Thesis for doctoral degree (Ph.D.)
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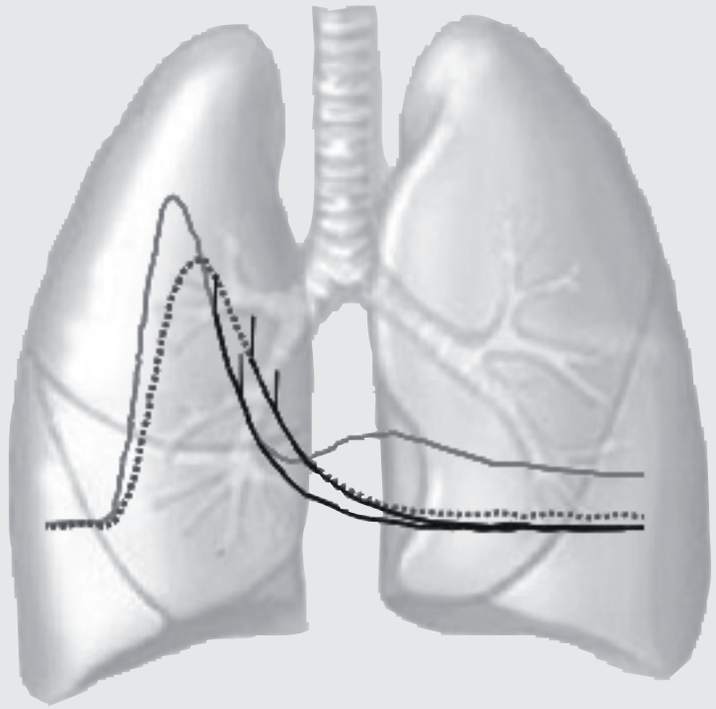
The role of the endothelin system in experimental acute lung injury

With special reference to the formation of extra-vascular lung water



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The role of the endothelin system in experimental acute lung injuryr

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THE ROLE OF THE ENDOTHELIN SYSTEM IN EXPERIMENTAL ACUTE LUNG INJURY

WITH SPECIAL REFERENCE TO THE FORMATION OF EXTRA-VASCULAR LUNG WATER.

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"All interest in disease and death
is only another expression of interest in life"
Thomas Mann

To My family
Ann-Sofie, Bella, Edvin and Axel

ABSTRACT

Acute lung injury is a major clinical challenge in the intensive care unit. Sepsis is the most frequent underlying cause of this pulmonary syndrome, which includs inflammation-induced diffuse alveolar damage and early stage high permeability edema. In spite of extensive research few therapies have reached the clinical arena, a fact that calls for additional interventional strategies.

The role of the endothelin system in pulmonary disease has been established, and recently it has also been implicated in the pathogenesis of acute lung injury. The 21-amino acid endothelin-1 (ET-1) has in addition to its vasoconstrictive properties also mitogenic and permeability increasing effects. Consequently, endothelin receptor antagonism might be a potential therapeutic strategy in acute lung injury.

The major aim of this thesis was to investigate the role of the endothelin system in the pathophysiology of endotoxin-induced acute lung injury, with special attention to edema formation. Moreover, we wanted to evaluate two different indicator dilution methods for assessment of extra-vascular lung water in relation to the well established gravimetrical method.

Endotoxin challenge induced a severe deterioration in pulmonary function and gas exchange along with increased extra-vascular lung water, pulmonary capillary pressure and pulmonary venous vascular resistance.

The molecular double indicator dilution method for measurement of extra-vascular lung water was unable to detect any change in this parameter despite a two-fold increase measured by gravimetry. The single thermal indicator method systematically overestimated extra-vascular lung water and was influenced by regional changes in ventilation and perfusion. This method relies on an empirically derived relationship between the intra-thoracic blood volume and the global end-diastolic volume. By simultaneous measurements of these parameters we found their relationship to be altered during endotoxemic conditions. However, the method was able to detect relative changes in extra-vascular lung water over time during endotoxemia.

The dual (ET_A/ET_B) endothelin receptor antagonist tezosentan abolished the endotoxin-induced increase in extra-vascular lung water, pulmonary capillary pressure and pulmonary venous resistance and improved gas exchange. Furthermore, *in vivo* challenges with ET-1 and the ET_B receptor agonist sarafotoxin increased pulmonary capillary pressure and pulmonary venous vascular resistance. Congruent results were obtained *in vitro* by the same agents. These findings point towards the importance of the ET_B receptor in endotoxin-induced pulmonary hypertension.

We conclude that during endotoxemia the endothelin system is involved in development of venous pulmonary hypertension and edema formation which may be prevented by dual ET receptor antagonism. When we tried to estimate pulmonary edema with either molecular double indicators or a single thermal indicator we found inaccuracies, which raise the question of their use in the clinical situation. Our results together with growing evidence showing the potential of ET receptor antagonists to reduce inflammation, permeability and possibly pulmonary fibrosis, suggest that we might have come to a point where clinical studies could be of interest.

Key words: Acute lung injury, endothelin-1, extra-vascular lung water, pulmonary capillary pressure, sepsis, pulmonary edema, endotoxin

List of Publications

I. Rossi P, Oldner A, Wanecek M, Leksell L G, Rudehill A, Konrad D, Weitzberg E.
Comparison of gravimetric and a double-indicator dilution technique for assessment of extra-vascular lung water in endotoxaemia.
Intensive Care Med. 2003 Mar;29(3):460-6.

II. Rossi P, Wanecek M, Rudehill A, Konrad D, Weitzberg E, and Oldner A.
Comparison of a single indicator and gravimetric technique for estimation of extravascular lung water in endotoxemic pigs.
Crit Care Med 2006 May; 34(5) 1437-43.

III. Rossi P, Wanecek M, Konrad D, Oldner A.
Tezosentan counteracts endotoxin-induced pulmonary edema and improves gas exchange.
Shock. 2004 21(6): p.543-548.

IV. Rossi P, Persson B, Boels P, Arner A, Weitzberg E, Oldner A.
Endotoxemic pulmonary hypertension is largely mediated by endothelin receptor-induced venous constriction.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

$^{2}H_2O$	Deuterium (heavy water)
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
DSt	Down slope time
ET	Endothelin
ET_A	Endothelin receptor subtype A
ET_B	Endothelin receptor subtype B
EVLW	Extra-vascular lung water
EVLWI	Extra-vascular lung water index (mL/kg)
GEDV	Global end-diastolic volume
HR	Heart rate
ITBV	Intra-thoracic blood volume
ITTV	Intra-thoracic thermal volume
MAP	Mean arterial pressure
MDID	Molecular double indicator dilution
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-κB
PaO_2	Arterial partial pressure of oxygen
PAOP	Pulmonary arterial occlusion pressure
Pcap	Pulmonary capillary pressure
P-F or P/F	Partial pressure of oxygen in arterial blood divided by inspired fraction of oxygen
P_{IF}	Interstitial pressure
P_{eff}	Effective filtration pressure
π_{IF}	Interstitial oncotic pressure
π_C	Capillary oncotic pressure
PTV	Pulmonary thermal volume
RM-ANOVA	Repeated measure Analysis of variance
STID	Single thermal indicator dilution

INTRODUCTION

In 1967 Ashbaugh *et al.*[12] firstly described the clinical course and specific radiological findings in 12 patients with respiratory failure that initially was to be defined as the adult respiratory distress syndrome[153], later renamed acute respiratory distress syndrome (ARDS) since it occurs in children as well. The criteria of ARDS were expanded in 1988[136] and further reviewed in 1994 by the American-European consensus committee[18]. This report introduced the concept of extra-pulmonary and pulmonary ARDS and introduced a severity grading of the syndrome based on the degree of hypoxemia relative to inspired fraction of oxygen. Not until the late 1990's was it demonstrated that the distinction of extra-pulmonary and pulmonary ARDS was valid regarding pathological mechanisms and therapeutic responsiveness[68]. At the 1998 consensus conference it was suggested that the ARDS terminology should be substituted with the term acute lung injury (ALI) and that it should be accompanied with a detailed severity score, for better correspondence with the continuous development of the syndrome[11]. Although the initial criteria from 1994 (table 1) have been questioned and others have been proposed, they are still in use[56,57,177,214]. The term ALI denotes a pulmonary inflammatory syndrome involving diffuse alveolar damage and high-permeability edema induced by various insults.

Table 1. 1994 consensus criteria for ALI and ARDS[18]

Hypoxemia	P/F ratio < 300 mmHg (ALI), P/F ratio < 200 mmHg (ARDS)
Radiological finding	Bilateral infiltrates
Onset	Acute onset
Left ventricular failure	PAOP< 18 mmHg or no clinical suspicion

ALI constitutes a major clinical problem and the incidence have been reported to vary between 18-80 per 100 000 subjects and year. The mortality is in the range of 38-68 % in recent studies[114,116,120,167]. The great variability in these figures could be a reflection of the crudeness of the initial criteria[57]. Regardless of the criteria, sepsis is one of the major underlying causes. Since ALI is a syndrome secondary to many different underlying diagnoses such as sepsis, pneumonia, trauma and massive blood transfusion improvements in therapy of these conditions could be expected to decrease the incidence of ALI[123].

