The role of the renin-angiotensin system in acute lung injury
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Chapter 4
Acute respiratory distress syndrome leads to reduced ratio of ACE/ACE2 activities and is prevented by Angiotensin-(1-7) or an Angiotensin II receptor antagonist

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Abstract

Acute respiratory distress syndrome (ARDS) is a devastating clinical syndrome. Angiotensin-converting enzyme (ACE) and its effector peptide angiotensin (Ang) II have been implicated in the pathogenesis of ARDS. A counter-regulatory enzyme of ACE, i.e. ACE2 that degrades Ang II to Ang-(1-7), offers a promising novel treatment modality for this syndrome. As the involvement of ACE and ACE2 in ARDS is still unclear, this study investigated the role of these two enzymes in an animal model of ARDS.

In rats, ARDS was induced by intratracheal administration of LPS followed by mechanical ventilation. During ventilation animals were treated with saline (placebo), losartan (Ang II receptor antagonist), or with a protease-resistant, cyclic form of Ang-(1-7) [cAng-(1-7)].

In bronchoalveolar lavage fluid (BALF) of ventilated LPS-exposed animals, ACE activity was enhanced, whereas ACE2 activity was reduced. This was matched by enhanced BALF levels of Ang II and reduced levels of Ang-(1-7). Therapeutic intervention with cAng-(1-7) attenuated the inflammatory mediator response, markedly decreased lung injury scores and improved lung function, as evidenced by increased oxygenation.

These data indicate that ARDS develops, in part, due to reduced pulmonary levels of Ang-(1-7) and that repletion of this peptide halts the development of ARDS.
Introduction

Acute respiratory distress syndrome (ARDS) is a common and often devastating condition. In the USA alone, each year about 200,000 patients suffer from ARDS, resulting in approximately 75,000 deaths per year (Ware and Matthay, 2000; Wheeler and Bernard, 2007; Rubentfeld and Herridge, 2007; Phua et al., 2009). ARDS is characterized by acute onset of respiratory distress and severe hypoxemia. The pathophysiological mechanisms playing a central role in the acute phase of ARDS include diffuse alveolar damage and an acute inflammatory mediator response. In ARDS, a complex network of cytokines and chemokines initiates and maintains the inflammatory response (Ware and Matthay, 2000; Wheeler and Bernard, 2007; Matute-Bello et al., 2008). To date, only supportive ventilation strategies for ARDS are available as a therapeutic intervention.

The role of the renin-angiotensin system (RAS) in the pathogenesis of ARDS is well established. In bronchoalveolar lavage fluid (BALF) from ARDS patients, an enhanced activity of the primary enzyme of the RAS, angiotensin-converting enzyme (ACE), is found (Idell et al., 1987). Furthermore, there is an association between an ACE insertion-deletion polymorphism, the susceptibility to develop ARDS, and the outcome of patients with ARDS (Jerng et al., 2006; Marshall et al., 2002). The frequency of the DD genotype (leading to higher ACE activity compared to the II genotype) is increased in patients with ARDS compared to controls. In addition, the D allele correlates with mortality in the ARDS group.

ACE degrades the decapeptide angiotensin (Ang) I to the octapeptide Ang II. Ang II predominantly exerts its activity via a type 1 (AT1) receptor maintaining blood pressure homeostasis, as well as fluid and salt balance (Baudin, 2002). Via the same receptor, Ang II also triggers inflammation, fibrosis and apoptosis (Suzuki et al., 2003; Wang et al., 1999; Wösten-van Asperen et al., 2008; Li et al., 2003). In experimental animal models of ARDS, inhibition of ACE by captopril, or blocking the Ang II receptor by the antagonist losartan, reduced the inflammatory response and lung injury (Hagiwara et al., 2009; Wösten-van Asperen et al., 2010; Imai et al., 2005).

Until recently, ACE was considered the key enzyme in the RAS, but this was challenged by the discovery of the enzyme ACE2 (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 converts Ang II to Ang-(1-7), thus counteracting the effects of ACE. Using ACE2 knockout mice, Imai et al. showed that failure to degrade Ang II resulted in more severe ARDS (Imai et al., 2005). Recent in vitro studies, however, have revealed that Ang-(1-7), via its Mas receptor, may intervene with the signaling cascade triggered by Ang II, providing an alternative (but not mutually exclusive) explanation for the results with the ACE2 knockout mice (Gallagher et al., 2008; Su et al., 2006).

