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Original Article

Crosstalk between ACE2 and PLGF regulates vascular permeability during acute lung injury

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Abstract: Angiotensin converting enzyme 2 (ACE2) treatment suppresses the severity of acute lung injury (ALI), through antagonizing hydrolyzing angiotensin II (AngII) and the ALI-induced apoptosis of pulmonary endothelial cells. Nevertheless, the effects of ACE2 on vessel permeability and its relationship with placental growth factor (PLGF) remain ill-defined. In the current study, we examined the relationship between ACE2 and PLGF in ALI model in mice. We used a previously published bleomycin method to induce ALI in mice, and treated the mice with ACE2. We analyzed the levels of PLGF in these mice. The mouse lung vessel permeability was determined by a fluorescence pharmacokinetic assay following i.v. injection of 62.5 μg/kg Visudyne. PLGF pump or soluble Flt-1 (sFlt-1) pump was given to augment or suppress PLGF effects, respectively. The long-term effects on lung function were determined by measurement of lung resistance using methacholine. We found that ACE2 treatment did not alter PLGF levels in lung, but antagonized the effects of PLGF on increases of lung vessel permeability. Ectogenic PLGF abolished the antagonizing effects of ACE2 on the vessel permeability against PLGF. On the other hand, suppression of PLGF signaling mimicked the effects of ACE2 on the vessel permeability against PLGF. The suppression of vessel permeability resulted in improvement of lung function after ALI. Thus, ACE2 may antagonize the PLGF-mediated increases in lung vessel permeability during ALI, resulting in improvement of lung function after ALI.

Keywords: Acute lung injury (ALI), Angiotensin converting enzyme 2 (ACE2), placental growth factor (PLGF), vessel permeability

Introduction

Acute lung injury (ALI) is characterized with expiratory dyspnea, refractory hypoxemia and non-cardiogenic pulmonary edema, the deterioration of which could result in the aggravation of ALI into highly lethal Acute Respiratory Distress Syndrome (ARDS) [1-10]. The cellular pathology of ALI is comprised of loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration and release of pro-inflammatory cytokines, e.g. interleukin (IL)-6, tumor necrosis factor (TNF)-α and CXCL1 [1-4]. Injuries of both pulmonary endothelial cells (PECs) and alveolar epithelial cells occur after ALI, resulting in loss of respiratory capacity [1-4]. Among all these pathological processes, the increases in lung vessel permeability are critical and play a non-redundant role in the pathogenesis of ALI, which allows penetration of neutrophils cross the vascular epithelia to cause the pathological changes [1-4]. Excessive and prolonged activation of neutrophils results in basement membrane destruction and increased permeability of the alveolar-capillary barrier [1-4]. Moreover, neutrophils also release pro-inflammatory and pro-apoptotic cytokines to injure adjacent cells to create ulcerating lesions to aggravate the damages of the alveolar-capillary barrier [1-4].

The vessel permeability is mainly regulated by a coordination of a series of pro-angiogenic and anti-angiogenic factors. Placental growth factor (PLGF) is a member from vascular epithelial growth factor (VEGF) family [11], and is a potent pro-angiogenic factor that is recently defined to play a critical role in the increases in lung vessel permeability in ALI [12]. PLGF exerts its function by binding to its unique receptor, VEGF receptor 1 (VEGFR1, or Flt-1). Soluble Flt-1 (sFlt-1) is thus an inhibitor for PLGF signaling. In

renin-angiotensin system, renin induces the production of angiotensin I (Ang I), which is converted to an important vasoconstrictive peptide Ang II by Ang I-converting enzyme (ACE) [13]. Angiotensin-converting enzyme 2 (ACE2) is the homolog of ACE but counterbalances the ACE activity through induction of degradation of Ang II [13]. Recently, it has been shown that ACE2 has therapeutic effects on ALI, seemingly through different mechanisms, e.g. suppression of apoptosis of PECs [14-20]. However, a role of ACE2 in antagonizing PLGF-mediated increases in lung vessel permeability in ALI has not been acknowledged.

In the current study, we examined the relationship between ACE2 and PLGF in ALI model in mice. We found that ACE2 treatment did not alter PLGF levels in lung, but antagonized the effects of PLGF on increases of lung vessel permeability. Ectogenic PLGF abolished the antagonizing effects of ACE2 on the vessel permeability against PLGF. On the other hand, suppression of PLGF signaling mimicked the effects of ACE2 on the vessel permeability against PLGF. The suppression of vessel permeability resulted in improvement of lung function after ALI.