PATHOPHYSIOLOGY OF ALI

The initial description of autopsy findings included atelectasis, hemorrhage and pulmonary edema with protein rich hyaline membranes[12], subsequent studies later revealed extensive pulmonary microthrombosis formation and fibrin deposition[22]. Experimental studies and increasing knowledge concerning molecular mechanisms has added to the pathophysiological picture. Disappointingly, so far, only one therapeutic strategy (i.e. the ARDSnet study on reduced tidal volumes) convincingly improving survival has emerged[1]. This fact underlines the complexity of the ALI pathophysiology. The hallmark of early ALI is the inflammatory-induced high permeability edema in the lung, described specifically in the following sections. However, many of the suggested therapeutic strategies have targeted pathophysiological changes secondary to this inflammation and they will firstly be described below.

Atelectasis

The formation of atelectasis during ALI occurs as a consequence of small airway collapse present in inflamed lungs. Another mechanism is by resorption of trapped air distally to obstructed airways. Airway obstruction occurs both by stagnant secretion but also by inflammatory changes in the mucosa of the terminal airways[40]. Furthermore the intubation procedure in patients already breathing high fractions of oxygen further adds to the formation of atelectasis both by the inevitable prolonged period of apnea and enhanced gas resorption in obstructed airways. In the last decade the importance of opening these atelectatic regions has become more evident[6,103]. Mechanical ventilation-induced repetitive opening and closure of atelectatic lung regions (atelecto-trauma) as well as over distention increases the pulmonary production of pro-inflammatory mediators that may contribute further to pulmonary damage[197].

Lung stretch

Although ventilator-induced lung injury is beside the scope of the current thesis this entity deserves some attention since mechanical ventilation is so intimately linked to ALI. It is now obvious that mechanical ventilation can cause lung injury by stretch-induced release of proinflammatory mediators from the alveolar epithelium and endothelium. It has furthermore been suggested that these pro-inflammatory mediators, originating from the pulmonary tissue, may cause injury to distant organs, possibly providing an explanatory model for ALI-induced multi organ failure[111]. These mechanisms may to some extent explain the favorable outcome of low tidal volume strategy in the ARDS network study[1].

Ventilation perfusion mis-match

In physiological conditions the overall perfusion of the lung is well matched to the ventilation. A regional vertical distribution of different pulmonary ventilation-perfusion ratios has since long been acknowledged with relatively more ventilation to the apical parts and the opposite with relatively more perfusion to the basal parts of the

lungs[212]. More recently, the importance of a sagittal distribution pattern has attracted much attention in the investigations of the mechanisms behind the potential beneficial effects of prone positioning of patients[5,71,134]. In animal models the blood flow has been shown to be diverted from edematous areas suggesting an important role of hypoxic vasoconstriction[24]. The findings of regionally disturbed pulmonary perfusion in ALI patients found by Pistolesi *et al.* supports this notion[155]. However, it is known that hypoxic pulmonary vasoconstriction might be blunted in certain conditions such as endotoxemia which in part might explain the increased venous admixture observed in ALI[43,121,195]. In contrast, a recent study of pulmonary perfusion in ALI patients surprisingly showed no difference in perfusion pattern compared to healthy control subjects[178]. These findings illustrate the difficulties in comprehending the complex regulation of pulmonary perfusion in different pathophysiological conditions.

Pulmonary hypertension

Considering the vascular physiology of the pulmonary circulation, increased pulmonary venous vascular resistance elevates the pulmonary capillary pressure and promotes edema formation more, when compared with an isolated increase in pulmonary arterial resistance.

In ALI pulmonary hypertension is often present[221]. It is well documented experimentally that this is largely due to an increase in pulmonary venous vascular resistance both in ALI and septic models[54,79,193]. Suggested mechanisms of increased pulmonary vascular resistance are leukocyte sequestration in pulmonary capillaries and venules together with formation of microthrombi and vasoconstrictive substances released during inflammation[126]. Furthermore, precapillary vasoconstriction mediated by hypoxic pulmonary vasoconstriction definitively plays a role in ALI.

In chronic pulmonary hypertension increases in both arterial and venous pulmonary vascular resistance has been noted[58,90,185], possibly mediated by a slow remodeling of the pulmonary vasculature[51,75]. In acute inflammatory states like endotoxemia, anaphylaxia and smoke inhalation injury increases in predominantly venous pulmonary vascular resistance has been noted[54,62]. These increases have been attributed to various substances like histamine, thromboxane and platelet activating factor[14,31,51,67,75,198].

However, the regulation of the longitudinal distribution of pulmonary vascular resistance and its impact on edema formation in various pathological conditions is complex. An illustrating example of this is how precapillary vasoconstriction can promote edema formation as in high altitude pulmonary edema. Hypoxic pulmonary vasoconstriction is an intrinsic system that diverts regional blood flow by means of precapillary vasoconstriction from hypoxic- to normoxic regions. This defense mechanism increases the pulmonary arterial vascular resistance in these regions and at a certain degree of hypoxia overall pulmonary vascular resistance and even mean pulmonary arterial pressure are increased. As pulmonary hypoxic vasoconstriction is of major importance in the development of high altitude pulmonary edema it has been debated how precapillary vasoconstriction can induce pulmonary edema. It has been suggested that the pulmonary hypoxic vasoconstriction is not evenly distributed

throughout the pulmonary circulation allowing the increased pressure to be transmitted to regions of less precapillary vasoconstriction resulting in increased pulmonary capillary pressure and subsequent edema formation[15].

PULMONARY EDEMA

Fluid filtration.

Fluid filtration across any capillary ($\dot{V}$) bed is determined by the factors in the classical theory of Starling, expressed in the below equation[187].

$$\dot{V} = K \times (Pcap + \pi_{IF} - P_{IF} - \pi_C)$$

Under physiological conditions the filtration coefficient (k) is constant, but different for various capillary beds. The effective transmural filtration pressure P_{eff} is the difference between the pulmonary capillary pressure (Pcap) and the pressure of the interstitial fluid (P_{IF}) of the surrounding tissue. Since the P_{IF} is smaller than Pcap the P_{eff} is the hydrostatic driving force of fluid filtration. In analogy, the effective transmural oncotic pressure (π_{eff}) is the difference in oncotic pressure between the capillary (π_C) and the interstitium (π_{IF}). The π_C is larger than the π_{IF} resulting in an oncotic force counteracting the hydrostatic filtration force to some extent. As the Pcap decline downstream the capillary, and the π_C increases due to fluid filtration, the net forces shift direction and a resorption of fluid occurs. Since filtration exceeds resorption the lymphatic drainage balances the equation.

Hydrostatic edema

An isolated increase in Pcap results in an increase in fluid filtration which up to a point exceeds the draining capacity of the lymphatic system whereby a hydrostatic edema develops. This is the case in isolated cardiogenic pulmonary edema. With increasing Pcap structural changes in the pulmonary blood-gas barrier occurs with transcellular breaks in the endothelial and the type I alveolar cells as well as in the basal membrane[211]. This has been noted to start with P_{eff} as low as 24 mmHg and considering that the normal P_{IF} is subatmospheric[130] one gets a notion how delicate this balance is. This initially produces an interstitial edema that can progress to leakage, subsequently flooding of the alveoli.

Inflammatory edema

Inflammation is normally induced by noxious stimuli such as tissue damage or host invasion by microbes. The noxious stimuli trigger a very complex pro-inflammatory cascade with the aim of isolating and removing injured tissue as well as invading microbes. In order to be locally self confining, the process also involves anti-inflammatory properties. These anti-inflammatory processes are also important in converting the process into healing of tissue and restoring organ function. It is now

evident that both pro- and anti-inflammatory processes starts almost at the same time and if the ensuing balance is disturbed severe organ dysfunction may result, either as a consequence of failed healing, or prolonged overwhelming pro-inflammatory activity. Furthermore, distant organs not primarily affected can become engaged by such a prolonged inflammatory activity[135].

Some times the noxious stimuli are overwhelming as in severe multiple trauma, severe burn injuries and exaggerated bacterial invasion like fecal peritonitis or severe pneumonia and sepsis. In addition, certain microbes have the ability to form so called super-antigens by which they trigger a rapid overwhelming and uncontrolled systemic inflammatory reaction although the microbial inoculation is not massive[4].

In the case of pulmonary aspiration, pneumonia and lung laceration the primary noxious stimuli occur in the lung and the development of ALI might not be surprising. When the primary injury occurs to some other part of the human body the lung is frequently the first distant organ to be secondarily affected, probably since all the blood containing various inflammatory mediators, draining from the injured region, will enter the pulmonary capillary circulation. It has been suggested that primary and secondary ALI have different pathophysiological mechanisms[151]. As in general with many previously described clinical syndromes, growing knowledge will probably lead to subdivision into many separate entities[166].

Oncotic relationships

With critical illness a reduction in plasma protein concentration is invariably noted, but in the absence of pulmonary inflammation the reduction in capillary oncotic pressure will not induce pulmonary edema. With local inflammatory reactions follows a fragmentation of the interstitial matrix with a rise in interstitial oncotic pressure. When occurring in the lung these two changes act synergistically in promoting edema formation together with the increased permeability[130,138].

Leukocytes

In septic conditions the pro-inflammatory mediators tumor necrosis factor-α, interleukin-8 and platelet activating factor are all increased and it has been shown that they induce changes in the cytoskeleton of the leukocytes whereby their deformability decreases leading to sequestration in the pulmonary microcirculation. This sequestration occurs within an hour after onset of endotoxemia and has been suggested to explain the drop in leukocyte counts observed in early ALI. Leukocyte sequestration occurs mainly in the capillaries and venules and adds to the increase in pulmonary vascular resistance. The role of adhesion molecules in this process is not yet fully clarified, but it is clear that they play an important role in ALI by mediating leukocyte activation and migration into the lung tissue. Chemokines produced by macrophages, leukocytes, endothelial- and epithelial cells and other chemo-attractants like complement C5a and leukotriene B_4 also play an important role in leukocyte activation and migration in the pulmonary inflammatory process of ALI[165].