As the involvement of the different components of the RAS in ARDS remains unclear, the present study investigated the role of ACE and ACE2 in an animal model of ARDS. We
hypothesized that the development of ARDS is determined by the balance between levels of Ang II and Ang-(1-7) that depends on the balance between ACE and ACE2 activity within the lung. Furthermore, we addressed whether therapeutic intervention with a protease-resistant, cyclic form of Ang-(1-7) [cAng-(1-7)] or blocking the AT1 receptors attenuate lung injury by dampening the inflammatory response.

Materials and Methods

Animals
The experiments were performed in male Sprague-Dawley rats weighing 314 ± 17 g (Harlan CPB, Zeist, the Netherlands). All animal procedures were approved by the Ethical Committee for animal experiments of the Erasmus Medical Center (Rotterdam, the Netherlands).

A tracheostomy was performed under inhalation anesthesia and the carotid artery was catheterized. Anesthesia was maintained by hourly intraperitoneal injections of pentobarbital sodium (60 mg kg⁻¹, Nembutal®, Algin BV, Maassluis, the Netherlands). Muscle relaxation was obtained with 2 mg kg⁻¹ pancuronium bromide (Pavulon®, Organon, Boxtel, the Netherlands) administered intramuscularly hourly.

Animals were mechanically ventilated for 4 h in a pressure-controlled mode: peak inspiratory pressure (PIP) 26 cmH₂O (Servo ventilator 300. Siemens-Elema, Solar, Sweden) with a FiO₂ of 0.5 and a positive end-expiratory pressure (PEEP) of 5 cmH₂O (tidal volume ±18 ml/kg). Respiratory rate was set at a frequency of 30 per min (inspiratory/expiratory ratio of 1:2) and, to maintain normocapnia, adjusted when necessary. Blood gas analyses and blood pressure were recorded just before and hourly after randomization.

Experimental acute lung injury protocol
From a group of 48 animals, 24 received 5 mg kg⁻¹ LPS (Escherichia coli, serotype 0111:B4, Sigma-Aldrich) intratracheally 24 h prior to the ventilation period. Ventilated animals (n = 6/group) were randomly assigned to three treatment groups during 4 h of mechanical ventilation. Group 1 received intravenously (i.v.) normal saline (placebo), Group 2 received i.v. 2.5 mg/kg/h losartan (a generous gift from Merck & Co, New Jersey, USA; dose adopted from Jeng et al. (2007)) and Group 3 received i.v. 2.4 μg/kg/h cAng-(1-7) (BiOMaDe Technology Foundation, Groningen, the Netherlands: dose results from dose-response experiments, Supplementary Figure 1). Non-ventilated unexposed (n=6) and non-ventilated LPS-exposed animals (n=6) served as controls.

Animals were sacrificed with an overdose of intra-arterial administered pentobarbital sodium. Bronchoalveolar lavage (BAL) was performed twice in the left lung with normal saline (5 ml per lavage). The recovery of lavage fluid between and within the groups was
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≥ 90%. The retrieved BALF was pooled, centrifuged (300 x g at 4°C for 10 min) and the supernatant was stored in aliquots at -80°C. After this procedure, the right lung was dissected and recruited by a positive airway pressure of 10 cmH₂O. The right lung was fixed in 4% buffered formalin and embedded in paraffin.

