Online supplement

Role of the renin-angiotensin system in ventilator-induced lung injury: An in vivo study in a rat model

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Methods

Reagents

Captopril, PD123319, lipopolysaccharide (LPS) from Escherichia coli (serotype 055:b5), and, unless otherwise indicated, all other chemicals were purchased from Sigma (Sigma Chemical, St. Louis, MO). Losartan, a specific AT1 receptor antagonist, was a gift from Merck (Merck & Co., Inc., Whitehouse Station, NJ). Antibodies to rat TNF-α, ACE, ACE2, AT1, AT2, or rat NF-κB p65 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies to rat I-κB or phosphorylated I-κB were purchased from Cell Signaling (Beverly, MA).

Animal preparation, mechanical ventilation protocol, and drug treatment

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

Male Sprague-Dawley rats weighing 200-250 g were cared for, 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 mechanical ventilation was applied. Tracheostomy was performed, followed by arterial and venous catheterization. The tracheostomy tube was then connected to a volume-controlled ventilator for small animals (New England Medical Instruments, Inc., Medway, MA) and the animal ventilated according to the study design protocol. Immediately before starting mechanical ventilation, the animals were given intravenous pancuronium (4 mg/kg).

MV was applied using methods modified from the protocol described elsewhere [1, 2] that a very high tidal volume (40 ml/kg) was used to create injurious inflation of the alveoli. The animals were divided into the following three experimental groups: 1) non-ventilated controls; 2) treated with MV with a high tidal volume (40 ml/kg tidal volume, 3 cmH_2O of positive end-expiratory pressure [PEEP], 20 breaths/min, room air); 3) treated with MV with a low tidal volume (7 ml/kg tidal volume, 3 cmH_2O of PEEP, 100 breaths/min, room air). The control group only received the intraperitoneal injection of urethane. In the low-tidal volume and high-tidal volume groups, MV was applied for 4 hours and the peak airway pressure was monitored throughout. Blood samples (0.1 ml) were taken from the left femoral artery via the cannula immediately before starting ventilation and every 60 minutes thereafter. Arterial blood gas analysis was performed on site immediately after blood sampling using a portable analyzer

(i-STAT, Abbott Laboratory, Abbott Park, IL, USA) according to the manufacturer's recommendations. After ventilation, the animals were given a lethal dose of intraperitoneal pentobarbital, then, after flushing the pulmonary vessels with intracardiac injection of normal saline, the lungs were removed en block. The right lung was immediately frozen in liquid nitrogen and stored at -80˚C for further analysis, while the left lung was processed for further studies described below. As a positive control for lung injury and inflammation, another group of non-ventilated rats received E. coli LPS instilled transtracheally [0.2 mg/100 g body weight dissolved in 0.5 mL of phosphate-buffered saline (PBS)] . The LPS solution was dispersed in the trachea just above the level of tracheal bifurcation.

Additional groups of rats received captopril pre-treatment before MV or were treated with losartan during MV. For the captopril-treated group, 50 mg/kg of captopril was added to the drinking water (500 mg/l) for the three days preceding mechanical ventilation. For the losartan- or PD123319-treated, losartan (10 mg/kg) or PD123319 (10 mg/kg) was injected intravenously via a pump during the 4 hours of MV. All medicated animals underwent the same ventilatory strategies described above.

Histological studies

The left 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, dehydrated with a graded alcohol series and embedded in paraffin at 52°C. Sections were prepared and stained with hematoxylin and eosin for histological evaluation. Each lung section was scored for lung injury by a board-certified pathologist using previously published criteria [3]. Briefly, ALI was scored based on: 1) alveolar capillary congestion; 2) hemorrhage; 3) infiltration or aggregation of neutrophils in the air space or the vessel wall; and 4) thickness of the alveolar wall/hyaline membrane formation. Each item was graded according to the following five-point scale: 0 = minimal (little) damage; 1 = mild damage; 2 = moderate damage; 3 = severe damage; and 4 = maximal damage. The average sum of each field score was compared among groups.The total lung injury score for each animal was given as the mean of the scores for five lung sections.