Permeability

The inflammatory process induces the liberation of several endogenous substances from various cell types capable of promoting increased permeability. The most important being: bradykinin, histamine, vascular endothelial growth factor, thrombin, reactive oxygen species and tumor necrosis factor-α, the latter three are considered of major importance in ALI. Some of these mediators act locally whereas others enter the circulation.

Thrombin is inactivated by activated protein-C and is short lived in plasma, nevertheless since thrombin is concentrated in fibrin clots (up to 1000-fold) it can be subsequently released during fibrinolysis in the later phases of ALI[106,176].

Reactive oxygen species are generated by activated leukocytes in ALI and mediate increased permeability through several effects on the endothelium like lipid-peroxidation, endothelial cell contraction and activation of phospholipases and protein kinases[28].

Tumor necrosis factor-α is a key mediator in sepsis and is released from activated monocytes and macrophages. Tumor necrosis factor-α can affect endothelial permeability by multiple mechanisms like activation of protein kinase-C_{α} and p38- mitogen-activated protein kinase. Furthermore, evidence suggests that tumor necrosis factor-α enhances the thrombin-induced increase in permeability and edema formation[126,174].

Exogenous substances such as endotoxin can increase permeability both locally and systemically if entering the circulation[147,159]. Endotoxin activates the release of several cytokines like tumor necrosis factor-α by transcriptional mechanisms involving NF-κB, through Toll-like receptor-4 activation. In addition, endotoxin induces Rho-A-dependent endothelial contraction, reorganization of actin filaments and protein tyrosine phosphorylation, all being suggested mechanisms of endothelial cell injury leading to increased permeability.

Thrombosis formation

Fibrin deposition and formation of microthrombi has been acknowledged features of ALI for a long time[22]. The growing knowledge of the importance of inflammatory mechanisms involved in ALI fits well with the notion that coagulation and inflammation interact in ALI. Furthermore, data emerging show that mechanical ventilation might induce changes in coagulation, contributing to ventilator-induced lung injury[106].

Tissue factor is released at sites of tissue damage as in ventilator-induced lung injury but release is also induced by cytokines like tumor necrosis factor-α, interleukin-1 and interleukin-6 produced during inflammation. Furthermore, these mediators stimulate the release of plasminogen activators leading to decreased fibrinolysis. Tissue factor pathway inhibitor, anti-thrombin and activated protein C all decline in sepsis due to increased breakdown and reduced production. All these effects promote thrombus formation and fibrin deposition which are seen in ALI[94,176].

Edema counteracting mechanisms

The pulmonary lymphatic system can increase its draining capacity several-fold during certain conditions such as endotoxemia[39]. With increasing edema the P_{IF} increases initially which reduces the P_{eff} and the edema formation rate whereby a new steady state can be established if the rise in Pcap is transient and inflammation does not ensue. The formation of alveolar edema can in part be counteracted by active absorption of water from the alveoli by both type I and type II alveolar cells, this clearance is enhanced by increases in intracellular cyclic adenosine mono-phosphate, and the mechanism involves active transport of sodium and water by means of specific sodium channels and aquaporines[126,173]. The accumulation of fluid in the pleural spaces is also considered a defense mechanism against pulmonary edema formation. Since the pleural spaces can harbor only a small amount of fluid without compressing the lung, this mechanism is of limited value in the ALI setting.

MEASUREMENT OF EXTRA-VASCULAR LUNG WATER

Since edema formation is a major pathophysiological feature of ALI it has long been considered an important parameter to monitor[188]. A monitoring technique should allow multiple repetitive measurements at the bedside with acceptable accuracy. Chest x-ray have been used for a long time but is considered too blunt in the intensive care setting[192]. Scanning technologies like magnetic resonance imaging and computer-tomographical x-ray can be used but does not allow for repetitive measurements in the clinic. For the repetitive quantification of extra-vascular lung water, indicator dilution techniques have been developed. They all share the same principle in that they are based on the determination of the mean transit time, through the pulmonary circulation, of a given amount of indicator and the simultaneous determination of flow. The indicator is injected into a central vein and detected in arterial blood. Specific features of the techniques used in this thesis are further outlined in the material and methods section below.

Double indicator dilution techniques

The initial molecular double indicator dilution (MDID) technique described by Chinard included the use of radioactive isotopes as indicators of the intra- and extra- vascular lung compartments[33]. The use of radioactive isotopes, difficult sampling and the need of post sampling analysis of radioactivity hampered further development of this methodology. The introduction of indocyanine green and heavy water (^2H_2O) allowed for spectrophotometric, high resolution determination of the dilution curves without using radioactive substances[108].

In the mid sixties the thermal indicator was introduced as a diffusible indicator in combination with indocyanine green and the so called thermo-dye technique was established[149], it was further developed and commercialized in the early seventies. This device did not allow visual inspection of the dilution curves and consequently

the inclusion of corrupt curves in the results together with inferior thermistor technology might explain the variable results obtained. In the early nineties a new commercial thermo-dye device, which allowed curve inspection was introduced. This newer thermo-dye technique has been evaluated extensively regarding influence of variations in ventilation and perfusion[16,20,63,213]. Nevertheless, this device has not gained widespread clinical use due to its invasiveness (detector located in aorta) and difficulty to calibrate. It has also been suggested that the thermal indicator in contrast to $^{2}H_{2}O$ can not discriminate between changes in flow and changes in volume of distribution[200]. The device is no longer available on the market as the manufacturer has chosen to promote their new single indicator system instead.

Single thermal indicator dilution technique

The single thermal indicator dilution (STID) technique is also an indicator dilution technique using a sole indicator consisting of a thermal bolus. The technique measures the change in temperature over time by means of a thermistor at the site of detection (distal aorta). The change in temperature is induced by a bolus injection of cold saline solution. The commercially available device has gained widespread use because of its relatively low invasiveness, and its validated ability to determine other parameters (e.g. cardiac output). In addition to determination of mean transit time of the indicator and flow, the methodology for determination of extra-vascular lung water is based on a principle introduced by Newman[140] and an assumption of linear relationship between two intra-thoracic volumes. This methodology has only to a limited extent been evaluated in comparison to the gravimetrical method as well as regarding the possible influence from changes in regional ventilation and perfusion of the lung.

THERAPEUTIC STRATEGIES AGAINST ALI

Early diagnosis and rapid intervention has improved survival considerably for myocardial infarction, trauma and septic shock and probably contributed to a decreased incidence of ALI[123].

The insight that mechanical ventilation could induce pulmonary damage through mechanisms such as shear stress, over distension and repetitive opening and closure of the peripheral airways[103,162] has contributed to major improvements in ventilatory strategies. The normalization of arterial blood gases as a goal has been substituted with limits of mechanical stress and the acceptance of relative hypoxemia combined with permissive hypercapnia[1]. Further experimental and clinical studies have shown that redistribution of ventilation, increased compliance and reduced pleural pressures probably accounts for the beneficial effects on gas exchange observed when using the prone position[69,134,137,152].

Negative fluid balance improve general intensive care outcome[131] and this strategy has been extrapolated to ALI although convincing evidence of beneficial effect is still lacking. Continuous furosemide infusion has proven to improve oxygenation both in the experimental setting[164] and lately also in a clinical trial also addressing the role

of correcting hypoproteinemia[122]. The underlying strategy here is of course to try to reduce extra-vascular lung water.

The role of steroid treatment in ALI has been debated in the literature for several decades. Previous studies have failed to prove beneficial effects of high dose steroid treatment in the early phases of ALI[19,21,115,186,207]. Recent studies, however, suggests that low dose steroid treatment for prolonged time might be useful[8,38,127] but the dosage, timing and duration of such a therapy remains to be clarified.

The therapeutic expectations of a ventilation-guided vasodilator like inhaled nitric oxide were initially great. Indeed, improvement in oxygenation has been demonstrated with inhaled nitric oxide in ALI patients but it failed to increase survival in two randomized multicenter trials[46,117]. Furthermore, inhalation of prostacyclin has been shown to improve gas exchange[201,223] but not survival. Consequently, neither inhaled nitric oxide nor inhaled prostacyclins are currently used for the routine treatment of ALI.

Although pulmonary hypertension is a main feature of ALI pathophysiology, surprisingly few studies have addressed whether reducing pulmonary hypertension, or pulmonary capillary pressure in particular, might influence outcome. The endothelin (ET) system has been demonstrated to be of major importance in pulmonary hypertensive disorders, and outside the ALI setting, ET receptor antagonists have been shown to be efficacious in reducing pulmonary hypertension[133]. Furthermore, in experimental studies they have been able to reduce endotoxin-induced pulmonary hypertension[203].

THE ENDOTHELIN SYSTEM

The existence of an endothelium-derived vasoconstrictive factor was first described by Hickey *et al.* in 1985[81]. Three years later the peptide endothelin-1 (ET-1) was isolated and characterized by Yanagisawa *et al.*[218]. Three different isopeptides of ETs (1-3) have been described of which ET 1-3 are encoded by three separate genes[86,169]. It is considered that ET-1 is the most significant of the ETs in humans. The following discussion will only concern ET-1 and also be limited to its potential role in various aspects of ALI. ET-1 is one of the most potent endogenous vasoconstrictors known and it is established that it plays a role in regulation of blood pressure[34,217]. Since the initial description it has become apparent that ET-1 is produced in many types of organs and tissues, and that ET-1 might be involved in numerous pathophysiological processes and diseases (table 2).