Measurement of BALF RAS components and inflammatory mediators
ACE and ACE2 activity was measured by monitoring the degradation of the fluorogenic peptide substrate Mca-R-P-G-F-S-A-F-K(Dnp)-OH or Mca-Y-V-A-D-A-P-K(Dnp)-OH, respectively (R&D Systems, Uithoorn, the Netherlands), as described previously (Wösten-van Asperen et al., 2010; Vickers et al., 2002; Wysocki et al., 2006). For Western blotting, equal volumes of BALF were analyzed and rabbit polyclonal anti-ACE2 antibody (a kind gift of Millennium Pharmaceuticals Inc, Cambridge, USA) and rabbit polyclonal anti-mouse ACE antibody (R&D systems) were used for immuno-detection. BALF was subjected to a lipid extraction (Bligh and Dyer, 1959). Peptides from the aqueous phase were purified using a C18 column (Varian) as follows. C-18 columns were prepared by washing with 1 ml [100% MeOH + 0.1% formic acid] and subsequently with 1 ml [100% MQ + 0.1% formic acid]. After loading the sample on the column a washing step followed with 2 ml [5% MeOH + 0.1% formic acid]. Peptides were eluted from the column with 800 μl [100% MeOH + 0.1% formic acid]. Angiotensin I was used as internal standard. Levels of Ang II and Ang-(1-7) were measured via liquid chromatography-mass spectrometry (Triple Quadrupole LC/MS/MS Mass Spectrometer, API 3000, Applied Biosystems), as described previously (Kluskens et al., 2009).

BALF levels of CINC-3, IL-6, IL-1β, TNF-α, IL-10 and GM-CSF were measured using rat fluorokine MAB assay (R&D) and quantified with a BioRad Bioplex 100. Total protein was measured by the Bradford method (BioRad assay, Munich, Germany) using bovine serum albumin as a reference.

Histology and immunohistochemistry
To determine the lung injury score, 4-μm thick sections were cut and stained with hematoxylin and eosin. The sections were scored for injury, including: 1) alveolar and capillary edema, 2) intravascular and peri-bronchial influx of inflammatory cells, 3) thickness of the alveolar wall, and (4) hemorrhage. The items were semi-quantitatively scored as none, minimal, light, moderate or severe (score 0, 1, 2, 3 or 4, respectively) by a pathologist blinded to the experimental group. The values allocated for each of the four parameters analyzed were added. The final lung injury score was obtained by averaging the score from the animals within each group.

Immunohistochemical staining for ACE and ACE2 in lung tissue was performed using a monoclonal mouse anti-ACE antibody (clone 2E2, dilution 1:100, Santa Cruz Biotech, Santa Cruz, USA) and a polyclonal rabbit anti-ACE2 antibody (dilution 1:750, a kind
gift of Millennium Pharmaceuticals Inc, Cambridge, USA), respectively. For optimal immunostaining, tissue pretreatments were needed with heat-induced antigen retrieval using Tris/EDTA, pH 9.0. ACE staining was visualized with 3,3’-diaminobenzidine (Dako, Glostrup, Denmark) as chromogen. For ACE2, staining was visualized using 3-amino-9-ethylcarbazole (AEC). Negative controls, replacing the primary antibodies with irrelevant immunoglobulins (same isotype and concentration), were included.

To identify the AT1- and Mas-positive cells in the alveolar septa, a sequential triple immunostaining was performed combining a polyclonal rabbit anti-AT1 (clone sc-579, dilution 1:1000, Santa-Cruz Biotech, sc-579) and a monoclonal mouse anti-Mas (dilution 1:50, a generous gift from Robson A.S. dos Santos, Brazil; for detailed description of the production of the antibody see ref (Becker et al., 2007)) antibodies with mouse anti-rat macrophage marker ED-1 (dilution 1:200, Serotec, Poole, England) or mouse anti-pan-cytokeratin (i.e. epithelial) marker (clone A1+A3, dilution 1:200, ThermoFisher/Labvision, Fremont, CA, USA) (van der Loos, 2008; van der Loos 2010). Briefly, after dewaxing the paraffin tissue sections, graded alcohols and blocking of endogenous peroxidase activity, tissue sections were pretreated with Tris-EDTA pH 9.0 heat-induced antigen retrieval buffer in the PTModule (ThermoFisher) for 20 min at 98 °C. First AT1 (rabbit) and Mas (mouse) were incubated as antibody cocktail (overnight 4 °C) and detected by a 1:1 polymer cocktail consisting of: Bright Vision anti-mouse IgG/alkaline phosphatase (AP) and Bright Vision anti-rabbit IgG/horseradish peroxidase (HRP) (both ImmunoLogic, Duiven, the Netherlands) including 10% normal rat serum (60 min, room temperature). The enzymatic activities of AP and HRP were visualized in blue and brown, respectively, using Vector Blue (Vector Laboratories, Burlingame, CA, USA) and Bright DAB (ImmunoLogic). After an intervening heat step (TrisEDTA pH 9.0, 10 min, 98 °C), to remove all immuno-reagents from the first staining sequence (van der Loos and Teeling, 2008), the anti-ED-1 (mouse) or anti-AE1/AE3 (mouse) antibody was detected with Bright Vision polymer anti-mouse IgG/AP (including 10% normal rat serum) (30 min, room temperature) and AP activity was visualized in red using Vector Red (Vector Labs). Tissue sections were counterstained with 1:5 diluted eosin ensuring a faint staining of all tissue elements. Negative controls, replacing the primary antibodies with irrelevant immunoglobulins (same isotype and concentration), were included. Results of the individual antibody stainings are shown in Supplementary Figure 2.

