal model.

RAS may play an important pathogenic role in the inflammatory process associated with VILI is consistent with previous reports that pulmonary RAS and angiotensin II can be associated with various pathophysiologic conditions (26-28). Previous reports supporting the active role of angiotensin in lung inflammation and injury were mainly based on studies on cultured cells. Our findings may provide further *in vivo* evidence of the involvement of the RAS and VILI. The pathogenic role of RAS in the inflammatory process and VILI is confirmed by the attenuation of VILI by captopril treatment, suppression of TNF-α, MIP-2, NF-κB, angiotensin II over-expression with significant animal survival benefit in this study.

Our findings suggest that the NF-κB pathway may be the down-stream response pathway of the angiotensin II action in the inflammation process. Although similar findings have been shown in other systems that angiotensin II can activate NF-κB (29), other studies show that inhibition of NF-κB also can attenuate the activity of the RAS (30). Therefore a positive feedback system may be suggested, but remains to be confirmed. This may explain the RAS mediated and ventilator-induced lung inflammation can develop rapidly. Angiotensin was also associated with apoptosis and fibrogenic process in lung injury (19, 20), whether this is associated with VILI is uncertain.

Our finding that captopril treatment can attenuate VILI, suppress the RAS, angiotensin and the inflammation process with significant survival benefit in the animal model may have important clinical implications. It may allow development of specific anti-inflammatory therapeutic agents for the treatment or prevention of VILI. ACE inhibitor treatment was associated with a 19% lower risk of pneumonia, and the effect was more significant among Asian patients, with 47% risk reduction (31). Polymorphism of the ACE gene is also associated with the risk of acute respiratory distress syndrome (32). Mice deficient for ACE showed markedly improved disease in acid aspiration-induced acute lung injury (33). In our study, the lung inflammation action by angiotensin II may be mediated through both the AT1 and AT2 receptors. Angiotensin receptors AT1 and AT2 can activate NF-κB and trigger a variety of inflammatory process in other tissues (16, 29). NF-κB can be inactivated by administration of ACE inhibitor or AT1/AT2 receptor blockades (34). The activation of NF-κB associated with VILI can also be partially blocked by corticosteroids (11).

This study may have several limitations. This was a short-term study and data regarding the long-term effects of MV are lacking. But it did show that active inflammation in the lungs can develop early in injurious mechanical ventilation, and VILI can be attenuated by ACEI. This study involved mechanical ventilation as the single insult in originally health lungs. This is different clinically where most patients suffer from other initial insults, such as pneumonia or sepsis before receiving mechanical ventilation. The local angiotensin II and ACE activity were not determined in the lungs in this study because of the short half-life of angiotensin II.

In conclusion, the expression of macrophage inflammatory protein-2 can be significantly up-regulated by injurious mechanical ventilation, and the renin-angiotensin system plays an important role in the pathogenesis of this expression and the inflammatory process in the lungs and ventilator-induced lung injury. Treatment with angiotensin-converting enzyme inhibitor can attenuate the expression of MIP-2 as well as ventilator-induced lung injury in the animal model.

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FIGURE LEGENDS

Figure 1:
A. Histologic changes of rat lungs in ventilator-induced lung injury. H & E staining of lung sections were performed for non-ventilated control group (a), low-tidal-volume (7 ml/kg) ventilation group (b), high-tidal-volume (40 ml/kg) ventilation group (c), and high-tidal volume group pretreated with captopril (d). **B**. Pathologic lung injury score of different groups with or without captopril pretreatment. LV=low tidal volume; HV=high tidal volume **C**. Myeloperoxidase (MPO) activities were measured in the control, low-volume and high volume groups with or without pretreatment with captopril (500 mg/l) for three days (n= 3 for each group). High-volume ventilation (HV) significantly increases the MPO activity in the lungs (** $p < 0.05$) compared with the control group, and can be attenuated by pretreatment with captopril (** $p < 0.05$).

Figure 2:
Effect of mechanical ventilation on the expression of pro-inflammatory cytokines in rat lungs. Lung expressions of TNF-α and MIP-2 mRNAs were determined by RT-PCR. **A**. Chronologic pattern of TNF-α in rat lung treated with 4 hours of high-tidal-volume (40 ml/kg) ventilation; **B**: Effects of different tidal volumes on the expression of TNF-α mRNA in rat lung after 4 hr of MV (n=6 for each group); **C**: Effects of different tidal volumes on the expression of MIP-2 mRNA in rat lung after 4 hr of MV (n=6 for each group). LV: low tidal volume group (7 ml/kg); HV: high tidal volume group (40ml/kg) (* $p<0.05$; ** $p<0.01$). LV=low tidal volume; HV = high tidal volume.

Figure 3:
Effect of captopril on the activation of nuclear factor-κB (NF-κB) in rat lungs induced by mechanical ventilation. **A**. Western blotting of cytosolic and nuclear portions of the p65 compartment of NF-κB in rat lungs. **B**. Western blotting of I-κB and phosphorylated I-κB of rat lung tissue. Representative findings are shown 4 hours after treatments. LPS: lipopolysaccharide-treated non-ventilated group; Ctrl: non-ventilated control group; LV: ventilation with low tidal volumes (7 ml/kg); HV: ventilated with high tidal volumes (40 ml/kg).

Figure 4:
Effect of mechanical ventilation of the expression of the RAS components. **A**. Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR) for determination of the mRNA expression of angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin II type 1 receptor (AT1) and type 2 receptor (AT2) in rat lungs. High-volume ventilation (40 ml/kg) significantly increases the mRNA expression of angiotensinogen, AT1 and AT2 (** $p < 0.01$) (n=3 for each group). **B**. Radioimmunoassay (RIA) of angiotensin II in the blood of rats shows significantly increase if angiotensin II level by high-volume ventilation (* $p < 0.05$) (n=3 for each group).