Table 2. Conditions with pathophysiological involvement of endothelin	
Acute lung injury[95]	Cardiomyopathy[219], Heart failure[196]
Primary pulmonary hypertension[85]	Subarachnoid hemorrhage[202]
Sepsis [204,210]	Hirschsprung's disease[124]
Asthma[72]	Prostate cancer[80]
Systemic sclerosis[36]	Ovarian cancer[80]
Renal disease[97]	Melanoma[156]

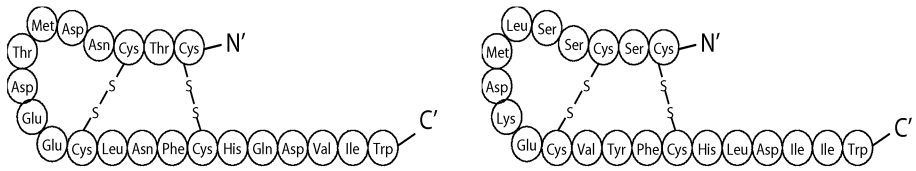

Figure 1. *Schematic illustration of endothelin-1 and sarafotoxin. Endothelin -1 (left) is a endogenous 21 amino acid peptide with two disulfide bindings. Sarafotoxin (right) is a exogenous selective* ET_B *receptor agonist differing in 10 amino acids from ET-1.*

Endothelin synthesis

ET-1 is a peptide with 21 amino-acids (fig. 1) which is continuously produced[76] but also stored in endothelial cell-specific storage granules released on stimulation[168]. ET-1 is generated by a two step conversion from the primary peptide pre-pro-ET-1 by an intracellular endopeptidase[105] to big-ET-1 which possesses biological activity but to a much lesser extent than ET-1 itself. Big-ET-1 is converted either intra- or extra-cellulary by different ET converting enzymes and chymases (fig. 2). ET-1 is mainly produced in endothelial cells but also in tissues like the lung, heart, kidney, liver and brain[60,87,144].

The synthesis and release of ET-1 is stimulated by several factors like endotoxin, vasoconstictors, thrombin, hypoxia, insulin, cytokines and growth factors[53,109,125]. The production of ET-1 is inhibited by atrial natriuretic peptide, estrogens and prostaglandins[83,158] and also indirectly by heparin and increased shear stress via nitric oxide[23,220]. ET-1 is mainly secreted abluminally from the endothelial cell towards the underlying vascular smooth muscle cells were it in a paracrine fashion exerts its primary action. Under physiological conditions only small amounts of ET-1 reach the circulation but in certain pathological conditions such as sepsis the circulating levels are dramatically increased[210].

The endothelin receptors

There are two different ET receptors, type A (ET_A) and type B (ET_B) which both belong to the hepta-helic G-protein-coupled receptor super family[44,109]. Both receptors are found on vascular smooth muscle cells and mediate contraction. In addition, the ET_B receptor is also located on endothelial cells, mediating vasodilation by means of release of nitric

oxide and prostacyclins[45]. This receptor subtype is also responsible for the clearance of circulating ET-1 via endocytosis[50,64,146]. The lung is the main site for removal of circulating ET-1, clearing up to 80% of the circulating ET-1 in a single passage through the pulmonary circulation[64]. ET-1 has equal affinity for both receptor subtypes[9,171]. ET receptors are also found on a variety of cell types in several organs where they also mediate different effects (fig. 3).

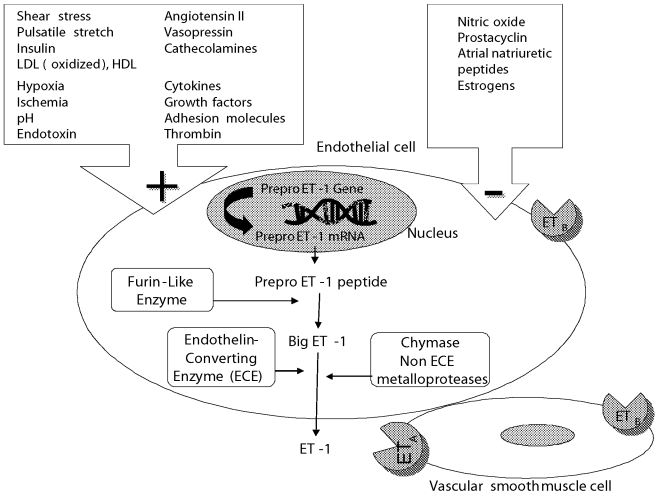

Figure 2. *Schematic illustration of human endothelin-1 synthesis. Endothelin-1 is synthesized in the endothelial cells and the production and release of ET-1 is stimulated by various stimuli. In addition ET-1 release and production is reduced by the indicated substances.*

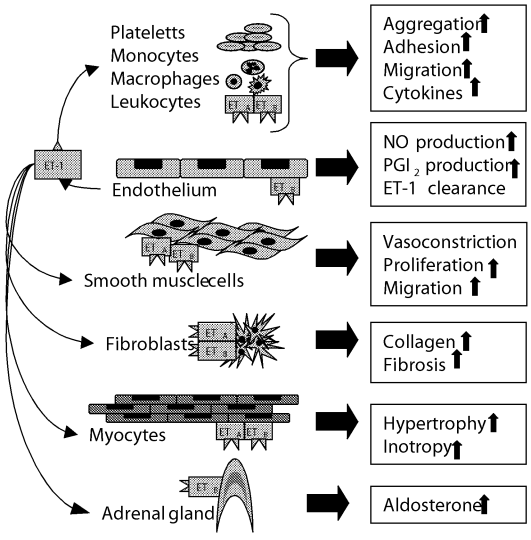

Figure 3. *Schematic illustration of ET-1 release from the endothelium and the effects mediated by the two ET-receptors on various cell types.*

Intracellular signaling

Both types of ET receptors activate the G-proteins and phospholipase-C which triggers a cascade of events mediated mainly by inositol triphosphate and diacylglycerol. This in turn activates calcium channels in the sarcoplasmic reticulum and a variety of cell membrane bound calcium channels resulting in a dramatic increase in cytosolic calcium[133](fig. 4). The calcium increase induces a contraction of the myofilaments and vasoconstriction ensues. The ET_B receptor also activates phospholipase A_2 with a subsequent production of prostaglandins and thromboxane, a known vasoconstrictor[10,183]. On the other hand stimulation of the endothelial ET_B receptors releases prostacyclin and induces nitric oxide production which mediates vasodilatation.

In addition, diacylglycerol and the increased calcium both contribute to the activation of protein kinase C which has emerged as a potential factor for increasing pulmonary capillary permeability. ET-1 also stimulates mitogen-activated protein kinases, both in a protein kinase-C dependant and independent way, which may explain the proliferative properties of this peptide[13,55,182,206]. Furthermore, it has been demonstrated that ET-1 can activate Rho-kinase A, which contributes to rearrangements of the cytoskeleton in vascular smooth muscle cells[206] and possibly to migratory properties of endothelial cells induced by cytoskeleton rearrangements[129]. These mechanisms of rearrangement may also contribute to increased permeability.

Endothelin and permeability

The role of ET-1 in modulating capillary permeability is far from clear. Several reports have been published suggesting that ET-1 increases permeability both directly by the ET_A receptor and indirectly by promoting release of permeability-increasing substances[27,52,59,77,99,113,157] (fig. 5). Clearly ET-1 seems to be involved in modulating microvascular permeability although the effects may depend on the specific capillary bed and the pathophysiological context studied.

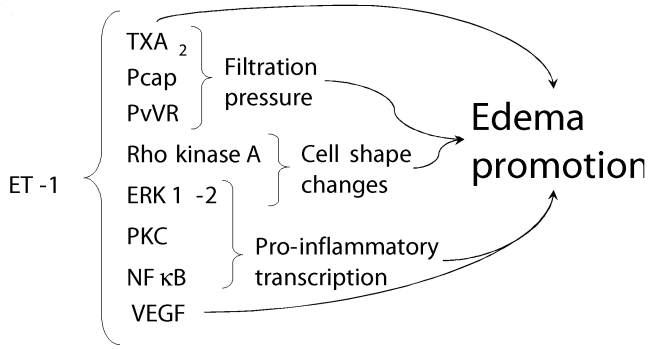

Figure 5. *Schematic illustration of edema promoting effects induced by endothelin-1. Tromboxane-A_2(TXA_2), Pulmonary capillary pressure (Pcap), Pulmonary venous vascular resistance (PvVR), extra-cellular signal-regulated kinases 1 and 2 (ERK 1-2), Protein kinas-C (PKC), Nuclear factor κ-B (NFκB), Vascular endothelial growth factor (VEGF).*