After the staining procedure, spectral imaging was used to identify co-localization of both receptor types on the cells in the alveolar septa. Briefly, Nuance™ Spectral Imaging System (Caliper Life Sciences/CRI, Woburn, MA, USA) enables to ‘unmix’ the signals based on their spectral characteristics into different component images, each displaying the distribution and abundance of the individual chromogen. The software (Nuance™ software version 3.0) facilitates visualization of these different images by creating a fluorescent-like composite image in good contrasting pseudocolors as well as
exclusive imaging of co-localization (van der Loos, 2008). Nuance™ 3.0 also facilitates quantification of the different co-localization combinations, as well as a pixel-based relative amount of each individual marker.

Statistical Analysis
All data are shown as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, USA). Group comparisons were evaluated by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls for pair-wise multiple comparisons, or the Kruskall Wallis test, where appropriate. Physiological parameters were evaluated by repeated measurements ANOVA. Log transformations of the variables were analyzed in case of lack of normality and/or homoscedasticity. A p-value < 0.05 was considered significant.

Results
Imbalance of the ACE/Ang II and ACE2/Ang-(1-7) pathways in a rat model of ARDS.
Rats were exposed intratracheally to LPS to induce lung injury. After 24 h, the animals were mechanically ventilated with moderate tidal volumes. Ventilation or administration of LPS caused some injury as evidenced by increased BALF protein levels and lung injury scores when compared with control animals. However, the combination of LPS exposure and ventilation resulted in substantial lung injury (Figure 1A) which was associated with decreased oxygenation (Figure 1B), increased vascular permeability (Figure 1C) and high lung injury scores (Figure 1D), indicative of ARDS.
Ventilation of LPS-exposed animals increased BALF ACE activity 9-fold compared to unexposed ventilated animals, whereas that of BALF ACE2 activity was decreased 30-fold, markedly reducing the ACE2 over ACE activity ratio (Figure 2A). By Western blotting we found that the increased ACE activity in ARDS BALF was paralleled by enhanced amounts of ACE protein (~140 kDa) (Figure 2B). Despite a reduced ACE2 activity in the LPS plus mechanical ventilation group, increased ACE2 protein (~80 kDa) was found compared to the control group. However, also in this LPS group, enhanced amounts of degraded ACE2 protein (~40 kDa; intact rat ACE2 is ~80 kDa) were found, whereas this was not detectable in the other experimental groups. Notably, levels of Ang II and Ang-(1-7) corresponded with ACE and ACE2 activities in BALF (Figure 2A). Ventilation of the LPS-exposed animals increased BALF Ang II levels 1.5-fold compared to the unexposed ventilated animals. BALF Ang-(1-7) levels decreased 9-fold, thereby reducing the Ang-(1-7) over Ang II ratio from 1.5 in the unexposed animals to 0.1 in the LPS-exposed animals.
Taken together, these data confirm that conditions that lead to ARDS are paralleled by increased ACE activity and reduced ACE2 activity in the pulmonary compartment. Immunohistochemical staining of ACE and ACE2 in rat lungs revealed (besides positive staining on endothelial cells) positive staining on alveolar epithelial cells and some infiltrating cells (macrophages, monocyte-like) in the alveolar walls (Figure 2C).

Attenuation of lung injury by cAng-(1-7) and losartan in a rat model of ARDS.