Figure 5:
Effect of losartan and PD123319 on the expression of MIP-2 in rat lungs treated with 4 hours of high-volume (40 ml/kg) mechanical ventilation. Reverse transcription-polymerase chain reaction (RT-PCR) shows attenuated expression of MIP mRNA in losartan-treated group (A) as well as in PD-123319-treated group (B). Enzyme-linked immunosorbent assay (ELISA) showed both losartan (C) and PD123319 (D) could attenuate the protein expression of MIP-2 in rat lungs treated with high-volume ventilation (n=3 for each group).

Figure 6:
Survival plotting of rats treated with 4 hours of mechanical ventilation with either low tidal volume (LV) or high tidal volumes. Rats were extubated after finishing the mechanical ventilation and observations were censored at the end of 7 days. Kaplan-Meier estimation was applied and a comparison between the groups was made by Log-Rank test. Significantly decreased survival was noted in high-VT group (n=10), as compared with low-VT group (n=10) (** $p < 0.01$), whereas pretreatment with captopril (n=15) tended to improve the survival of high-VT group (* $p = 0.062$).

TABLE

Table 1. Oligonucleotide sequences used as primers and probes for real-time RT-PCR

Gene Name	Oligonucleotide	Sequence	Position	Amplicon
Agt	Forward primer	GTGGAGGTCCTCGTCTTCCA	1239-1258	108
	Reverse Primer	GTTGTAGGATCCCCGAATTTCC	1346-1325	
Ace	Forward primer	CGGTTTTCATGAGGCTATTGGA	3080-3101	102
	Reverse Primer	TCGTAGCCACTGCCCTCACT	3181-3162	
Agtr1	Forward primer	GAAGCCAGAGGACCATTTGG	1260-1279	101
	Reverse Primer	CACTGAGTGCTTTCTCTGCTTCA	1360-1338	
Agtr2	Forward primer	GCCAACATTTTATTTCCGAGATG	528-550	81
	Reverse Primer	TTCTCAGGTGGGAAAGCCATA	608-588	

Agt: angiotensinogen; *Ace*: angiotensin-converting enzyme; *Agtr1*: angiotensin II receptor type 1; *Agtr2*: angiotensin II receptor type 2

FIGURES

Figure 1

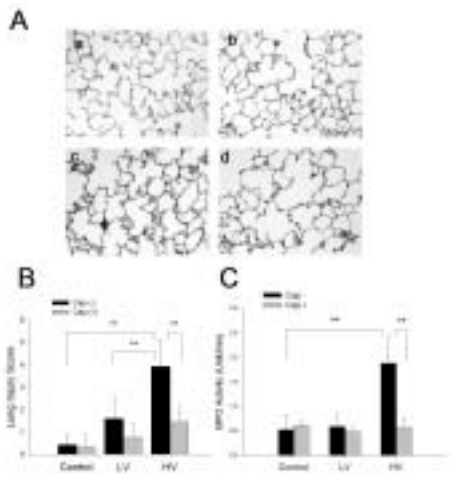

Figure 2

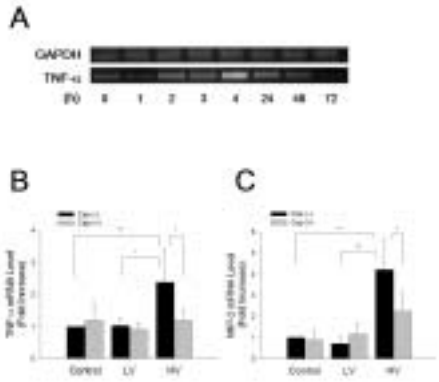

Figure 3

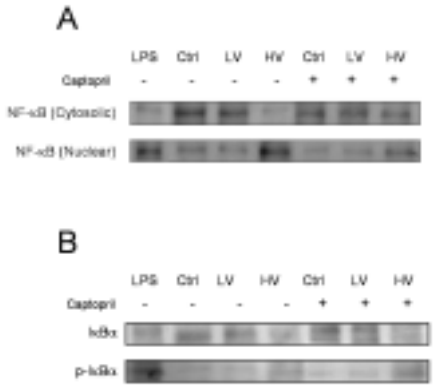

Figure 4

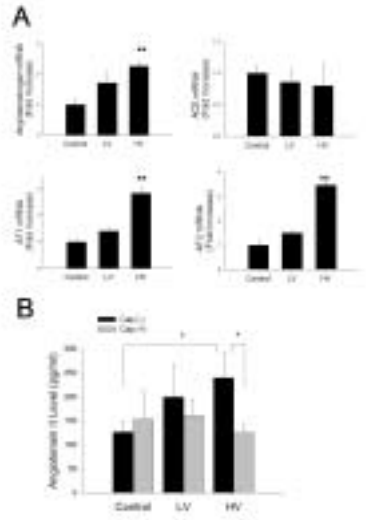

Figure 5

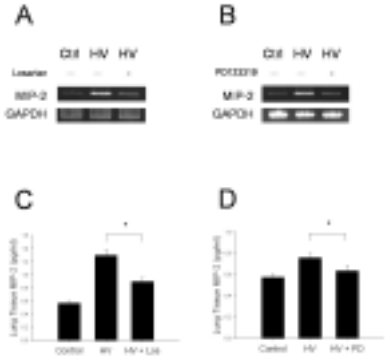

Figure 6