Myeloperoxidase assay

Myeloperoxidase (MPO) in the lung parenchyma was used as a marker enzyme for neutrophil infiltration into the lung [4, 5]. The left lungs were washed with saline and immediately homogenized in 10 mM potassium phosphate buffer, pH 7.4, containing 1.0 mM EDTA. The homogenate was centrifuged at 10,000 g at 4°C for 20 min and

the pellet resuspended in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% (vol/vol) hexadecyltrimethyl-ammonium bromide (Sigma), re-homogenized, and sonicated and the suspension centrifuged at 40,000 g at 4°C for 15 min. A sample of the supernatant (0.1 ml) was added to 2.9 ml of 50 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml of O-dianisidine hydrochloride (Sigma) and 0.0005% (wt/vol) hydrogen peroxide and the absorbance of the solution at 460 nm measured over 3 min. MPO activity was expressed as international units per gram of dry tissue, one unit of enzyme activity being defined as the amount of peroxidase that produced an absorbance change of 1.0 optical density unit/min at 25°C.

Bronchoalveolar lavage fluid protein measurement

Bronchioloalveolar lavage (BAL) was performed as described previously [6]. Briefly, after the right main bronchus was ligated, 4 ml of PBS at room temperature was slowly instilled into the trachea and the left lung via the tracheostomy using a gauge connected to a catheter, followed by inspiration with 1 ml of air. The lung fluid was drained by gravity by changing the position of the animal and collected on ice, and 30 μl of a 1 mg/ml solution of aprotinin (Sigma), 10 μl of a 10 mg/ml solution of PMSF in isopropanol, and 10 μl of 100mM sodium orthovanadate per ml of lung fluid was immediately added. The BAL fluid was centrifuged at 1000 g for 10 min at 4˚C and

the supernatant collected and stored at -80˚C for further analysis. Total protein in the BAL fluid was determined using a commercialized BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's protocol as described previously [7, 8].

Tissue RNA extraction

Total RNA from the right lung of rats after 4 h after treatment was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, lung tissue (0.5 g) was homogenized in liquid nitrogen and 1 ml of TRIzol added. One millilitre of the mixture was taken and 200 μl of chloroform added. The mixture was centrifuged at 13000 g at 4°C for 15 min, then the supernatant was removed and mixed with 0.5 ml of isopropanol and the mixture centrifuged at 13000 g at 4°C for 15 min. The supernatant was discarded and the pellet washed with 1 ml of 75% ethanol, then 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 electrophoresis (1% agarose-formaldehyde gel containing 20 mM morpholinosulphonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0).

Reverse transcription and polymerase chain reaction (RT-PCR)

TNF-α and macrophage inflammatory protein (MIP)-2 mRNA levels in the lung tissue were examined by the reverse transcription and polymerase chain reaction (RT-PCR) [9]. One microgram of RNA was subjected to first-strand cDNA synthesis in a 20 μl reaction mixture containing avian myeloblastosis virus reverse transcriptase (10 U), 2 μl of dNTP mixture (2.5 mM of each dNTP), 0.5 μl of RNase inhibitor, 1 μl of oligo(dT)12–18 primers (10 μM) and reaction buffer (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, then at 94°C for 2 min (enzyme denaturation step). The reverse transcription mixture was stored at -20°C for use in the PCR. All reagents were from Promega (Southampton, U.K.).

Four microliters of the reverse-transcribed products was used as the RT-PCR template. PCR amplification was performed using rTaq DNA polymerase (Takara, Shiga, JP) with an initial denaturation at 95°C for 5 min, amplification for 27-30 cycles of denaturation at 94°C for 30s, annealing at 55°C (GAPDH) or 60°C (all other transcripts) for 30 s and extension at 72°C for 45 s, and a final extension at 72°C for 7 min. The gene-specific oligonucleotide primers for TNF-α, MIP-2, and GAPDH have been described previously [9]. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Bands of each target transcript were

visualized by ultraviolet trans-illumination and captured using a digital camera. Each band was quantified by image analysis software AlphaImager System (Alpha Innotech Corporation). The level of gene expression for each transcript was normalized to that of the housekeeping gene, GAPDH.

