The role of the renin-angiotensin system in acute lung injury
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Summary and general discussion
Acute Lung Injury/Acute Respiratory Distress Syndrome and the RAS system

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are common conditions at both pediatric and adult intensive care units (ICUs). Although there are differences in incidence, morbidity and mortality between children and adults, patients with ALI and ARDS continue to be among those at the highest risk in ICUs, with longer lengths of mechanical ventilation, higher risk for nosocomial infections, and unknown long-term neurodevelopmental and respiratory morbidity (Khemani and Newth, 2010; Ware and Matthay, 2000; Rubenfeld et al., 2005). Independently of age, ALI/ARDS is characterized clinically by acute onset of respiratory distress and severe hypoxemia. This is accompanied by alveolar inflammation as reflected by enhanced amounts of inflammatory mediators and recruitment of inflammatory cells, and subsequently by lung tissue destruction, particularly diffuse alveolar damage (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.

Previously, the importance of the renin-angiotensin system (RAS) in the pathogenesis of ARDS has been shown. This is indicated by an enhanced activity of the primary enzyme of the RAS, angiotensin-converting enzyme (ACE), in bronchoalveolar lavage fluid (BALF) of ARDS patients (Idell et al., 1987). Moreover, in adults an association has been found between an ACE insertion-deletion (I/D) polymorphism and the susceptibility to develop ARDS and the outcome of this syndrome (Jerg 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 angiotensin (Ang) I to Ang II. Ang II triggers inflammation, fibrosis and apoptosis (Suzuki et al., 2003; Ruiz-Ortega et al., 2001; Wang et al., 1999; Li et al., 2003). These processes are known to play an important role in the pathophysiology of ALI/ARDS. The mechanism by which RAS contributes to ALI/ARDS is still far from clear.

In this Thesis, the role of RAS in these syndromes was studied by using experimental models of acute lung injury. Moreover, it was assessed whether RAS may have a role in Respiratory Syncytial Virus-induced acute lung injury in children.

Acute lung injury: battle between the two ACE’s?

The circulatory RAS plays a key role in maintaining cardiovascular function by regulating blood pressure and electrolyte homeostasis. Next to this systemic RAS, there is considerable evidence to support the existence of local RAS in a number of tissues,
including the lung (Campbell et al., 1995). This indicates that the RAS components in
the lung may be derived from local generation. Indeed, it has been shown that different
RAS proteins are present in cells of the alveolar compartment (Deszö et al., 1988; Li
et al., 2006). Furthermore, transcripts encoding RAS components, including ACE,
angiotensinogen, renin and Ang II receptors are present in the lung (Ohkubo et al., 1986;
Kakar et al., 1992; Baudin, 2002; Li et al., 2006; Wang et al., 1999). These findings are
indicative of local production of the RAS components in the lung, although this still does
not exclude that they originate from the circulation.

The RAS in the lung could influence the pathogenesis of lung injury. Indeed, it was
shown that injurious (high-pressure amplitudes) mechanical ventilation leads to increased
BALF activity of ACE (Chapter 2). This was accompanied by increased BALF levels of
Ang II, inflammatory mediators and increased numbers of apoptotic cells. Inhibition of
ACE by captopril or blocking the Ang II receptor by the antagonist losartan reduced
the inflammatory response and apoptosis. Mechanical ventilation of animals with lungs
pre-injured by intratracheal instillation of the bacterial component lipopolysaccharide
(LPS) also resulted in markedly enhanced BALF ACE activity and in increased levels of
Ang II and inflammatory mediators. In addition, expression of the Ang II receptor, AT1,
was enhanced in the lungs (Chapter 3). BALF inflammatory mediator production
was attenuated after administration of the Ang II receptor antagonist, losartan. This reduction
was more pronounced when compared to the decrease that was observed after blocking
ACE with captopril. Taken together, these results indicate that Ang II plays an important
role in the inflammatory injury observed in ALI/ARDS.

Until recently, ACE was considered to be the key enzyme in the RAS, but this classical
view was challenged by the discovery of the enzyme ACE2 (Donoghue et al., 2000; Tipnis
et al., 2000). The major role for ACE2 is the conversion of Ang II to Ang-(1-7), hereby
counteracting the effects of ACE. ACE2 knock-out mice were shown to have enhanced
vascular permeability, increased lung edema, neutrophil accumulation and worsened
lung function (Imai et al., 2005). Apparently, ACE2 has a protective effect. This effect
may be due to degradation of Ang II, formation of Ang-(1-7) or both. In an experimental
ARDS model an imbalance was shown between ACE and ACE2 (Chapter 4). Lung injury
induced by LPS followed by mechanical ventilation was associated with an increased
ACE and decreased ACE2 activity in the lung. As a consequence, levels of Ang II and
Ang-(1-7) were increased and decreased, respectively. This imbalance was associated
with higher lung injury scores and an exaggerated inflammatory mediator production.
These effects were attenuated after blocking the AT1 receptor or by administration of
a protease-resistant, cyclic form of Ang-(1-7). As a result, lung function improved as
evidenced by increased oxygenation. Taken together, these findings strongly suggest
that ACE functions as a lung injury-promoting factor via Ang II and its AT1 receptor. In
contrast, ACE2 functions as a lung protective factor via Ang-(1-7) and its Mas receptor.
Mechanisms explaining the interplay between RAS, inflammatory mediators and lung injury

