

Losartan attenuates microvascular permeability in mechanical ventilator-induced lung injury in diabetic mice

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Abstract Mechanical ventilation can cause direct injury to the lungs, a type of injury known as ventilator-induced lung injury (VILI). VILI is associated with up-regulates angiotensinogen and AT1 receptor expression of in the lung. This work explored effects of losartan on VILI in diabetic mice. Ninty-six C57Bl/6 mice were randomly divided into six groups, control group (C group), diabetes group (D group), diabetes mechanical ventilation group (DV group), losartan control group (L + C group), losartan treatment group in diabetic mice (L + D group) and losartan treatment group in mechanical ventilation diabetic mice (L + DV group). Lung W/D, myeloperoxidase (MPO) activity, microvascular permeability, blood–gas analysis, Ang II concentrations and AT-1R protein expression were measured. Compared with D group, DV group increased Ang II concentrations, AT-1R protein expression, W/D ratio, MPO activity, and microvascular permeability. PaO₂ were significantly lower in the DV group than D group or control group. Compared with DV group, L + DV group attenuates ventilator-induced lung injury in diabetic mice and prevented the increase Ang II concentrations, AT-1R protein expression and microvascular permeability caused by ventilation in diabetic mice. This

study provides in vivo evidence that losartan attenuates microvascular permeability via down-regulates Ang II and AT-1R expression in mechanical ventilator-induced lung injury in diabetic mice.

Keywords Ventilator-assisted lung injury · Diabetes mellitus · Microvascular permeability · Losartan

Introduction

It has been shown that some mechanical ventilation strategies can cause ventilator-induced lung injury (VILI) [1, 2], which is currently known to present as nonspecific physiological and morphological changes in the lung parenchyma. Changes in the balance of fluids in the lungs, diffuse alveolar injury, increased endothelial permeability and increased epithelial permeability have been described in animals submitted to mechanical ventilation [3]. However, the detailed mechanism behind “endothelial dysfunction” in VILI in diabetic mice is not well understood yet.

Diabetes is associated with increased risk of morbidity and early mortality as a result of its chronic microvascular complications [4]. Recently, Renin-angiotensin system (RAS) blockade induces diabetes protective effects is further supported by a meta-analysis of randomized clinical studies [5]. Circulating and tissue RAS outlines the physiological functions of the RAS major active substance, angiotensin II (ANG II). Circulating ANG II is generated from angiotensin I (ANG I) by carboxypeptidase angiotensin-converting enzyme (ACE) expressed in the pulmonary endothelial cells [6]. Local RASs play a multitude of autocrine, paracrine and/or intracrine roles in the regulation of specific tissue and organ functions compared with

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circulating RASs. The existence of a pulmonary RAS and high ANG II concentrations have been demonstrated in normal rat lung [7]. In addition, angiotensinogen and ANG II type 1 receptors (AT1) are also expressed in the lung tissue [8]. Furthermore, it has been demonstrated that losartan, a selective inhibitor of subtype AT1 receptors for angiotensin II, can relieve acute lung injury caused by high volume ventilation [9].

Local Ang II synthesis increases vascular permeability by promoting the expression and secretion of vascular endothelial growth factor (VEGF) [10]. Therefore, we hypothesized that VILI could be promoted by local ANG II action in diabetic mice. We tested this hypothesis by investigating the pulmonary expression of the angiotensinogen and AT-1R protein expression in a model of VILI in diabetic mice. Meanwhile, we assessed the effect of losartan, a selective inhibitor of AT1 receptor, on the pulmonary microvascular permeability induced by VILI in diabetic mice.

Materials and methods

We obtained institutional review board approval to perform this study. All animal experiments were performed in accordance with Wuhan University Guide regarding the care and use of animals for experimental procedures.

Animals

Normal female C57Bl/6 wild-type (Wt) mice with weights ranging from 18 to 22 g, 6–8 weeks of age, were obtained from the experimental animal center of Wuhan University School of Medicine. The animals were housed in individual cages in a temperature, humidity, and light-controlled room (12 h light and 12 h dark cycle), and acclimated for at least 7 day prior to use in experiments.

Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma Chemical Co., St. Louis, Mo.) at a dose of 150 $\mu\text{g/g}$ body wt in 50 μl of sterile 0.05 mol/l sodium citrate (pH 4.5). The control group received an equal volume of vehicle. At 48 h and 10 days after streptozotocin injection, the blood glucose level was measured with a Glucometer (Abbott Diabetes Care Inc., Alameda, CA). All STZ-induced diabetic mice developed stable hyperglycemia (STZ 22.8 ± 3.1 vs. controls 8.5 ± 0.6 mmol/l, $P < 0.001$). The experimental protocol was approved by the Animal Experimentation of Wuhan University School of Medicine.

Groups and experimental protocols

Ninety-six C57Bl/6 mice were randomly divided into the following experimental groups: control group (C group),

diabetes group (D group), diabetes mechanical ventilation group (DV group), losartan control group (L + C group), losartan treatment group in diabetic mice (L + D group) and losartan treatment group in mechanical ventilation diabetic mice (L + DV group). Animals from each group were divided equally into two subgroups for specific studies: one half of the animals were used for pulmonary microvascular leakage determination, and the other half of animals were used for biochemical analysis. All mice were placed in a supine position and connected to a ventilator with a TOPO small animal ventilator (kent scientific, Torrington, USA). A Y-tube connector was surgically inserted into the trachea under general anesthesia with 100 mg/kg ketamine, 0.2 mg/kg medetomidine, and 1.0 mg/kg atropine (i.p.). Maintenance anesthesia consisted of 36 mg/kg ketamine (Hengrui medicine co., LTD, Jiangsu, china), 0.04 mg/kg medetomidine (Hongfangde medicine co., LTD, jinan, china), and 0.075 mg/kg atropine (tianjing pharmaceutical group xinzheng Co., tianjing, china). Maintenance mix was administered via an intraperitoneal catheter (PE 10 tubing; BD, Breda, the Netherlands) every hour. To correct for hypovolemia, 0.9 % NaCl solution was administered via the intraperitoneal catheter every 30 min. DV group mice were ventilated with an inspiratory pressure of 18 cm H₂O for 4 h (resulting in injurious VT ~ 15 mL/kg). Respiratory rate was set at 70 breaths/min. Positive end-expiratory pressure was set at 2 cmH₂O. FIO₂ was kept at 0.5. The inspiration-to-expiration ratio was kept at 1:1 throughout the experiment. Control and D group mice, receiving equate the dose of induction anesthesia, were instrumented, but not ventilated. L + C group, L + D group and L + DV group mice received intraperitoneal injections of Losartan (Merck - Sharp & Dohme Corp, USA) 30 mg/kg 30 min before mechanical ventilation. Animals were killed at the end of the experiment. The unventilated control group was killed immediately after tracheotomy in an identical way.

Sample preparation

Plasma samples were collected and stored at -20 °C. Tissue samples were frozen and stored at -70 °C for index assessment.

Analysis of lung water

Lung tissues were taken from the right upper lobes, rinsed in saline, and weighed immediately then dried at 80 °C for 24 h and re-weighed until the weight became constant. Lung W/D was used as an index of pulmonary edema formation.

Myeloperoxidase assay

One of the left lung lobes was frozen at -70°C until assay. Frozen tissue samples were later thawed, homogenized in 0.2 mmol/L potassium phosphate buffer (PPB, pH 7.4) with homogenizer ($\sim 3,400$ rpm on ice) and centrifuged at $4,000\times g$ for 30 min at 4°C . The procedure of MPO content followed the instruction for the MPO assay kit from Jiancheng Co.