Since recent in vitro studies have shown that Ang-(1-7) may intervene with the Ang II-triggered signaling cascade, we aimed to assess the effect of Ang-(1-7) in ARDS (Gallagher et al., 2008; Su et al., 2006). Rats that had or had not been exposed to LPS were randomly allocated to three treatment groups. The groups were ventilated 24 h after LPS exposure and simultaneously treated with i.v. saline (placebo group), or with the Ang II type 1 receptor blocker losartan (positive control), or with Ang-(1-7). Previously, it was shown that Ang-(1-7) is rapidly degraded by ACE and other proteases (Allred et al., 2000). Cyclization of Ang-(1-7) [i.e. introducing a thioether bridge in Ang-(1-7)] increased its proteolytic resistance and thus its in vivo stability (Kluskens et al.,...
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![Graphs showing BALF ACE activity, ACE2 activity, Ang-(1-7) levels, and BALF Ang-(1-7)/Ang II ratio in unexposed and LPS-exposed non-ventilated (spontaneously breathing) and mechanically ventilated (MV) animals.](image)

**Figure 2.** Imbalance of the ACE/Ang II and ACE2/Ang-(1-7) pathways in a rat model of ARDS. (A) BALF ACE activity, ACE2 activity and ACE2/ACE ratio and BALF levels of Ang II and Ang-(1-7) in unexposed and LPS-exposed non-ventilated (spontaneously breathing) and mechanically ventilated (MV) animals. n = 6 per group. *p<0.05 compared with control; **p<0.0001 versus all other groups; †p<0.0001 versus control; ‡p<0.0001 versus MV and control. (B) Western blot for ACE and ACE2 protein in equal volumes of BALF from unexposed and LPS-exposed mechanically ventilated animals. (C) Immunohistochemistry for ACE and ACE2 in lung tissue from unexposed and LPS-exposed mechanically ventilated animals. Diffuse staining was found on endothelial cells and also on epithelial cells (see insert) and infiltrating cells (macrophages, monocyte-like cells) in the alveolar walls. Representative images are shown. Original magnification 20x, inlay 100x.
2009). Moreover, because cAng-(1-7) more effectively interacts with its receptor, the Mas receptor (Kluskens et al., 2009), in the present study cAng-(1-7) was used.

In unexposed ventilated animals, treatment with cAng-(1-7) or losartan did not influence BALF ACE or ACE2 activity, or Ang II or Ang-(1-7) levels (Figure 3A & B). However, in LPS-exposed animals, BALF ACE2 activity and Ang-(1-7) levels were significantly higher in both treatment groups compared to the placebo group. In line with this, the Ang-(1-7)/Ang II ratio was comparable to that found in unexposed ventilated animals.

In unexposed ventilated animals, PaO2 levels remained stable throughout the 4 h ventilation period, irrespective of treatment (Figure 4A). In contrast, PaO2 levels of LPS-exposed animals decreased over time during ventilation. However, treatment with...

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**Figure 3.** Restoration of the imbalance of the ACE/Ang II and ACE2/Ang-(1-7) pathways by cAng-(1-7) and losartan. (A) BALF ACE activity, ACE2 activity and ACE2/ACE ratio and (B) BALF levels of Ang II and Ang-(1-7) and Ang-(1-7)/Ang II ratio of unexposed and LPS-exposed non-ventilated (spontaneously breathing) and mechanically ventilated (MV) animals treated with saline (placebo), cAng-(1-7) or losartan. n = 6 per group. *p<0.05 compared with non-LPS-exposed animals; **p<0.01 compared with LPS-exposed animals treated with saline; ***p<0.001 versus LPS-exposed animals treated with saline.