Materials and methods

ALI model in mice and ACE2 treatment

All mouse experiment protocols were approved by the Animal Research and Care Committee at Fourth Hospital of Hebei Medical University. All experiments were performed in accordance with the guidelines from the Animal Research and Care Committee at Fourth Hospital of Hebei Medical University. Mouse manipulations were performed in accordance with the principles of laboratory care, supervised by a qualified veterinarian. The me-thods were carried out in accordance with the approved guidelines.

Specific pathogen free (SPF) Balb/c mice (aged 10 weeks, weight 18-22 g) were supplied by Laboratory Animal Center of Shanghai Academy of Sciences, Chinese Academy of Sciences, China. After acclimatization for 1 week, a group of mice were used as the no-injury group and received no treatments (NT). The lung injury model was induced in another groups of mice, by intraperitoneal administration of the bleomycin solution (10 mg/mL, Sigma-Aldrich, St Louis, MO, USA) at 20 mg/kg body weight, as has been applied and described before [14-19]. This ALI model is stable and easy to be built. No death was observed in the current study. After successful induction of ALI, the mice were further randomly divided into groups to receive single tail vein injection of either saline (termed ALI), or ACE2 (R&D systems, Shanghai, China) at a dose of 1 mg/kg body weight (termed ALI+ACE2). Ten mice were used in one experimental group.

PLGF or sFlt-1 pump treatment

To increases PLGF levels or suppresses PLGF, 10 μg recombinant PLGF (Sigma-Aldrich) or 1 μg recombinant mouse sFlt-1 (R&D systems, Minneapolis, MN, USA) was dissolved in PBS and placed in mini-osmotic pumps (Alzet osmotic pumps, Cupertino, CA, USA), which were implanted subcutaneously to the mice, at the time of ACE2 treatment. Mini-pumps containing PBS only were implanted in the mice (CTL pump) as a control. The pumps keep releasing solution for 7 days. The mice were analyzed after 7 days.

Assessment of vessel permeability

The mouse was anaesthetized and a left-sided thoracotomy was performed through the 4th intercostal space to allow the left lung mobilized under the microscope. A left cervical incision was performed to cannulate the external jugular vein. Visudyne (Novartis, Hettlingen, Switzerland) was dissolved in PBS and injected of 62.5 μg/kg. Spectrofluorometric measurement of the Visudyne distribution was performed at different time points using an optical fiberbased spectrofluorometer. The detected Visudyne signal was analyzed by an imaging spectrometer and analyzed by computer. The mean fluorescence signal after removing the underlying autofluorescence was plotted for three areas in each animal.

Lung function

Mice were anesthetized, after which a small incision was made to expose the trachea, and a cannula was inserted to connect to an inline nebulizer and ventilator. Mice were then challenged with aerosolized PBS followed by increasing doses of methacholine (Sigma-Aldrich). Airway resistance was determined by analysis of pressure and flow waveforms.

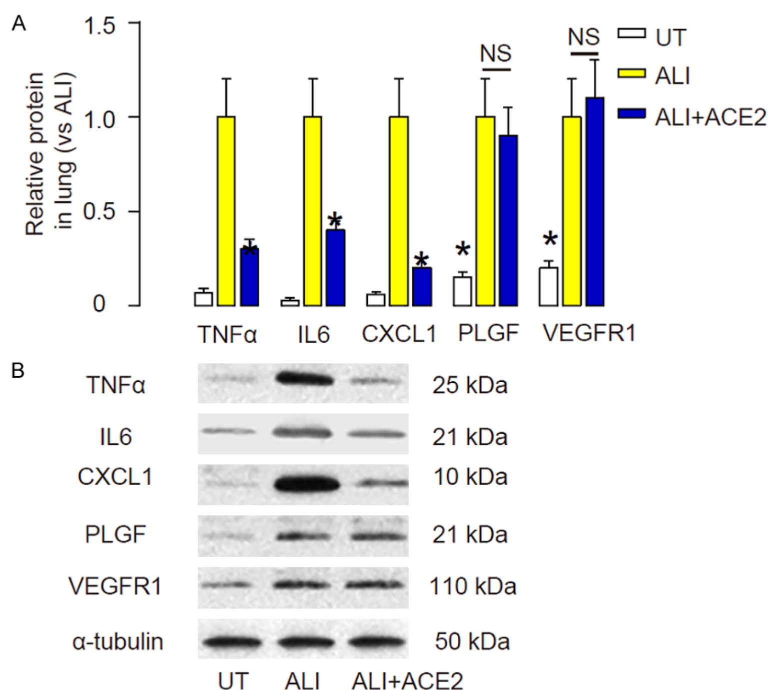

Figure 1. ACE2 does not alter PLGF levels in ALI mouse lung. We isolated protein from the lung of the mice treated with bleomycin only (ALI) and ACE2 after bleomycin (ALI+ACE2). We then analyzed the inflammatory cytokine levels by Western blot, compared to the mice without treatment (UT). (A, B) The levels of TNFα, IL6, CXCL1, PLGF and VEGFR1 were analyzed, shown by quantification (A), and by representative images (B). *$p<0.05$. NS: non-significant. N=10.