Evaluation of microvascular permeability

Pulmonary microvascular permeability was measured according to the method described by Chen [11]. Fluorescein isothiocyanate (FITC)-labeled bovine albumin 10 mg/kg (Sigma-Aldrich, St. Louis, MO) was intravenously administrated to animals 4 h before the end of mechanical ventilation. Eight mice from each group were sacrificed and the pulmonary vascular permeability was determined. At the end of the experiments, mice were exsanguinated by cardiac puncture and serum was collected. The lungs were lavaged in situ three times with phosphate-buffered saline. The bronchoalveolar lavage (BAL) fluid was centrifuged and the supernatant was collected for FITC fluorescence measurement. FITC fluorescence in the serum and BAL fluid was measured using a fluorescence spectrometer (BX-400; Ker Machinery & Engineering Co. Ltd, Wuhan, china) with excitation at 494 nm and emission at 520 nm. The BAL-to-serum FITC fluorescence ratio was calculated and taken as a measure of pulmonary microvascular permeability.

Blood–gas analysis

Arterial blood was assayed immediately after being taken from the carotid artery when the animal was killed by exsanguinations. Arterial blood samples were analysed with a clinical blood gas analyser (I-STAT Portable Clinical Analyser, I-STAT, USA) for partial pressures of oxygen (PaO_2).

Microscopy observation

One of the left lung lobes were cut into 1-mm-thick slices, immersion-fixed in 2.5 % buffered glutaraldehyde at 0 to 4°C for 60 min, buffered in phosphate-buffered saline, and fixed with 1 % osmic acid, then washed with distilled water, and dehydrated by dimethylketone. After being embedded in Epon-812, they were cut into ultrathin sections (70 nm) by an ultramicrotome (Dupont, Newtown, Conn) and stained with uranyl acetate and plumbum citrate. Sections were examined under a Hitachi H-600 electron microscope (Hitachi, Tokyo, Japan).

Enzyme Immunoassay for Ang II

Extraction of peptides from lung tissues or plasma was performed on the basis of instruction provided by the company in the kit (Phoenix Pharmaceuticals, Burlingame, CA). Briefly, homogenize the tissues in ice bath. The homogenate was centrifuged at $1,300\times g$ for 30 min at 4°C . The supernatant was collected, and the total protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). The supernatant or plasma was mixed with *buffer A* at the ratio of 1:3 and centrifuged at 3,500 rpm for 15 min at 4°C . Sep-column containing 200 mg of C18 was equilibrated by washing once with 1 ml of *buffer B* and three times with 3 ml of *buffer A* for each time. The supernatants were loaded onto the pretreated C18 sep-column. The peptides were slowly eluted with *buffer B* (3 ml, once). The extracts from lung tissue or plasma were reconstituted with $1\times$ assay buffer and assayed with the Ang II Enzyme Immunoassay Kit (SPI Bio, Massy, France). The concentrations of Ang II are expressed as Ang II picograms per milligram protein for lung tissue or milliliters for plasma.

Western blotting for AT-1R protein expression

Western blotting for AT-1R was performed. All samples were kept on ice in lysis buffer (1 % NP-40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate) and homogenised until the solution turned clear. The total protein concentrations of the samples were measured by the bicinchoninic acid (BCA) method. Equal amounts of protein extract (30 μg) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, CA, USA) and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked with 5 % non-fat dried milk or 5 % bovine serum albumin in Tris buffered saline Tween (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1 % Tween 20). The membrane was incubated overnight at 4°C with primary anti AT-1R antibody (Santa Cruz Biotechnology, CA, USA), and the bound antibody was visualized using the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.). The immune complex was visualized by using enhance chemiluminescence. The relative densities of AT-1R, and β -actin bands were quantified by an alpha imager 2000 analyzer.

Statistical analysis

All data were presented as mean \pm standard deviation. Analysis of variance (ANOVA) and the Student–Newman–Keuls q test were used for statistical analysis to compare values among all groups. A significant difference was presumed for a probability value of <0.05 .