Real-time reverse transcription-polymerase chain reaction

Real-time RT-PCR was used to quantitatively measure mRNA levels for RAS components [10]. Oligonucleotide primers for rat angiotensinogen, ACE, ACE2, and the AT1 and AT2 receptors were designed from the GenBank databases (NM 012544 and NM 134432) (Table 1) 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 manufacturer's instructions. In brief, reverse transcription was carried out in a total volume of 25 μl consisting of 5 μl of the RT sample (250 ng), 12.5 μl of QIAGEN SYBR Green Master Mix (QIAGEN, Valencia, CA), 1 μl of each of the forward and reverse primers (5 μM) and 5.5 μl of H_2O. The thermocycling conditions were 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. The amounts of mRNA were determined by comparing the cycle threshold of each sample to those of RT standard curves. The

mRNA levels for each RAS component was normalized to the GAPDH mRNA levels in each sample and expressed as a fold increase compared to the control group.

Measurement of lung tissue angiotensin II levels

Lung tissue levels of angiotensin II were determined with EIA following the methods described previously[11]. Lungs were homogenized 10 100% methanol at 4°C, lyophilized, and resuspended in EIA assay buffer (Peninsula Loboratories, Belmont, CA, USA). Columns were pretreated with one volume of methanol and then two volumes of H_2O. Following sample application, columns were washed with one volume each for H2O, hexane and chloroform and bound material eluted in 90% methanol. Eluted samples were lyophilized, resuspended in assay buffer, and quantitated by EIA (Peninsula Laboratories). Developed EIA plates were read on a plate reader, and data were then analyzed. All samples were run in duplicate.

Preparation of cytosolic and nuclear proteins from rat lung tissue and Western blot analysis for NF-κB and I-κB

To investigate the involvement of the NF-κB pathway in VILI, we performed Western blotting to assess the amounts of NF-κB, I-κB and phosphorylated I-κB in the rat lungs. A sample of the left lung (0.5 g) was homogenized and washed with

cold PBSOK. For extraction of cytosolic and nuclear proteins, 200 µl of cold buffer A (10 mM HEPES-KOH, 1.5 mM $MgCl_2$ and 10 mM KCl) containing 10 µl/l of a protease inhibitor mixture (Sigma Protease Inhibitor, Sigma-Aldrich) was added and the sample placed on ice for 10 minutes, then centrifuged at 8000 g for 1 minute at 4°C. The supernatant was collected as the cytosolic protein fraction and the pellet resuspended in cold buffer C (20 mM HEPES, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA and 25% glycerol), placed on ice for 10 min and centrifuged at 13000 g for 2 min at 4°C. The supernatant was collected as the nuclear protein fraction.

For analysis of NF-κB p65 and I-κB, 20 µg of cytosolic or nuclear protein was electrophoresed on a 10 % SDS-PAGE gel and transferred to a polyvinylidine difluoride membrane (Amersham, Arlington Heights, IL), which was then blocked for 1 h at room temperature (RT) in 5 % non-fat dried milk in TBST [0.24 % (w/v) Tris, 0.8 % (w/v) NaCl, 0.05 % (v/v) Tweeņ 20, pH 7.6] and incubated for 1 h at RT with monoclonal antibody against rat NF-κB p65 or rat I-κB. After three washes in TBST, the blots were incubated with peroxidase-conjugated-goat anti-rabbit IgG antibody for 1 h at RT and washed for 3 x 10 min with TBST. Bound antibody was then detected using ECL reagent (Amersham) and the immunoreactive bands analysed on an AlphaImager 2200 System (Alpha Innotech Corporation). Antibodies to proliferating cell nuclear antigen (PCNA) or c-Jun N-terminal kinase 1 (JNK1) were used to detect

these proteins, used as loading controls for the nuclear and cytosolic samples, respectively.