The mechanisms that are responsible for activation of the RAS are poorly understood. In addition, it is unclear whether the effects on pulmonary ACE and ACE2 in ALI/ARDS are due to the alveolar RAS and/or that from the circulation. It has been shown in vitro that alveolar macrophages and epithelial cells are capable of expressing the genes encoding RAS components (Wang et al., 1999; Uhal et al., 2007). This and the presence of several RAS components at the surface of these cells (Chapter 3 and 4; Gembardt et al., 2005; Hamming et al., 2004) support the existence of a local (pulmonary) RAS. The involvement of the pulmonary RAS in ALI/ARDS follows from the changes in the activity of ACE and ACE2 observed in BALF. Moreover, the apparent cross talk between Ang II and Ang-(1-7) in ALI/ARDS are suggestive of cellular co-localization of the AT1 receptor and Mas receptor, as indeed is manifest on the alveolar macrophages and epithelial cells (Chapter 4). This, however, merely suggests the involvement of the pulmonary RAS in ALI/ARDS. But final proof for a distinct role should be obtained. For instance, in situ hybridizations as well as further immunohistochemistry should provide evidence for a role of a local RAS. It can, however, not be excluded that ACE and ACE2 leak from the circulation into the alveolar compartment during mechanical ventilation and are taken up by the alveolar macrophages.

The mechanism that links the RAS system to the inflammatory response and subsequent lung injury in ARDS is unknown. Ang II has been shown to activate the transcription factors nuclear factor (NF)-kappaB and activator protein (AP)-1, which translocates to the nucleus and increases transcription of pro-inflammatory genes in renal, vascular, and mononuclear cells (Ruiz-Ortega et al., 1998; Han et al., 1999; Pueyo et al., 2000; Kranzhöfer R et al., 1999; Wolf et al., 2002; Ruiz-Ortega et al., 2002; Esteban et al., 2004; Ruiz-Ortega et al., 2001). NF-kappaB regulates, among others, the expression of ICAM-1, IL-1β, IL-6, IL-8 and TNF-alpha (Fan et al., 2001; Christman et al., 2000). In agreement, inhibition of ACE in experimental lung injury models was associated with an inhibition of the activity and attenuation of the nuclear translocation of NF-kappaB and AP-1 transcription factors (Ortiz et al., 2002; Jerng et al., 2007). As a consequence, the inflammatory mediator response was significantly decreased in the lung.

The signaling pathways by which Ang II affects the activation of the transcription factors NF-kappaB and AP-1 are not fully understood (Figure 1). It has been found in several cell types that Ang II induces phosphorylation of three members of the mitogen-activated protein kinase (MAPK) family: extracellular signal-related kinase (ERK), Jun amino terminal kinases (JNK)-1 and JNK-2, and p38 kinase (Ushio-Fukai et al., 1998; Eguchi et al., 2001; Wamsley-Davis et al., 2004; Tian et al., 1998; Kudoh et al., 1997; El Bekay et al., 2003; Su et al., 2006; Morrel et al., 1999). This effect was prevented by incubating
cells with an Ang II type 1 (AT1) receptor blocker. A similar effect was observed in an experimental ARDS model (cecal ligation and puncture) (Shen et al., 2009). These data indicate that Ang II activate the MAPK proteins via the AT1 receptor. Interestingly, the p38 intracellular signaling pathway activated by Ang II is also activated in alveolar cells by mechanical stretch (Oudin and Pugin, 2002) and has been shown to attenuate post-transcriptional mRNA degradation. Previous studies with lung epithelial cells have shown that an attenuated mRNA degradation results in an exaggerated production of inflammatory mediators (van den Berg et al., 2005). The synergy between the RAS components, inflammatory stimuli and ventilator-induced lung cell stretching observed in animals and in in vitro models may thus be explained by both an increased transcription of target inflammatory genes due to cooperation between transcription factors, and by a stabilization of messenger RNAs encoding inflammatory mediators, allowing for

Figure 1. Mechanisms explaining the interplay between RAS, inflammatory mediators and lung injury
Abbreviations: ACE = angiotensin-converting enzyme; Ang = Angiotensin; AT1 = Angiotensin II type 1 receptor; MAPK = mitogen-activated protein kinase; ERK = extracellular signal-related kinase, JNK = Jun amino terminal kinase; NF-kB = nuclear factor-kB; AP-1 = activator protein-1
Summary and general discussion