**Figure 4.** Attenuation of lung injury by cAng-(1-7) and losartan in a rat model of ARDS. (A) Physiological parameters (PaO2, levels, PaCO2, levels and blood pressure) of unexposed and LPS-exposed mechanically ventilated animals treated with saline, cAng-(1-7) or losartan. n = 6 per group. †p<0.05 compared with animals treated with cAng-(1-7) or losartan, ‡p<0.01 compared with placebo group and animals treated with losartan, ‡p<0.05 compared with animals treated with cAng-(1-7). (B) Lung injury scores. †p<0.05 compared with unexposed animals. ‡p<0.05 compared with ventilated LPS-exposed animals treated with saline (placebo). (C) Lung histopathology. Representative images are shown. Original magnification 20x (D) BALF levels of total protein content, †p<0.05 compared with unexposed and non-ventilated LPS-exposed animals. (E) BALF levels of CINC-3, interleukin (IL)-6, IL-1β, TNF-α, IL-10 and granulocyte macrophage-colony stimulating factor (GM-CSF) in BALF from unexposed and LPS-exposed non-ventilated (spontaneously breathing) and mechanically ventilated (MV) animals treated with saline (placebo), cAng-(1-7) or losartan. n ~6 per group. *p<0.05 compared with unexposed and non-ventilated LPS-exposed animals. **p<0.01 compared with ventilated LPS-exposed animals treated with saline.
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cAng-(1-7) or losartan resulted in significantly higher values at the end of 4-h ventilation compared to the placebo group (Figure 4A). There was no significant difference in PaCO$_2$ levels between the experimental groups (Figure 4A). Notably, losartan, but not cAng-(1-7), caused a significant decrease of blood pressure in LPS-exposed ventilated animals during ventilation compared to the other treatment groups. In parallel with the markedly improved lung function parameters, treatment with cAng-(1-7), and to lesser extent for losartan, was associated with a marked reduction of lung injury in ventilated LPS-exposed animals (Figure 4B and C).

Increased vascular permeability and enhanced inflammatory mediator response are hallmarks of ALI/ARDS. In the present study, treatment with cAng-(1-7), and to a lesser extent by losartan, resulted in reduced BALF protein levels in unexposed and LPS-exposed ventilated animals; however, the difference was not significant (Figure 4D). Yet, there was a significant attenuation of the inflammatory mediator production in the intervention groups. Except for IL-6 and IL-1β, treatment with cAng-(1-7) or losartan prevented the increase of inflammatory mediators seen in LPS-exposed ventilated animals (Figure 4E).

Mas and Ang II type 1 receptor co-localize on alveolar macrophages and epithelial cells.

Thus, cAng-(1-7) proved to be an effective and fast-working intervention in experimental ARDS, since it led to near-normal respiratory and inflammatory parameters 4 h after its application to ventilated LPS-exposed rats. It has been shown that alveolar epithelial cells and alveolar macrophages play a pivotal role in the inflammatory mediator response observed in the acute phase of ARDS by producing pro-inflammatory cytokines, chemokines and other factors (Thorley et al., 2007). To examine whether these cells may be affected by cAng-(1-7), expression of the Mas receptor in lung tissue was assessed. Our group has shown that alveolar macrophages and epithelial cells express the Ang II type I receptor (AT1) (Wösten-van Asperen et al., 2010). In the present study, immunohistochemistry showed that the Mas receptor co-localizes with AT1 on alveolar epithelial cells (Figure 5A and C) and alveolar macrophages (Figure 5B and D). In the non-exposed animals, 10% of the alveolar epithelial cells express both the AT1 and Mas receptor. LPS exposure increases this percentage to 35%. In non-exposed animals, 30% of the alveolar macrophages express both receptor types. LPS exposure increases the total number of alveolar macrophages whereas the percentage of macrophages expressing both receptor types remains the same. Thus these cells may be targeted by cAng-(1-7) and thereby attenuate the inflammatory mediator production.
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Discussion

ARDS is a devastating disorder of overwhelming pulmonary inflammation leading to hypoxemia and respiratory failure, for which there is no effective therapeutic option in most of the cases. Previously, it has been shown that the RAS plays an important role in the pathogenesis of ARDS (Hagiwara et al., 2009; Wösten-van Asperen et al., 2010; Imai et al., 2005). ACE and ACE2 are homologues with different key functions in the RAS. ACE cleaves Ang I to generate the activator Ang II, whereas ACE2 acts as a negative regulator of the system by inactivating Ang II and generating Ang-(1-7). Human and animal studies have shown that BALF ACE activity is enhanced in ARDS. This is paralleled by accumulation of its effector peptide Ang II, which triggers inflammatory responses and lung injury via the AT1 receptor, as it is inhibited by its antagonist, losartan.