Western blot for ACE, ACE2, AT1 and AT2 in the lung tissue

Protein levels of the RAS components in the rat lung were determined by Western blotting. After the rat lungs were removed, they were be homogenized in lysis buffer (50 μM Tris-HCl, 3 mM sucrose, 0.1% Triton X-100, and 1mM protease inhibitor cocktail; Calbiochem-Novabiochem, La Jolla, CA, USA). The supernatant was removed, and protein content was estimated using a Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). Aliquots from lung homogenates were diluted in reducing sample buffer (0.5 M Tris-Cl, 2% β-ercaptoethanol, 87% glycerol, 10% SDS, and 1% bromophenol blue). Protein (40 μg/well) was loaded in 6% polyacrylamide gels, separated by electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada), and then blocked for nonspecific binding in a 7% skimmed milk solution. Membranes were incubated with primary rabbit antibodies against ACE, ACE2, AT1 and AT2 (all from Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at a 1:500 dilution. Membranes were washed and incubated for 1 hour with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImunoResearch, Bio/Can Scientific, Mississauga, Ontario, Canada). After

repeated washing, membranes were incubated with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and placed in a Fluor-X Max Imager, where the image was captured, and bands were analyzed by densitometric analysis. Results on all samples were normalized to control samples as well as the β-actin densities.

Enzyme-linked immunosorbent assay (ELISA) for macrophage-inflammatory protein-2 (MIP-2)

For the MIP-2 assays, 0.3 g of frozen right lung was added to 2 ml of a cold acid-ethanol mixture (93% ethanol, 2% concentrated HCl) containing 85 μg/ml of phenylmethylsulphonyl fluoride and 5 μg/ml of pepstatin A, homogenized for 1 min with a polytron homogenizer and left overnight at 4ºC. The samples were then centrifuged at 10,000 g at 4°C for 20 min and the supernatant collected and assayed for MIP-2 using a rat MIP-2 ELISA kit (Biosource International, Camarillo, CA). Each sample was run in duplicate according to the manufacturer's instructions.

Statistical analyses

All continuous data are expressed as the mean + SD. Comparisons of continuous variables between groups were performed using the t test and one-way analysis of

variance using SPSS 10 software (SPSS, Inc, Chicago, IL) according to the instructions. A p value of < 0.05 was considered significant.

Results

Lung injury scores and TNF-α mRNA expression in rats treated with losartan or PD123319

Concomitant infusion of losartan or PD123319 significantly reduced the lung injury scores of rats ventilated with high tidal volumes (Fig 1 A) ($p=0.001$ for losartan, and 0.03 for PD123319). The lung tissue mRNA levels of TNF-α were also reduced by losartan or PD123319 in the high-volume ventilation group (Fig 1B).

References

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Figure legend

Figure 1. Lung injury scores (panel A) and lung TNF-α levels (panel B) in different groups of VILI. A. Concomitant intravenous infusion of losartan (10 mg/kg) during mechanical ventilation significantly attenuated the lung injury score of the rat lung samples from the high-volume group (p=0.001). Similar finding was noted in the high-volume group treated with concomitant PD123319 (10 mg/kg) (p=0.03). B. Reverse transcription-polymerase chain reaction of the rat lung RNA samples showed a reduction of mRNA expression by either losartan or PD123319 in the high-volume ventilation groups.

Tables

Table 1. Oligonucleotide 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	
Ace2	Forward primer	ACCCTTCTTACATCAGCCCTACTG	755-778	74
	Reverse Primer	TGTCCAAAACCTACCCCACATAT	828-806	
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; *Ace2*: angiotensin-converting enzyme 2; *Agtr1*: type 1 angiotensin II receptor; *Agtr2*: type 2 angiotensin II receptor.