Western blot

The protein from the mouse lung or cultured cells was extracted using RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000× g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-tumor necrosis factor α (TNFα), anti-IL6, anti-CXCL1, anti-PLGF, anti-VEGFR1 and α-tubulin (all purchased from Cell Signaling, St Louis, MO, USA). α-tubulin was used as a protein loading control. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to loading controls, and then normalized to experimental controls.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean ± standard deviation from 5 individuals and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bon-ferroni correction, followed by a Fisher's Exact Test, as necessary.

Results

ACE2 does not alter PLGF levels in ALI mouse lung

The mouse bleomycin-ALI model has been performed as has been described before. As a routine quality control, we isolated protein from the lung of the mice treated with bleomycin only (ALI) and ACE2 after bleomycin (ALI+ACE2). We then analyzed the inflammatory cytokine levels by Western blot, compared to the mice without treatment (UT). We found that ALI induced significantly increases in TNFα, IL6 and CXCL1 levels, by quantification (**Figure 1A**), and by representative images (**Figure 1B**). These data confirmed the successful establishment of

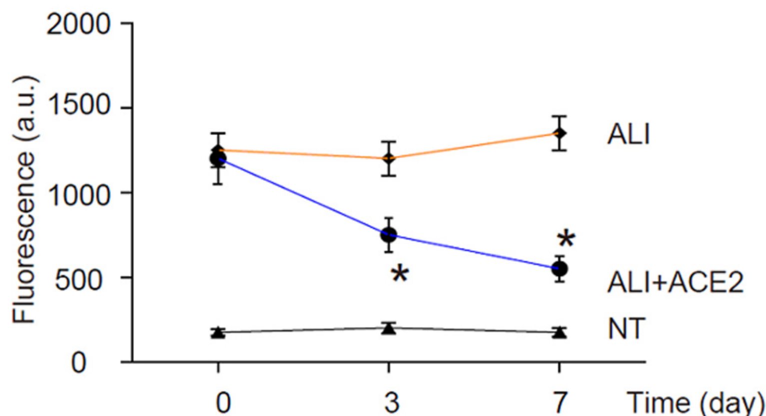

Figure 2. ACE2 treatment attenuates ALI-induced increases in lung vessel permeability. We examined the effects of ACE2 on lung vessel permeability at 0 day, 3 days and 7 days after ACE2 treatment. The fluorescence pharmacokinetics profile of the photosensitizer was measured 15 minutes after i.v. injection of 62.5 μg/kg Visudyne. We found that ALI significantly increased lung vessel permeability, while ACE2 alleviated the ALI-induced increases in lung vessel permeability. *p<0.05. N=10.