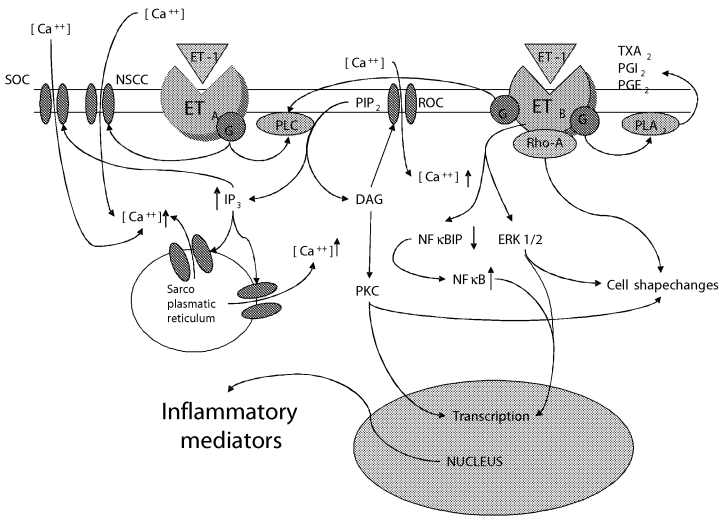

Figure 4. *Schematic illustration of endothelin receptor mediated intracellular signalling.*
By binding to the endothelin receptors G-proteins (G) are activated which in turns activates non specific cationic ion-channels (NSCC) in the cell membrane allowing calcium inflow. This leads to a depolarization which opens voltage dependent calcium channels (not illustrated). In addition, the G-proteins activates Phospholipase C (PLC) which hydrolyses phosphatidyl inositol 4,5 biphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG opens receptor operated calcium channels (ROC) and IP_3 both induces calcium mobilization from the sarcoplasmic reticulum and activates store-operated calcium channels (SOC). This cascade results in a large increase in intracellular calcium which induces smooth muscles cell contraction. The elevated cytosolic calcium levels together with the increase in DAG activate protein kinase C (PKC) which modulates gene transcription and induces changes in cell shape. The ET receptors activates extra-cellular signal-regulated kinases 1 and 2 (ERK 1/2) and inhibits the production of nuclear factor–κB inhibitory protein (NF-κBIP) which further modulates gene transcription. Furthermore, the ET receptors activate Rho kinase-A which together with ERK 1 and 2 also mediates cell shape changes. The ET_B receptor in addition, activates phospholipase A_2 that induces the release of thromboxane, prostaglandin E_2 and prostaglandin I_2 (i.e. prostacyclin). Regarding the endothelial ET_B receptor it is known to induce the release of prostaglandins and thromboxane through activation of PLA_2 but also to increase cyclic guanosyl-monophosphate an thereby induce nitric oxide production.

ENDOTHELIN AND THE LUNG

The lung is a major production site of ET-1[208]. Although ET-1 production is mainly attributed to endothelial cells it has been demonstrated that various cell types in different organs can produce this peptide. In the lung ET-1 is produced in the endothelium as well as by alveolar type II cells, alveolar macrophages, bronchial epithelium and vascular smooth muscle cells. In addition to the aforementioned association between ET-1 and permeability it has been suggested that ET-1 is a chemo-attractant for both monocytes and neutrophils and that this property could be blocked by the dual receptor antagonist

bosentan[2,42,216].These findings further support that ET-1 might be of importance in inflammatory states. In analogy, pulmonary ET-1 production has been suggested to be of importance in asthma and pulmonary fibrosis[36,72,194]. Furthermore, ET-1 is involved in pulmonary vascular remodeling in primary pulmonary hypertension as well as in chronic hypoxia[93]. These effects of ET-1 are thought to be attributed to its mitogenic properties. In addition, it has been shown that pulmonary vascular smooth muscle cells can produce ET-1 and it has therefore been suggested that this peptide might play a role in the late fibroproliferative stage of ALI[132,215]. Accordingly, the dual ET receptor antagonist bosentan is now used for treatment of patients with pulmonary hypertension[30].

Increased circulating levels of ET-1 have been observed in both septic and ALI patients[104,210]. Furthermore, ET-1 mRNA and ET_B receptor mRNA levels changes in endotoxemic experimental models[61,181]. ET receptor antagonists during endotoxemia reduce pulmonary hypertension and improve gas-exchange which further supports a role for the ET system in sepsis-induced ALI[204,209]. In addition, it has most recently been shown that high plasma levels of ET-1 were associated with increased extra-vascular lung water in septic patients[102].

Considering the properties of ET-1, which clearly have the potential of contributing to the pathophysiology of ALI, it is relevant to further investigate the role of the ET system in experimental models of ALI.

AIMS OF THE THESIS

The overall aim of the present thesis was to evaluate clinically applicable methods for the sequential determination of extra-vascular lung water in experimental endotoxemic conditions, and to investigate the role of the ET system in the pathophysiology of experimental acute lung injury.

The specific aims were:

- To establish an experimental model of endotoxin-induced acute lung injury including increased extra-vascular lung water.
- To evaluate the molecular double indicator dilution technique for assessment of extra-vascular lung water formation in endotoxin-induced acute lung injury.
- To evaluate the single thermal indicator technique for assessment of extra-vascular lung water formation in non-endotoxemic conditions and during endotoxin-induced acute lung injury.
- To evaluate the influence of regional changes in ventilation and perfusion on the accuracy of the single thermal indicator dilution technique.
- To determine if dual ET receptor antagonism during established endotoxin-induced acute lung injury influences pulmonary function and extra-vascular lung water formation.
- To study the effects of ET-receptor subtype agonism on pulmonary arteries and veins *in vitro* and *in vivo*.
- To determine the effects of endotoxin on pulmonary capillary pressure, pulmonary arterial and venous vascular resistances in the current model.
- To evaluate the effect of dual ET receptor antagonism on pulmonary capillary pressure, pulmonary arterial and venous vascular resistances during endotoxemia.

MATERIAL AND METHODS

THE PORCINE ENDOTOXIN MODEL

Several different experimental ALI models have been described differing both in insult and in species. The insults are often divided into primary pulmonary insults and non-pulmonary insults (table 3).

Table 3. Different experimental models of ALI and their clinical correlates

Primary ALI	Clinical correlate	Secondary ALI	Clinical correlate
Bronchio-alveolar lavage	ALI in the newborn	Gut ischemia and reperfusion injury	Reperfusion injury
Inhalation of toxic gases	Inhalation injury	Endotoxin infusion	Sepsis
Tracheal instillation of live bacteria	Pneumonia	Cecal ligation and puncture	Sepsis
Tracheal instillation of hydrochloric acid	Aspiration	Oleic acid	

All of these models, except the oleic acid model have their obvious correlate in the clinical setting. In addition, ventilation with high tidal volumes can be used in isolation or superimposed to mimic ventilator-induced lung injury. For the study of ALI related to sepsis, which still is the most common cause of ALI[49,116], the cecal ligation and puncture model is not as reproducible as the endotoxin model which hampers its use in large animal experiments. The methodologies used in this thesis for the determination of EVLW and Pcap were all adapted from the clinical setting and did not allow for use of smaller animals. In addition, the choice of species was also done based on the physiological resemblance, including the ET system, when compared with humans[190].

In order to induce an ALI we infused Escherichia coli lipopolysaccharide (serotype 0111:B4) during 4-5 hours. In all experiments included in the thesis, we used Swedish landrace pigs, which were anesthetized and mechanically ventilated. The animals were sacrificed at the end of the protocol when still anesthetized, by means of injections of pentobarbital in ethanol.

THE MYOGRAPH EXPERIMENTS

Freshly harvested swine lungs were purchased and rinsed at an abattoir and transported on ice to the experimental laboratory. Pulmonary arteries and veins were identified and vessel rings were prepared. Pairs of arteries and veins from each animal were mounted on a myograph in an oxygen perfused and heated organ bath. Viability of the vessels was established by inducing contraction to potassium. The viability of the endothelium was tested by its ability to mediate smooth muscle cell relaxation through endothelial nitric oxide production, which was induced by adding acetylcholine after contracting the vessel rings with prostaglandin $F_{2\alpha}$. Contractions to stimuli with ET-1, phenylephrine (a selective α_1-receptor agonist) and sarafotoxin (a selective ET_B receptor agonist), were determined and expressed in relation to maximal contraction induced by potassium (80 mM). Differences between arteries and veins in concentration-response curves were then analyzed. The myograph methodology was used in paper IV.

BIOCHEMICAL ANALYSES

In paper III, we analyzed the ET-1 concentration in plasma by determining the ET-1 like immuno-reactivity in a radioimmunoassay as described by Hemsén[78].

EVLW MEASUREMENTS

The gravimetric method

The gravimetrical method is the reference method for determining extra-vascular lung water. The method demands extraction of the lungs and can thus, only provide a single value at the end of an experiment. A blood sample is used to determine the concentration of hemoglobin, hematocrit and wet/dry weight ratio of the blood. The lungs are removed immediately after the circulation ceases and are drained of blood and weighed. The corresponding weight in water is added to induce hemolysis. The lungs are homogenized with the added water using a commercial blender and a homogenizer. Half of the homogenate is used to determine its wet-dry weight and the other half is centrifuged at 30 000 G for 1h at 4º Celsius. The supernatant is separated and its hemoglobin concentration and wet-dry weight is determined. The dry weights of the blood, homogenate and the supernatant are determined after 3 days incubation in a heat chamber of 85º Celsius. This process is in accordance with the modification[180] of the original procedure developed by Pearce[148]. The main purpose of this method is to account for residual blood in the lungs. EVLW is then calculated and indexed to bodyweight (for calculations see appendix). The gravimetrical method was used in paper I-III.

The MDID method

The molecular double indicator dilution techniques for the assessment of EVLW are based on the simultaneous injection into a central vein of two indicators with different volumes of distribution. The diffusible indicator has a volume of distribution which includes both the pulmonary tissue water and the intra-vascular compartment. In contrast the non-diffusible indicator has the same volume of distribution as plasma, i.e. the intra-vascular compartment.