Figure 5. Localization of AT1 and Mas receptor in alveolar compartment. (A) Spectral imaging analyzed triple immunohistochemistry for Ang II type 1 receptor (AT1), developed by Vector Blue, blue pseudocolor, the Mas receptor (for Ang-(1-7); developed by diaminobenzidine, green pseudocolor) and epithelial cell marker AE1/AE3 (developed by Vector Red, red pseudocolor), and eosin marking all tissue elements (grey pseudocolor) in lung tissue of unexposed animals. Top left shows overview of original RGB image. Top middle shows triple staining after spectral imaging in pseudocolors. Top right shows co-localization of all three markers (yellow pseudocolor) combined with grey pseudocolor. Lower panel shows staining for separate markers after spectral unmixing combined with eosin in grey pseudocolor. (B) As (A) but now with macrophage marker ED-1 (developed by Vector Red) instead of epithelial cell marker. (C) and (D) As (A) and (B), respectively, but now of lung tissue of mechanically ventilated LPS-exposed animals.
present study extends these findings by showing that in a rat model of ARDS the ACE2 activity is reduced, paralleled by low amounts of Ang-(1-7). Since ACE2 degrades Ang II, the reduced ACE2 activity is in line with the Ang II-mediated ARDS pathophysiology. However, treatment with a protease-resistant, cyclic form of Ang-(1-7), cAng-(1-7), restored the balance between ACE and ACE2 activity and amounts of Ang II and Ang-(1-7), attenuated the inflammatory response, decreased lung injury scores, and improved lung function as reflected by increased oxygenation. These data indicate that Ang-(1-7) plays a pivotal role in ARDS development and offers a novel treatment approach for this syndrome.

BALF ACE2 activity is markedly decreased in mechanically ventilated LPS-exposed rats, which contrasts the enhanced amounts of soluble ACE2 protein in these samples as observed by Western blot analyses (Figure 2B). It is unlikely that this discrepancy is related to the Western blot analysis of equal volumes of BALF. Both the activity and the Western blot analyses were performed on the same BALF samples using similar volumes of fluid. This enables direct comparison of the amount and the activity of the protein. One could argue that ACE2 in the Western blot analysis is underestimated in the samples from the other groups as we did not normalize BALF for protein content. In line with other studies, we have not corrected for protein content as an enhanced protein exudation is a hallmark of ALI/ARDS, and thus correction for protein content would lead to an underestimation of the measured parameters. If, however, we would correct for the 2-3 fold higher protein content in BALF from the MV + LPS group, this would not change the conclusion of the experiment. The reduction of ACE2 activity may, at least in part, be due to proteolytic degradation of ACE2, as was seen in BALF from ventilated, LPS-exposed animals only. An alternative, not mutually exclusive, explanation is that in BALF from these rats, an ACE2 inhibitor is present. The enhanced amounts of Ang II and reduced amounts of Ang-(1-7) in BALF from ventilated LPS-exposed rats correspond with the respective ACE activities. This relationship also applies to the interventions with losartan and cAng-(1-7), suggesting that ACE and ACE2 activities are the major enzymes controlling the local amounts of Ang II and Ang-(1-7). However, it cannot be excluded that additional peptidases might also have contributed to the (im)balance between the various angiotensin peptides.