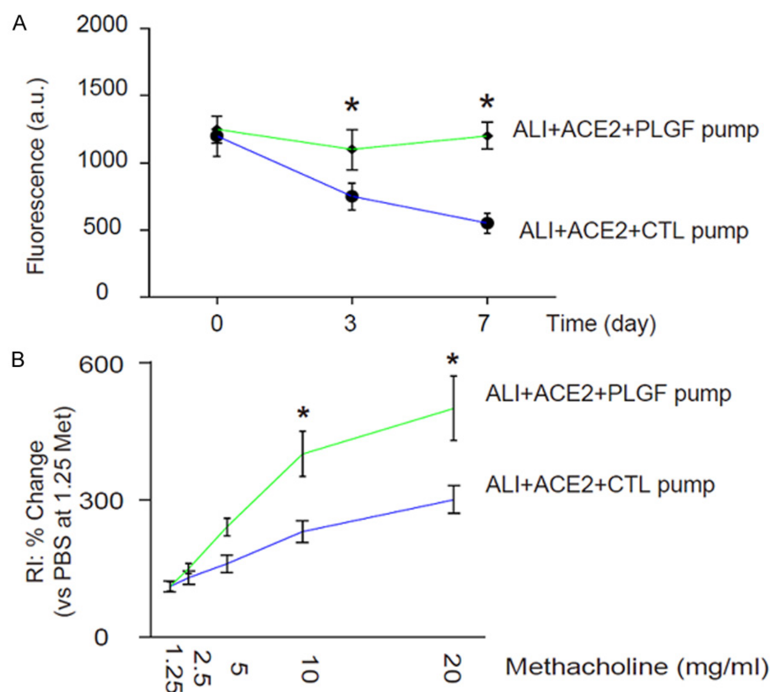

Figure 3. Ectogenic PLGF abolishes the antagonizing effects of ACE2 on the vessel permeability against PLGF. We implanted a releasing pump of PLGF at the time of ACE2 treatment (ALI+ACE2+PLGF pump). Mini-pumps containing PBS only were implanted in control mice (ALI+ACE2+CTL pump). A. Provision of ectogenic PLGF abolished the antagonizing effects of ACE2 on the vessel permeability against PLGF. B. The effects of ACE2 on lung function protection in a methacholine response test for lung resistance (RI). *p<0.05. N=10.

mouse ALI model by bleomycin and the therapeutic effects of ACE2 in bleomycin-induced ALI. Then, we examined the effects of PLGF and VEGFR1, and we found that the levels of PLGF and VEGFR1 in mouse lung were significantly increased by bleomycin treatment, but ACE2 did not altered PLGF and VEGFR1 levels.

ACE2 treatment attenuates ALI-induced increases in lung vessel permeability

Next, we examined the effects of ACE2 on lung vessel permeability at 0 day, 3 days and 7 days after ACE2 treatment. The fluorescence pharmacokinetics profile of the photosensitizer was measured 15 minutes after i.v. injection of 62.5 μg/kg Visudyne. We found that ALI significantly increased lung vessel permeability, while ACE2 alleviated the ALI-induced increases in lung vessel permeability (**Figure 2**). Since PLGF is a major trigger for increasing vessel permeability and ACE2 did not alter PLGF levels, these data suggest that ACE2 may antagonize the effects of PLGF-mediated increases in lung vessel permeability.

Ectogenic PLGF abolishes the antagonizing effects of ACE2 on the vessel permeability against PLGF

In order to confirm that ACE2 may antagonize the effects of PLGF-mediated increases in lung vessel permeability, we implanted a releasing pump of PLGF at the time of ACE2 treatment (ALI+ACE2+PLGF pump). Mini-pumps containing PBS only were implanted in control mice (ALI+ACE2+CTL pump). We found that provision of ectogenic PLGF abolished the antagonizing effects of ACE2 on the vessel permeabil-

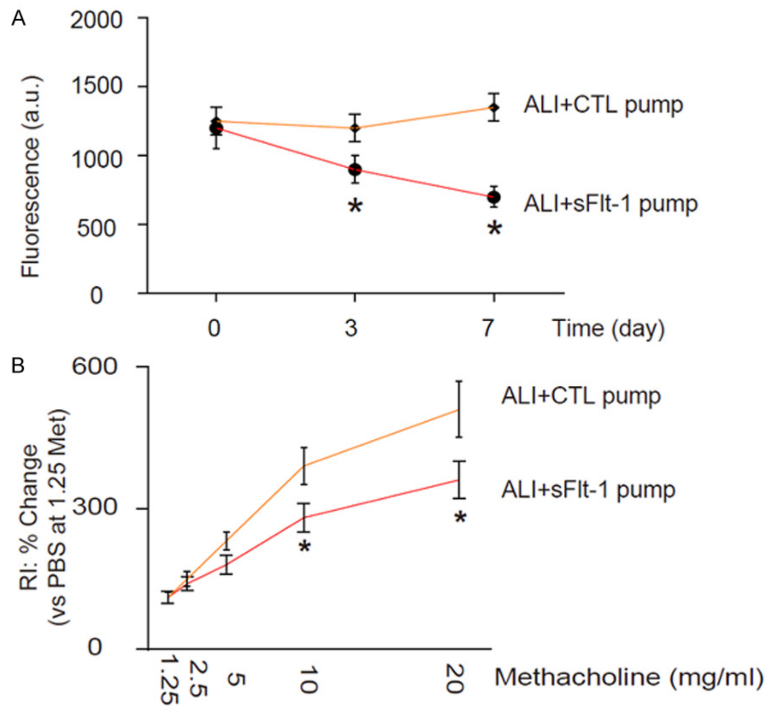

Figure 4. Suppression of PLGF signaling mimics the effects of ACE2 on the vessel permeability against PLGF. We implanted a releasing pump of a PLGF signaling inhibitor, aFlt-1, after induction of ALI (ALI+sFlt-1 pump). Mini-pumps containing PBS only were implanted in control mice (ALI+CTL pump). A. Provision of PLGF signaling inhibitor sFlt-1 mimicked the effects of ACE2 on the vessel permeability against PLGF. B. The effects of ACE2 on lung function protection in a methacholine response test for RI. *p<0.05. N=10.

ity against PLGF (**Figure 3A**), and then the effects of ACE2 on lung function protection in a methacholine response test for lung resistance (RI) (**Figure 3B**). Hence, this loss-of-function experiment suggests that ectogenic PLGF may abolish the antagonizing effects of ACE2 on the vessel permeability against PLGF, resulting in aggravated lung function.