After injection in a central vein simultaneous and repetitive determination of the concentrations of the two indicators in arterial blood, provides time dependent curves of concentrations. These are separated in time if the volumes of distribution of the two indicators are different. By subtracting the volumes of distribution one can calculate the EVLW.

Molecular indicators used previously have been radioactively labeled and their concentrations determined by scintillation counting of serial blood samples collected in rotating cuvette holders. The method used in paper I is based on spectrometric analysis of the non-radioactive substances indocyanine green and deuterium (heavy water, ^2H_2O). Blood is pumped through the detector system from the arterial side and back to the venous side. ^2H_2O is a suitable permeable indicator since it distributes in the same manner as water. It is non-toxic in trace amounts and can be detected spectrometrically. The concentrations of the two indicators are determined 1,500 times every minute, resulting in smooth and precise time dependent concentration curves.

To determine the area under the curve (AUC) and flow for the indicators the last portion of the decay is substituted by Stewart-Hamilton extrapolation[74,189], which accounts for recirculation of the indicators. The mean transit time defined as the point in time, which divides the AUC in half, is determined for each curve as described by Zierler[222](fig. 6). EVLW was calculated as the flow rate for ^2H_2O multiplied by the difference in mean transit times for the ICG and ^2H_2O curves, and indexed to body weight to obtain EVLWI. At least two sets of curves were used to calculate a mean value for each measurement. The method has been described in detail earlier[199].

The indicator dilution curves for indocyanine green and ^2H_2O were presented on a computer and corrupt curves were discarded. Corrupt curves were defined as: 1) slow washout curves caused by very early recirculation causing a failure of the concentrations of the indicators to fall to at least one half of the peak concentration[25,29] which in turn gives curves without a semi-exponential decay form; 2) early recirculation curves which have a semi-exponential decay but with enlarged area under the curve[179] , 3) technically distorted curves, i.e., signal distortion due to microbubbles of air or microthrombi.

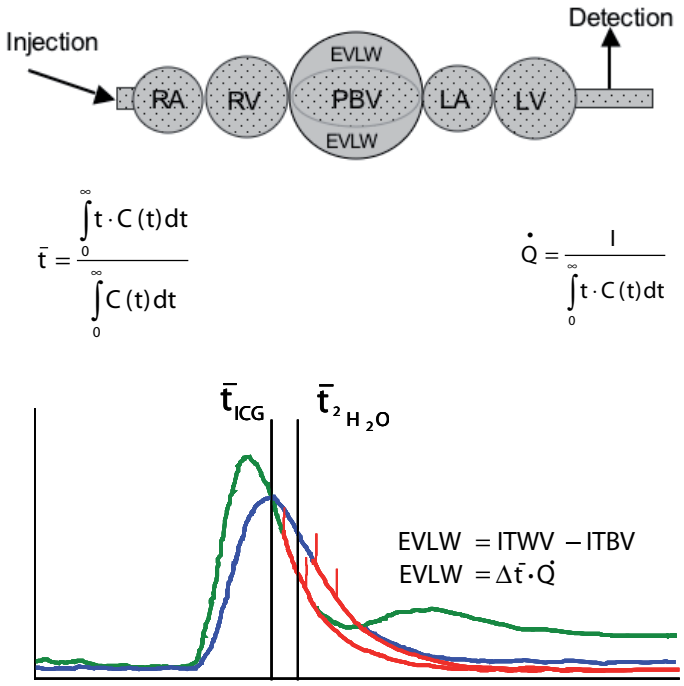

Figure 6. *Schematic illustration of the molecular double indicator dilution technique for the determination of extra vascular lung water (EVLW).The distribution volume for indocyanine green is termed intra-thoracic blood volume (ITBV) and is depicted with grey dotted filling. The distribution volume of heavy water is denoted intra-thoracic water volume (ITWV) and is depicted in grey filling. Green and blue curves are dilution curves for indocyanine green and heavy water respectively. The red curves are Stewart-Hamilton extrapolations derived from the original curve data between the red "flags". Mean transit time and flow are calculated according to the above equations using changes in concentration (C) and intensity (I) over time (t). Hence, EVLW is calculated as the difference in mean transit time for the two indicators times the flow. Right atrium (RA), right ventricle (RV), pulmonary blood volume (PBV), left atrium (LA) and left ventricle (LV).*

The STID method

By using standard equations (the same calculations as for the MDID-method) the system calculates the flow ($\dot{Q}$) and the mean transit time ($\bar{t}$) for the thermal indicator[189,222]. When multiplying these two factors the system can determine the thermal distribution volume between the site of injection and the thermistor. This volume is denoted intra-thoracic thermal volume (ITTV) (fig. 7 and Eq. 1). In addition, the system measures the down slop time (DSt) of the logarithmically transformed dilution curve (fig. 8).

By multiplying the DSt with $\dot{Q}$ (Eq. 2) the system calculates the volume of the largest mixing-chamber in the serial system constituted by the heart chambers and the lungs, according to Newman *et al*.[140]. The largest mixing chamber for the thermal indicator is denoted pulmonary thermal volume (PTV) and is constituted by the pulmonary blood volume and the lung tissue. By subtracting the PTV from the ITTV

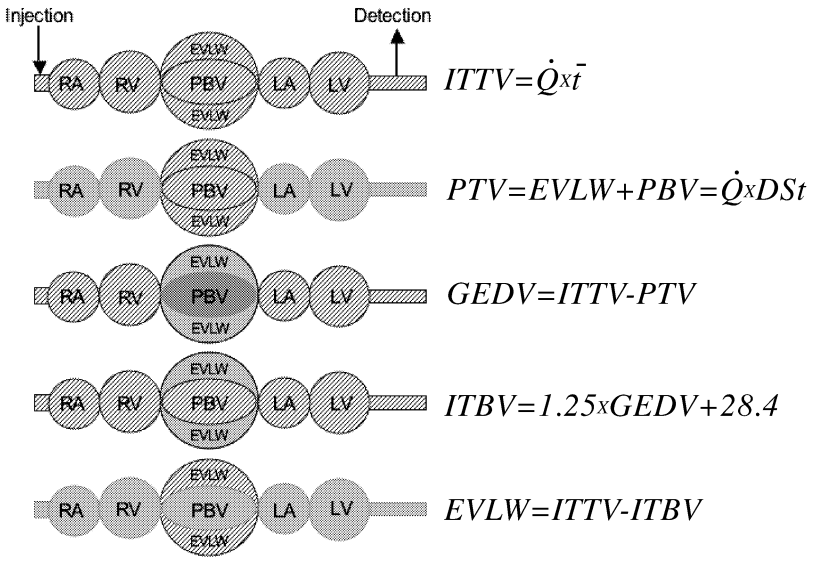

Figure. 7. *Schematic illustration of the single thermal indicator dilution technique for the determination of extra vascular lung water. Each entity explained by an equation is depicted graphically with oblique bars. The volume of distribution for the thermal indicator is denoted intra thoracic thermal volume (ITTV) and is determined by multiplying the mean transit time for the thermal indicator by flow. By multiplying the down slope time (DSt) with flow the largest mixing chamber denoted pulmonary thermal volume (PTV) can be determined. By subtracting the PTV from the ITTV the global end-diastolic volume (GEDV) is calculated. By using the empirical linear relationship between GEDV and the intra-thoracic blood volume (ITBV) the latter can be calculated. Finally by subtracting the ITBV from the ITTV the extra vascular lung water can be determined.*

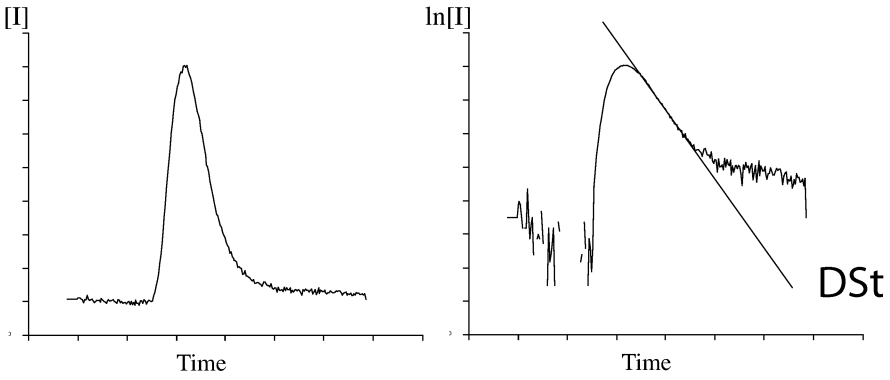

Figure 8. *Down slope time. The initial exponential decay of the thermal indicator dilution curve (left) is plotted logarithmically (right) and the linear down slope is determined and denoted down slope time (DSt). [I] indicator concentration.*

the composite extra-pulmonary blood volume between the site of injection and the thermistor is calculated and denoted; global end-diastolic volume (GEDV) (Eq. 3.). In order to calculate the EVLW from ITTV (Eq. 5.) the ITBV must be determined and the system uses an empirically established linear relation between the intra-thoracic blood volume and GEDV to calculate ITBV (Eq. 4.). The default relationship used by the PiCCO® system, ITBV = 1.25GEDV is based on a study by Sakka *et al.* who empirically found the relationship to be ITBV = 1.25GEDV –28.4 (mL)[170].