Table 1 Effects of losartan on PaO₂, W/D and MPO activity on VILI in diabetic mice ($n = 8$, $x \pm s$)

| Groups | PaO ₂ (mmHg) | W/D (g/g) | MPO (uU/mg prot) |
|---------|-------------------------|--------------------------|----------------------------|
| Control | 97 ± 2 | 4.02 ± 0.21 | 12.56 ± 2.21 |
| D | 94 ± 5 | 4.19 ± 0.25 | 18.68 ± 2.03* |
| DV | 76 ± 7*† | 5.11 ± 0.44*† | 35.11 ± 4.01*† |
| L + C | 98 ± 4 | 4.05 ± 0.23 | 12.09 ± 2.11 |
| L + D | 96 ± 5 | 4.17 ± 0.28 | 14.08 ± 2.64† |
| L + DV | 88 ± 6 [△] | 4.29 ± 0.35 [△] | 19.98 ± 2.39* [△] |

Compared with control group * $P < 0.05$, Compared with D group † $P < 0.05$, Compared with DV group [△] $P < 0.05$

Results

Lung wet-to-dry weight ratios and myeloperoxidase activity

We evaluated neutrophil accumulation in lung tissue by MPO assay, and evaluated organ edema by W/D. As shown in Table 1, The DV group had a significantly higher lung W/D and MPO than the D group. However, W/D and MPO significantly decreased in L + DV group compared with DV group.

Blood–gas analysis and histological examination

Ventilator-induced lung injury in diabetic mice was confirmed by arterial blood gas analysis (Table 1) and histological examination (Fig. 1). As shown in Table 1, DV

group mice had about ~20 % lower PaO₂ than C group and ~15 % lower PaO₂ than D group. L + DV group might protect against this decrease in PaO₂ of DV group. Under transmission electron microscope, we could observe the basement membranes of alveolar epithelium and capillary endothelium became swollen, widened; osmiophilic lamellar bodies emptied and the basement membrane were broke to varying degrees in DV group (Fig. 1, slide C). In comparison, L + DV group showed less lung damages and the changes of pathology (Fig. 1, slide D). C group has normal structure of mitochondria and lamellar bodies in alveolar epithelial cell (slide A).

Microvascular permeability

The group D and DV had significantly higher microvascular permeability than the control group. Pulmonary microvascular permeability was increased in DV group compared with D group (Table 2). After treatment of with losartan, this increase was dramatically attenuated.

Lung Ang II and AT-1R protein expression

The expression levels of AT1 receptor protein expression and concentrations of Ang II were significantly different between the Control, D, DV and L/P group. Diabetes increased plasma and lung Ang II levels (Table 2). AT1 receptor expression levels (Fig. 2) and concentrations of Ang II in the DV group significantly increased when compared with the Control and D groups. Mechanical ventilation pretreated with losartan significantly decreased

Fig. 1 Changes of pulmonary ultrastructure in each group mice. The basement membrane were broke to varying degrees in DV group, Losartan I treated mice showed little organelle changes. **a** Control group (original magnification ×8,000). **b** D group (×8,000). **c** DV group (×10,000). **d** L + DV group (×8,000)