The activation of the different signaling molecules by Ang II can be attenuated by Ang-(1-7) (Figure 1). This suggests the existence of a complex feedback system on a cellular level (Su et al., 2006; Tallant and Clark, 2003; Zhu et al., 2002). For instance, in rat proximal tubular cells Ang-(1-7) completely inhibited Ang II-stimulated phosphorylation of p38, ERK 1/2 and JNK (Su et al., 2006). It was concluded that local tissue generation of Ang-(1-7) could serve a protective role by counteracting the effects of locally generated Ang II. Paradoxically, it has also been shown that Ang-(1-7) has similar effects as Ang II by acting as a pro- rather than anti-inflammatory mediator. In vitro and in vivo experiments in mice have shown that Ang-(1-7), via its Mas receptor, aggravates the inflammatory response in experimental models of renal failure (Esteban et al., 2009). Infusion of Ang-(1-7) increased NF-kB activation in the kidneys of the investigated mice. Furthermore, it stimulated release of pro-inflammatory cytokines. In addition, studies have reported that the effects of Ang-(1-7) either may be blocked (Tallant et al., 1991; Jaiswal et al., 1992) or stimulated (Castro et al., 2005) by AT1 receptor antagonists under certain conditions, suggesting that Ang-(1-7) may also interact with AT1 receptor.

Together this has led me to propose that Ang II and Ang-(1-7) regulate inflammatory mediator production by influencing the cross-talk between the signaling pathways linked to the AT1 and the Mas receptors (Figure 1). In this model, Ang II drives transcription of inflammatory genes, and may act in addition or in synergy with other pro-inflammatory stimuli like LPS or TNF-α. Via p38, Ang II may also attenuate mRNA degradation further amplifying the inflammatory mediator production. Ang-(1-7) counteracts the effects of Ang II by inhibiting or reversing activation of the various kinases that are activated by Ang II. High concentrations of Ang-(1-7) may activate AT1 and therewith overcome the inhibitory effect of Ang-(1-7) via the Mas receptor. This model implies that Ang-(1-7) only attenuates the inflammatory mediator production by cells that co-express AT1 and the Mas receptor.

In conclusion, there is evidence for a delicate balance between the two effector peptides of the RAS: Ang II and Ang-(1-7). The overall regulation of the effects of the two peptides seems to be more complex than anticipated and may be dependent on cell type, concentration and interactive effects. Elucidation of the regulatory mechanisms of the different RAS components remains a key area of continuing research.
Potential mechanisms explaining the differences between pediatric and adult ARDS

There are striking differences between children and adults in incidence and outcome of ALI/ARDS. The reported incidence increases with age. The mean incidence in children is about 2-13 cases per 100,000 person-years (Zimmerman et al., 2009; Erickson et al., 2007; Dahlem et al., 2003; Kneyber et al., 2008). It increases to 16 cases per 100,000 person-years in the age between 15 and 19 years and it peaks in persons between 75 and 84 years of age at 306 cases per 100,000 person-years (Rubenfeld et al., 2005). Mortality also increases with age from 20 percent among pediatric patients to 60 percent among those of 85 years or older (Zimmerman et al., 2009; Flori et al., 2005; Albuali et al., 2007; Khemani et al., 2009; Rubenfeld et al., 2005). Although co-morbid conditions probably contribute to the higher mortality in elderly patients, middle-age adults still have a significantly higher mortality than children (Rubenfeld et al., 2005). This suggests pathophysiological differences in the development of ALI/ARDS in adults and children.

Recent animal studies have revealed possible explanations for the differences in incidence and outcome in pediatric and adult ALI/ARDS patients. Adult animals are more susceptible to ventilation-induced lung injury than newborn or young animals (Copland et al., 2003; Copland et al., 2004; Kornecki et al., 2005; Nin et al., 2008). This was associated with a relatively blunted inflammatory mediator response in the young animals (Kornecki A et al., 2005; Copland et al., 2004). In another study, a synergistic increase in pulmonary inflammation was shown in adult mice that had been exposed to LPS and subjected to moderate tidal volume mechanical ventilation (Smith et al., 2010). This synergistic increase was absent in juvenile mice. The differences between juvenile and adult animals may relate to an immature versus mature immune system such as a difference in the threshold for activation. Along similar lines, it may be argued that mechanical stress due to stretching differs between juvenile and adult animals, and/or the cross-talk between mechanical stretch and the immune response.