Equations:

$$ITTV = \dot{Q} \times \bar{t} \quad [1]$$

$$PTV = DSt \times \dot{Q} \quad [2]$$

$$GEDV = ITTV - PTV \quad [3]$$

$$ITBV = a \times GEDV + b \quad [4]$$

$$EVLW = ITTV - ITBV \quad [5]$$

DETERMINATION OF PULMONARY CAPILLARY PRESSURE

Capillary filtration pressure (P_{eff}) is the net resultant of the difference between the interstitial pressure and the Pcap. The former is not easily assessed but the Pcap can be measured. In paper IV we used the occlusion technique as described by Pellet *et al.*[150] who evaluated different algorithms for the determination of Pcap. To obtain as reliable results as possible it is important to determine the time point of occlusion, and that the occlusion occurs in systole. In order to satisfy these requisites we modified a conventional Swan-Gantz catheter with which we could register the pulmonary artery pressure both distally and 2 cm proximally to the balloon. Pellet *et al.* also found that the appropriate algorithms and time constants could vary depending on whether the vasoconstriction was predominantly arterial or venous. Furthermore, he found that using bi-exponential curve fitting between the time of occlusion and 4000 ms together with a time constant of 175 ms would be appropriate both for arterial and venous vasoconstriction. Hence, we used bi-exponential curve fitting and the 175 ms time constant (fig. 9). All occlusions maneuvers where performed in extended expirium. Only curves with a distinct occlusion point in systole and without major artifacts were analyzed.

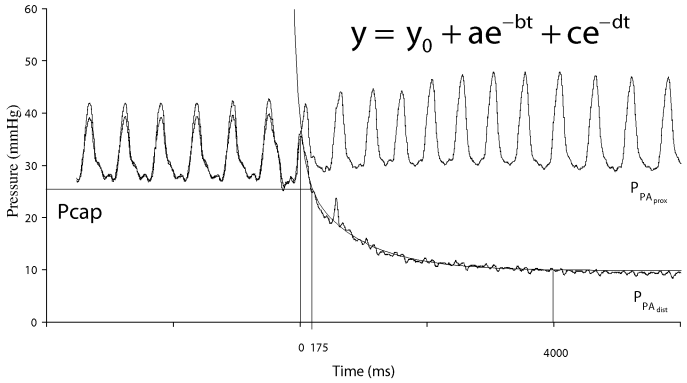

Figure 9. *Bi-exponential curve fitting for determination of pulmonary capillary pressure: Pcap: Pulmonary capillary pressure.* P_{PAprox}*: pressure tracing proximal to the occluding balloon.* P_{PAdist}*: pressure tracing distal to the occluding balloon. 0 = time of occlusion. 175ms = time constant for determination of Pcap on the fitted curve.*

PHARMACOLOGICAL INTERVENTIONS AND INSULTS

Endotoxin

Lipopolysaccharide from Escherichia Coli B0111:04 (Sigma, St. Luis, MO) was dissolved in warmed (35-37° C) saline solution and infused for five hours in all experiments except in paper IV where it was infused for four hours.

Tezosentan

The selective dual ET receptor antagonist tezosentan (Actelion, Allschwil, Switzerland) (fig. 10) was used in paper III-IV. Tezosentan has an affinity ratio between the ET_A-receptor and the ET_B-type receptor of 80/1. A minimal ratio of 100/1 is considered the limit for a selective ET_A receptor antagonist. Tezosentan is relative short acting and therefore suitable for intra-venous treatment in the acute setting[35,47,48]. Tezosentan was dissolved in saline solution and administered as a bolus infusion of 1 $mg \cdot kg^{-1}$ for ten minutes, immediately followed by a continuous infusion at a rate of 1 $mg \cdot kg^{-1} \cdot h^{-1}$. The selected dose has been used in other studies in various species. In a study published by Konrad *et al*. a higher dose of 10 $mg \cdot kg^{-1} \cdot h^{-1}$ in combination with endotoxin was associated with an increased mortality compared to the dosage used in paper III and IV[98].

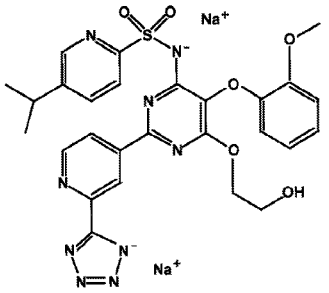

Figure 10. *The molecular formula of tezosentan. Tezosentan: [5-isopropyl pyridine-2-sulfonic acid6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2-1H-tetrazol-5-yl-pyridin-4-yl)-pyrimidin-4-ylamide]*

Endothelin receptor agonists and phenylephrine.

Endothelin-1.

ET-1 (American Peptide Co., Sunnyvale, CA) was used in paper IV both for *in vitro* and *in vivo* experiments. *In vitro* classical concentration response curves were constructed in the concentration ranges from 10^{-11}-$10^{-6.5}$ M. *In vivo* the average dose used was 1000 pmol/min. Due to variability in response the dosage was adjusted both up- and downwards in order to get increases in pulmonary vascular resistance that were significant but not overwhelming.

Sarafotoxin

Sarafotoxin is a 21 amino-acid peptide (fig. 1) found in the venom of the snake species *atractaspis engaddensis*. Sarafotoxin was further characterized as a selective ET_B-receptor agonist[184] and has subsequently been used in many experimental studies. We used the synthetic substance sarafotoxin S6c (American Peptide Co., Sunnyvale, CA). The dosages used in the study was 1000 pmol/min in average, again the dosage was adjusted to avoid deleterious effects.

Phenylephrine

Phenylephrine, (Apoteksbolaget, Stockholm, Sweden) a specific synthetic α_1-receptor agonist was used as reference substance for arterial vasoconstriction. The dosage used was 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$, a dose within the range of clinical use.

RESULTS AND COMMENTS

ENDOTOXIN-INDUCED EFFECTS

Severe systemic hemodynamic changes were induced by endotoxemia as noted by reductions in cardiac index, mean arterial pressure and mixed venous oxygen saturation and increases in pulmonary vascular resistance, mean pulmonary arterial pressure and heart rate (paper I). By increasing the volume-loading further to 30 $ml \cdot kg^{-1} \cdot h^{-1}$ including colloids these changes were partly attenuated (paper II and III). Furthermore, severe changes in pulmonary function were noted as reductions in oxygen partial pressure ($PaO_{2)}$, PaO_2-to the fraction of inspired oxygen ratio (P/F ratio) and compliance as well as increases in dead-space and alveolar to arterial difference in oxygen partial pressure. Both in paper II and III the severity of lung injury induced by endotoxemia was consistent with ALI except for the radiological criteria as no x-ray was performed in any of the experiments.

Endotoxemia also induced increases in Pcap both when estimated with the Gaar equation (Paper III) and when measured by the occlusion method (paper IV). Furthermore, we demonstrated in paper IV that the increase in pulmonary vascular resistance during endotoxemia was mainly of venous origin. The plasma ET-1-like immuno-reactivity increased several-fold during endotoxemia (paper III). Finally, extra-vascular lung water measured by the gravimetrical method was markedly increased in response to endotoxin challenge (paper I-III).

THE MDID METHOD VERSUS GRAVIMETRY (PAPER I)

The agreement in EVLWI measurements between the molecular double indicator dilution method and gravimetrically determined EVLWI was studied in two groups of animals. In the first group, only subjected to anesthesia (sham), the agreement of the methods was good. In the second group, where the measurements were performed after five hours of endotoxemia with severe deteriorations in pulmonary function, the agreement between the two methods for EVLW determination was flawed. The MDID method underestimated extra-vascular lung water with a mean bias of 5.8 mL/kg (fig. 11). This bias raises major concerns of the method since the absolute values in sham animals was 6.4 mL/kg.

In addition, the error of the MDID technique increased with increasing EVLW (measured gravimetrically). The most plausible explanation for the discrepancy between the two methods is that the MDID technique is sensitive to heterogeneities in pulmonary perfusion, often known to progress with the severity of sepsis-associated ALI. This might be explained by several factors such as formation of microthrombi and hypoxic vasoconstriction.

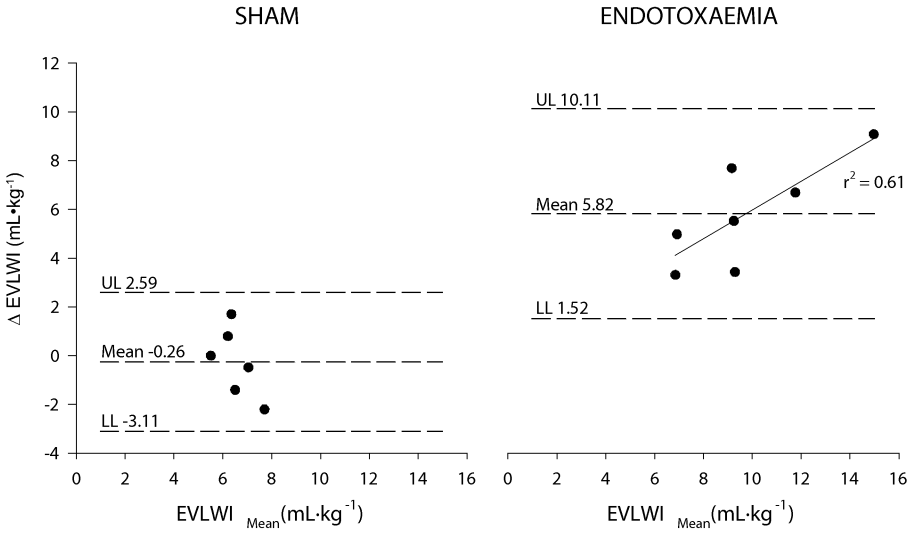

Figure 11. *Bland-Altman plot for EVLWI measurement by the MDID technique ($EVLWI_{MDID}$) and the gravimetrical method (EVLWIGrav) under endotoxemic and sham conditions. $\Delta EVLWI$ = EVLVIGrav - $EVLWI_{MDID}$. Upper limit of agreement (UL), lower limit of agreement (LL). $EVLWI_{Mean}$ = 0.5·(EVLWIGrav + $EVLWI_{MDID}$), Linear correlation between $\Delta EVLWI$ and $EVLWI_{Mean}$ under endotoxemic condition. r^2 =0.61. Regression line equation: $\Delta EVLWI$= 0.085 + 0.59·EVLWIMean*