The protective effect of Ang-(1-7) has been described in experimental liver, renal and cardiovascular diseases (Grobe et al., 2007; Mercure et al., 2008; Pereira et al., 2007). Although ACE2 is protective in experimental models of lung injury and fibrosis (Imai et al., 2005; Li et al., 2008), the role of Ang-(1-7) in lung injury has not been established. The rapid in vivo degradation of circulating Ang-(1-7) by ACE (abundantly present in the lung) and by other proteases, might explain why Ang-(1-7) has not yet been tested as a lung protective drug. In the present study, proteolytic degradation of Ang-(1-7) was circumvented by using cAng-(1-7). Also, the rats were treated with cAng-(1-7) for
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a 4-h period only, as opposed to several weeks in the earlier mentioned studies. Our findings indicate that cAng-(1-7) acts quickly, as various parameters were normal again 4 h after application, and PaO\(_2\) was affected even 1 h after application. However, the effect of cAng-(1-7) over a prolonged period remains to be elucidated. In vitro, Ang-(1-7) is known to inhibit Ang II-mediated phosphorylation of several MAPK kinases (i.e. p38, ERK1/2 and JNK), which mediate a range of cellular and inflammatory responses (Su et al., 2006). In fact, Ang-(1-7) was found to inhibit Ang II-induced downregulation of ACE2 mRNA (Gallagher et al., 2008). Thus, cAng-(1-7) may directly block the Ang II-induced phosphorylation of these MAPK kinases, preventing Ang II-mediated changes. Since alveolar epithelial cells and macrophages (Figure 5) co-express the receptors for Ang II and Ang-(1-7), it is likely that cAng-(1-7) acts via these cells. That not all cells are affected by Ang-(1-7) is also reflected by the differential effect of Ang-(1-7) on cytokine production (Figure 4E). More studies are needed to confirm this hypothesis.

There is current evidence to support the existence of a local RAS in the lung, as opposed to the systemic RAS. Lung fibroblasts, alveolar macrophages and epithelial cells are capable of expressing the genes encoding RAS components and synthesizing the RAS peptides (Wang et al., 1999; Li et al., 2006; Renzoni et al., 2004; Uhal et al., 2007). In line with these findings, our immunohistochemical analyses indicate that alveolar macrophages and epithelial cells express both ACE and ACE2, as well as the receptors AT1 and Mas, indicating that these cells express the major constituents of RAS. It is not clear whether ARDS involves the pulmonary RAS only and/or the systemic RAS. Establishing whether the pulmonary RAS steers the development of ARDS would imply that systemic treatment may be circumvented.

Losartan and cAng-(1-7) showed similar lung protective effects. However, in the LPS-exposed ventilated animals, losartan caused a significant decrease in blood pressure. In humans, losartan is reported to have serious side-effects (related to circulation) in patients with altered blood pressure (chronic or acute); however, no data are available on the effect of losartan in persons without altered blood pressure. This type of side-effect is important since ARDS is usually associated with severe hypotension. This is a major drawback for its use in the (critical) clinical situation.

A characteristic feature of the present study is that, restoring the equilibrium of ACE/ACE2 activity after induction of ARDS, actually mimics therapeutic options in the clinical situation. However, the present study has to be extended to other underlying etiologies of ARDS before it can be implemented in the clinical setting. Although studies by Imai et al. showed that ACE2 is also important for acid aspiration-induced ARDS, it is not known whether cAng-(1-7) reverses this form of ARDS (Imai et al., 2005). The same applies to sepsis or trauma-induced ARDS, both of which are important risk factors to develop ARDS.
In conclusion, our results show that the balance of ACE/ACE2 activity is disturbed in ARDS. Repletion with cAng-(1-7) almost fully normalized key parameters in experimental ARDS. These findings represent a paradigm shift in the understanding of the pathogenesis of ARDS. Administration of cAng-(1-7) has the potential to counteract the development of ARDS and may help to reduce of the high mortality rate associated with this syndrome.

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Supplementary Figure 1. Dose response experiments of cyclic Ang-(1-7)
Physiological parameters (PaO$_2$ levels (A) and blood pressure (B)) of LPS-exposed mechanically ventilated animals treated with cAng-(1-7) in three different doses. n = 6 per group. *p<0.05 compared with animals treated with 2.4 or 24 μg/kg/hr cAng-(1-7). Blood pressure of animals treated with 24 μg/kg/hr cAng-(1-7) was reduced compared with the other two doses, albeit the difference was not significant. However, these animals received several fluid boluses to keep a stable blood pressure. We therefore choose for 2.4 μg/kg/hr cAng-(1-7) as the treatment dose.
Ang-(1-7) or AT1 blocker attenuates inflammatory response in ARDS

Supplementary figure 2. Single antibody stainings for Ang II type 1 receptor (AT1; developed by Vector Blue), the Mas receptor (for Ang(1-7); developed by diaminobenzidine), the epithelial cell marker AE1/AE3 and macrophage marker ED-1 (developed by Vector Red) in unexposed and LPS-exposed mechanically ventilated animals. Original magnification 40x.