Suppression of PLGF signaling mimics the effects of ACE2 on the vessel permeability against PLGF

In a gain-of-function experiment, we implanted a releasing pump of a PLGF signaling inhibitor, sFlt-1, after induction of ALI (ALI+sFlt-1 pump). Mini-pumps containing PBS only were implanted in control mice (ALI+CTL pump). We found that provision of PLGF signaling inhibitor sFlt-1 mimicked the effects of ACE2 on the vessel permeability against PLGF (**Fig-ure 4A**), and then the effects of ACE2 on lung function protection in a methacholine response test for RI (**Figure 4B**). Hence, these data suggest that suppression of PLGF signaling may mimic the effects of ACE2 on the vessel permeability against PLGF, resulting in improved lung function. Together, our data suggest that ACE2 may antagonize the PLGF-mediated increases in lung vessel permeability during ALI, resulting in improvement of lung function after ALI.

Discussion

ALI and ARDS are severe forms of diffuse lung disease that impose a substantial health burden worldwide each year. Data from several population-based studies reveals a fairly consistent picture for the age, mortality, severity of illness, ratio of ARDS to ALI, and ratio of ALI to acute respiratory failure, yet there is almost a four-fold difference in the reported incidence of ALI/ARDS between studies [1-4]. The resolution from lung injury is not simply relief from injurious agents or factors, but rather reflects an actively regulated program involving removal of apoptotic neutrophils, remodeling of matrix, resolution of protein rich alveolar fluid, and engagement of numerous signaling pathways distinct from those involved in acute injury. Type II alveolar epithelial cells proliferate to cover the injured basement membrane and differentiate into the type I alveolar epithelial cells. Apoptosis is thought to be a major mechanism for the clearance of neutrophils from the injured lung. Among all these processes that may alleviate the severity of ALI, decreasing the lung vessel permeability appears to be a critical ones, since all inflammatory cells, pro-inflammatory cytokines and other factors that trigger the pulmonary pathological changes stem from the increases in lung vessel permeability.

The protective roles of ACE2 in acute and chronic lung diseases have been demonstrat-

ed, including anti-inflammation and anti-oxidation [14-17, 19]. In addition, a role of ACE2 in inhibiting cell apoptosis after ALI has been acknowledged [18, 20]. However, the effects of ACE2 on vessel permeability and its relationship with PLGF remain ill-defined.

Here, we used a previously published bleomycin method to induce ALI in mice, and treated the mice with ACE2. We then analyzed the levels of PLGF in these mice. The mouse lung vessel permeability was determined by a fluorescence pharmacokinetic assay following i.v. injection of Visudyne. We found that ACE2 treatment did not alter PLGF levels in lung, but antagonized the effects of PLGF on increases of lung vessel permeability. These effects may be conducted through competition on the modulation of intracellular downstream factors that regulate the changes in the endothelial-endothelial cell junctions, but not at the levels of receptor binding, since the PLGF levels were not altered during ACE2 treatment. Future studies may address the intracellular functional factor for this regulation.

In a loss-of-function and gain-of-function experiment, PLGF pump or a Flt-1 pump was given to increase or decrease PLGF effects, which was found to antagonize the effects of ACE2, or mimic the effects of ACE2, respectively. Thus, these data further confirm the antagonizing effects of ACE2 on PLGF-mediated increases in lung vessel permeability after ALI.

Furthermore, the long-term effects on lung function were determined by measurement of lung resistance using methacholine, confirming that the suppression of vessel permeability resulted in improvement of lung function after ALI.

ALI induced phosphorylation of a key factor SMAD2 of TGFβ receptor signaling [21-23], which could be dose-dependently inhibited by ACE2. Moreover, ACE2-induced suppression of miR-4262 induced the inhibition of the PEC apoptosis after ALI through Bcl-2. Together with the data shown here, it is suggested that ACE2 may have its therapeutic effects on ALI through different molecular signaling pathways. These studies may contribute to our understanding of using ACE2 as a potent treatment for curing ALI and for preventing ARDS.

Disclosure of conflict of interest

None.

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