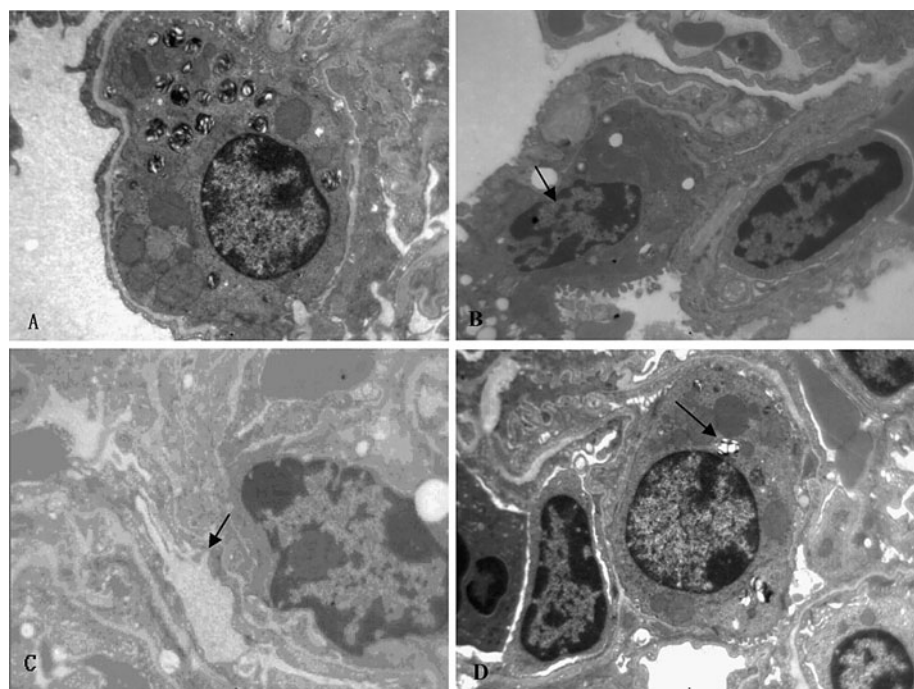


Table 2 Effects of losartan on BAL-to-serum FITC-albumin fluorescence ratio and Ang II levels on VILI in diabetic mice ($n = 8$, $x \pm s$)

| Groups | BAL/serum FITC ratio | Ang II (pg/ml) | |
|---------|----------------------------|----------------------------|---------------------------|
| | | plasmal | lung |
| Control | 13.51 \pm 1.49 | 460.8 \pm 23.9 | 1.15 \pm 0.14 |
| D | 20.31 \pm 2.02* | 960.3 \pm 54.8 | 4.33 \pm 0.39* |
| DV | 39.78 \pm 3.47*† | 1,650.6 \pm 79.7*† | 6.21 \pm 0.32*† |
| L + C | 13.96 \pm 1.33 | 459.5 \pm 27.5 | 1.18 \pm 0.16 |
| L + D | 15.24 \pm 2.04† | 614.9 \pm 33.2*† | 2.29 \pm 0.25*† |
| L + DV | 23.25 \pm 2.85* Δ | 639.2 \pm 40.7* Δ | 2.46 \pm 0.18* Δ |

Compared with control group, * $P < 0.05$; Compared with D group, † $P < 0.05$; Compared with DV group, $\Delta P < 0.05$

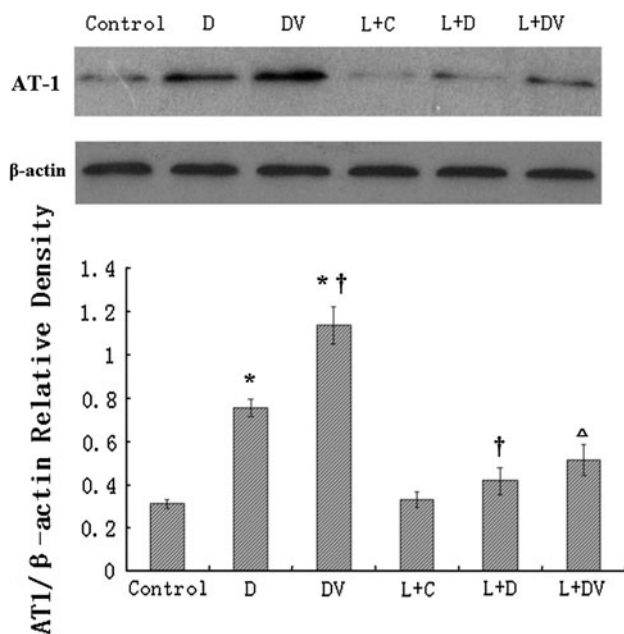


Fig. 2 Western blotting for AT1-R protein expression. AT1 receptor expression levels in the DV group significantly increased when compared with the Control and D groups. Mechanical ventilation pretreated with losartan significantly decreased AT1 Receptor protein expression in lung tissues. (Compared with control group, * $P < 0.05$; Compared with D group, † $P < 0.05$; Compared with DV group, $\Delta P < 0.05$)

the level of Ang II and AT1 receptor protein expression in lung tissues.