Notably, there are also age-dependent differences in the RAS. In adults, there is an association between the I/D polymorphism and the severity and outcome of ALI/ARDS (Jerem et al., 2006, Marshall et al., 2002). In contrast, an association between this polymorphism and RSV-induced ALI was not found in children (Chapter 5). This chapter described another difference between adults and children related to ALI/ARDS. A marked pulmonary inflammatory mediator response was found on the first day of admission of children. This hallmark of ARDS was already diminished after 48 hours. In contrast, levels of inflammatory mediators of adult ARDS patients peaked during the first 3 days of admission and returned to near normal values only after 14 days (Park et al., 2001). These data show that it is not appropriate to apply adult-based phenotype criteria for
ARDS in a pediatric context. Furthermore, the data underscore the need for clinical and experimental studies to further elucidate the differences in etiology, pathophysiology and outcome of ARDS in children and adults. These studies may lead to different treatment modalities for ARDS in children.

Experimental data indicate that aging is associated with increased renal ACE and AT1 production and decreased renal ACE2 production (Schulman et al., 2010). A significant age-specific decline of ACE2 production was also observed in the rat lung (Xie et al., 2006). There are also indications that there is an age-specific decline of ACE2 expression in the human lung. This is based on epidemiologic data that show that there is a predominance of young adult patients in severe acute respiratory syndrome (SARS) attacks (Liang et al., 2004; Poutanen et al., 2003; Booth et al., 2003). The corona virus that causes SARS uses ACE2 as its functional receptor. Taken together, the reduction of ACE2 production in time may cause an imbalance in the pulmonary RAS towards the ACE/Ang II pathway, thereby stimulating the inflammatory response. This could be the explanation for the differences in incidence and outcome of ARDS in adults and children.

Towards clinical use of the different RAS components

This Thesis and other studies have revealed intriguing possibilities for the use of RAS-modulating agents as novel targets for therapeutic treatment of ARDS. Especially, cyclic Ang-(1-7) is a promising candidate for treatment of this syndrome. However, there are still pathways and mechanisms to be explored and questions to be answered.

Nature of injury (direct and indirect lung injury). Pharmacological intervention during ARDS needs to be evaluated according to the predisposing condition. In this Thesis, the role of modulation of the RAS in direct lung injury was assessed. However, these studies have to be extended to other underlying etiologies of ARDS, such as sepsis-, acid-aspiration- and trauma-induced ARDS, before modulation of the RAS system should be considered in clinical practice for ALI/ARDS. Furthermore, the existence of an imbalance of the RAS during ALI/ARDS needs to be confirmed in humans.

Effect of aging. Experimental data indicate that aging is associated with a shift towards the ACE/Ang II pathway at the expense of the ACE2/Ang-(1-7) pathway. As a consequence, elderly patients may benefit more from modulation of the RAS towards the ACE2/Ang-(1-7) pathway than the younger patients. This has to be explored by comparative experimental studies between young and adolescents/adult animals. In addition, these studies need to be extended to humans.

Mode of action. The exact mode of action of the RAS components on the different cell types in the alveolar compartment needs to be elucidated. Accumulating evidence
supports that the overall regulation of the effects of the two effector peptides of the RAS seems to be more complex than anticipated and may be dependent on cell type, concentration and interactive effects (Esteban et al., 2009; Li et al., 2008; Brasier et al., 2000; Lambert et al., 2010). In vitro experiments studying the effects of Ang II and Ang-(1-7) on the inflammatory mediator response of alveolar epithelial cells and macrophages may help to unravel the mechanisms underlying the actions of the different RAS components in ALI/ARDS.

Phase of ARDS at the time of intervention. The development of ARDS consists of four phases: The first phase is defined as the at-risk phase. The second phase, known as the acute or exudative phase, takes place during the first 24 to 48 h. This phase is characterized by the activation and infiltration of inflammatory cells into the lung tissue. Subsequently, the disease progresses to phase 3, the proliferative phase, which occurs between day 2 and 7. Fibroblasts infiltrate and remodel the site of inflammation during this phase. Finally, the fibrotic phase ensues 7-14 days after the onset, resulting in consolidation and fibrosis of the pulmonary parenchyma. The success of drug therapy may depend on the phase of ARDS at the moment of treatment. However ACE inhibitors, AT1 receptor blockers and Ang-(1-7) not only result in attenuation of the anti-inflammatory response but also in inhibition of the fibrotic response (Li et al., 2003; Li et al., 2007; Li et al., 2008). This implies that therapy based on these agents can be applied both in phase 2 and phase 3 of ARDS and are thus attractive therapeutic candidates.

Taken together, the results described in this Thesis show that an imbalance in the (pulmonary) RAS plays an important role in the development of ALI/ARDS, at least in direct lung injury. If the development of such an imbalance is a pivotal step in the development of this syndrome, modulation by either blocking the lung injured ACE/ Ang II pathway or stimulating the lung protective ACE2/Ang-(1-7) pathway may offer a promising treatment option for this devastating syndrome.