Discussion

Although mechanical ventilation is an important therapy that is widely used in intensive care medicine, it can result in complications. One major complication is ventilator-induced lung injury.

Damages to the alveolar-capillary barrier, alveolar edema, and inflammation due to excessive mechanical stretch have been recognized as major pathophysiologic events of VILI [12]. This type of lung injury is manifested by increased endothelial and epithelial barrier permeability in the lung [13]. The mechanism of such an increase in permeability is not well understood. It was also reported that mechanical stretch promoted IL-8 synthesis with activation of p38 in HPMVECs. Moreover, cyclic stretch enhanced IL-6 and MCP-1 production and induced endothelial reorientation accompanied by actin polymerization [14]. The induction of a proinflammatory response in the lung epithelium is associated with an alteration in permeability in the local endothelium [15]. VEGF is involved in the regulation of vascular permeability and endothelial cell survival. A hypothetical protective effect of hydroxyethyl starch (HES) against VILI has been associated with the regulation of VEGF [16]. Rearrangement of the cytoskeleton regulates vascular endothelial barrier function and appears to be regulated by nonmuscle myosin light-chain kinase isoform (nmMLCK), which also has a regulatory role in angiogenesis, endothelial cell apoptosis, and leukocytic diapedesis [17, 18].

The present study reveals that, endothelial function of the microcirculation, which is usually evaluated through the vasodilator response to endothelium-dependent vasodilators or physiological stimuli, is characteristically impaired in patients with Type 1 diabetes [19]. Hyperglycaemia also increases endothelial nitric oxide synthase activity, resulting in increased nitric oxide release [20]. This, in turn, leads to microvascular endothelial dysfunction. However, although the endothelial dysfunction plays a fundamental role in the pathogenesis of ventilator-induced lung injury, there have been few *in vivo* studies during this pathological process. VILI cannot be distinguished from endothelial dysfunction that lead to hyperglycaemia, and VILI can worsen the initial profile when patients with diabetes are subjected to mechanical ventilation.

Blockade of the renin-angiotensin system (RAS) plays an important role in preventing organ injury associated with diabetes. Increasing evidence indicates that Ang II can directly enhance the expression of P-, E-, and L-selectins, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 on vascular endothelial and smooth muscle cells [21]. In addition, it was also found that ANG II induced oxidative stress by increasing the activity of NADPH oxidase in vascular endothelial cells. However, although the RAS plays a fundamental role in the ventilator-induced lung injury and diabetes, there have been few *in vivo* studies of alterations in the permeability in mechanical ventilator-induced lung injury in diabetic animal model. Therefore, we hypothesized that VILI might be

promoted by local ANG II action during diabetes and be alleviated by RAS blockade.

In the current study, we showed that mechanical ventilation with an inspiratory pressure of 18 cmH₂O in diabetic mice produced acute lung injury, as manifested by neutrophilia, and significantly decreased PaO₂. We ruled out the interference of anesthetic drugs on PaO₂ because our present data show that DV group mice had about ~20 % lower PaO₂ than control group and ~15 % lower PaO₂ than D group. Up-regulation of ANG II and AT1 receptor may contribute to VILI because of the role of ANG II and AT1 receptor in inflammatory response, oxidative stress, and lung cell epithelial apoptosis. We show that losartan was effective in preventing the development of vascular endothelium permeability to VILI in diabetic mice. Up-regulation of ANG II and AT1 receptor probably stimulates the release of inflammatory cytokines and chemokines from lung vascular endothelial cells during diabetes. However, the mechanism of up-regulation of angiotensinogen and AT1 receptor act on VILI remains to be further clarified. In the meantime, our experiments show that losartan can attenuate lung edema, neutrophil accumulation, increased microvascular permeability and alveolar septum fracture caused by VILI in diabetic mice. We suggest that the protection of losartan probably relates to blocking the action of ANG II and AT1 receptor.

In conclusion, our experiments demonstrate that losartan attenuates microvascular permeability via down-regulation of Ang II and AT-1R expression in mechanical ventilator-induced lung injury in diabetic mice.

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