THE STID METHOD VERSUS GRAVIMETRY (PAPER II)

The single indicator dilution technique overestimated EVLWI with a mean bias of 5.4 mL·kg^{-1} compared to the gravimetrical method (fig. 12), when using the default ITBV to GEDV relationship. By determining ITBV in endotoxemic animals with the MDID method the ITBV-GEDV relationship in endotoxemic pig was established. This relationship was found to be ITBV=1.52GEDV+49.7 instead of the default relationship of ITBV=1.25GEDV. When adopting this new relationship, the performance of the method improved in endotoxemic conditions. However, in non-endotoxemic conditions it produced an underestimation with a mean bias of 2.93 mL·kg^{-1}. Contrasting this possible pathophysiological dependency of the method, when trying to determine the EVLW in absolute values, was the finding that the STID method was able to detect changes over time in EVLW independently of the ITBV-GEDV relationship used.

The STID method was also demonstrated to be sensitive to induced changes in regional perfusion and ventilation but not to overall reduction in flow as demonstrated in paper II.

The findings described in paper II suggest that the STID method might not be able to distinguish changes in EVLW from changes in perfusion or ventilation. Furthermore, it indicates that the linear relationship between ITBV and GEDV, which is the foundation of the method, might actually vary between different pathophysiological condition and the species studied. The finding that it could detect changes in EVLW over time, suggests that the method may possibly be used as a trend monitor in the clinical situation.

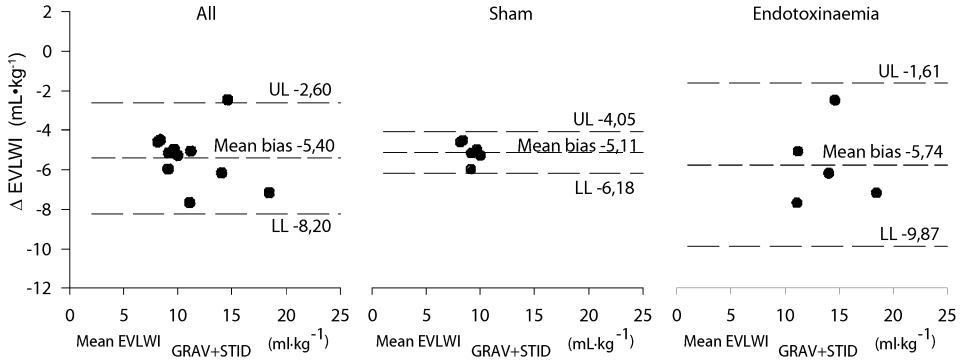

Figure 12. *Bland-Altman plots (of all measurements (n=11) and with sham (n=6) and endotoxemia (n=5) subgroup divisions) of extra-vascular lung water index determined by gravimetry ($EVLWI_{GRAV}$) and the single thermal indicator dilution method ($EVLWI_{STID}$) using the ITBV=1.25GEDV relationship. ITBV=intra thoracic blood volume, GEDV=global end-diastolic volume, UL= upper limit of agreement, LL=Lower limit of agreement. $\Delta EVLWI=EVLWI_{Grav}-EVLWI_{STID}$.*

TEZOSENTAN AND ENDOTOXEMIA (PAPER III-IV)

The most striking effect of tezosentan intervention during endotoxemia was that it abolished the endotoxin-induced increase in EVLW (fig. 13) (paper III). Furthermore, tezosentan improved pulmonary circulation by inhibiting a further rise in mean pulmonary arterial pressure, reducing venous admixture and dead space compared with non treated animals. Tezosentan-treated animals also improved in pulmonary compliance and gas-exchange.

Intervention with tezosentan after two hours of endotoxemia (paper III) induced a rise in cardiac index without any significant changes in HR or MAP. Furthermore, tezosentan counteracted the progressive fall in mixed venous oxygen saturation noted in endotoxemic control animals. In paper IV we reported on similar effects of tezosentan after three hours of endotoxemia but noted a reduction in mean arterial pressure and an increase in heart rate in response to intervention. These differences could possibly be explained by the lesser extent of fluid administration during these experiments.

In paper IV the effect of tezosentan on pulmonary vascular resistance and capillary pressure was analyzed. It was found that the endotoxin-induced increase in pulmonary venous resistance and capillary pressure was dramatically reduced by tezosentan intervention (fig. 14). The plasma ET-1-like immuno-reactivity increased notably when tezosentan was administered as a sign of significant ET_B-receptor antagonism.

Taken together these findings indicate that ET-1 is of great importance in endotoxemic pulmonary hypertension largely due to pulmonary venous constriction, possibly mediated via the ET_B receptor.

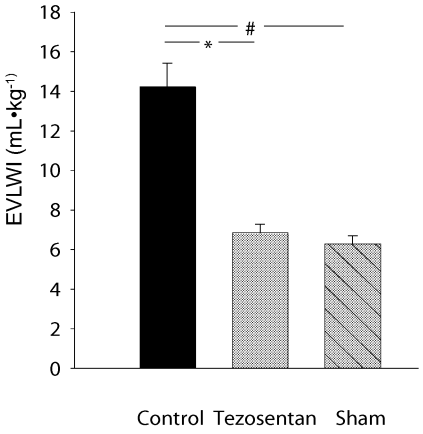

Figure 13. *Extra-vascular lung water index (EVLWI) in endotoxin control (black bar) and tezosentan-treated (grey bar) measured at 5h as well as non-endotoxemic sham animals (diagonally striped bar). Significant differences between groups analysed by Student's t-test for independent values symbolised by * = p < 0.001 for difference between control and tezosentan groups, and # = p < 0.001 for difference between control and sham groups.*

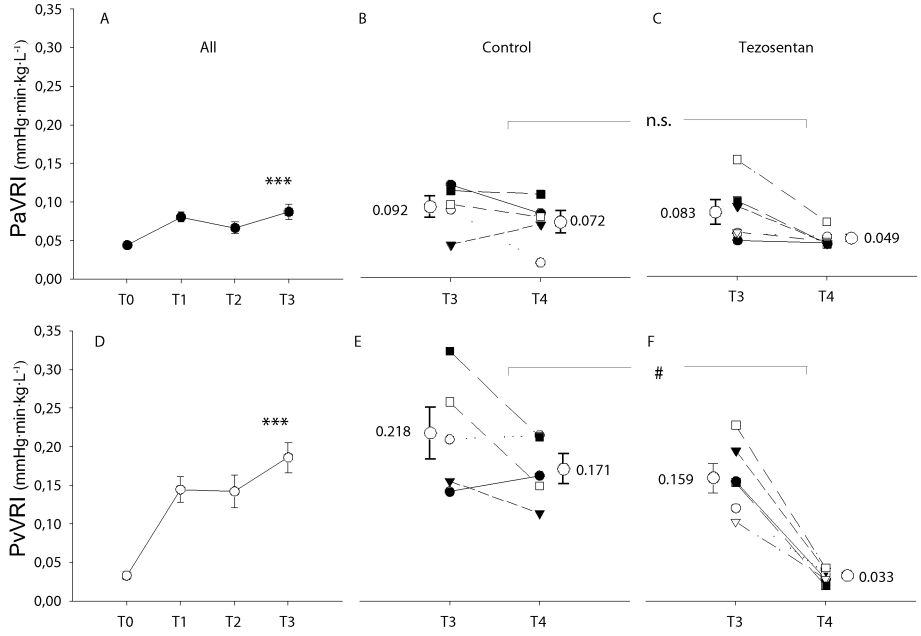

Figure 14. *Arterial and venous resistances during endotoxemia. Pulmonary arterial vascular resistance index (PaVRI, panels A-C) and pulmonary venous vascular resistance index (PvVRI, panels D-F). Panel A and D: All animals (n=12) from baseline (T0) to 3 hours (T3). Mean± SEM. Panel B and E: Individual values for control animals between 3 and 4 h (T4). Panel C and F: Individual values for tezosentan-treated animals between 3 and 4 h. Mean ± SEM for both time points indicated by numbers and larger open circles. Difference in time-effect interactions between PaVRI and PvVRI denoted by *** = p<0.001. Difference in time-effect interactions of tezosentan versus control calculated by RM-ANOVA and indicated by brackets with # for p< 0.05.*

PULMONARY VASCULAR REACTIVITY (PAPER IV)

In paper IV we report on pulmonary vascular reactivity to ET-1, sarafotoxin and phenylephrine both *in vivo* and *in vitro*. *In vivo* we observed that all three substances increased Pcap. Both ET-1 and sarafotoxin had a more pronounced effect on pulmonary venous resistance compared to arterial while the opposite was observed with phenylephrine (fig. 15).

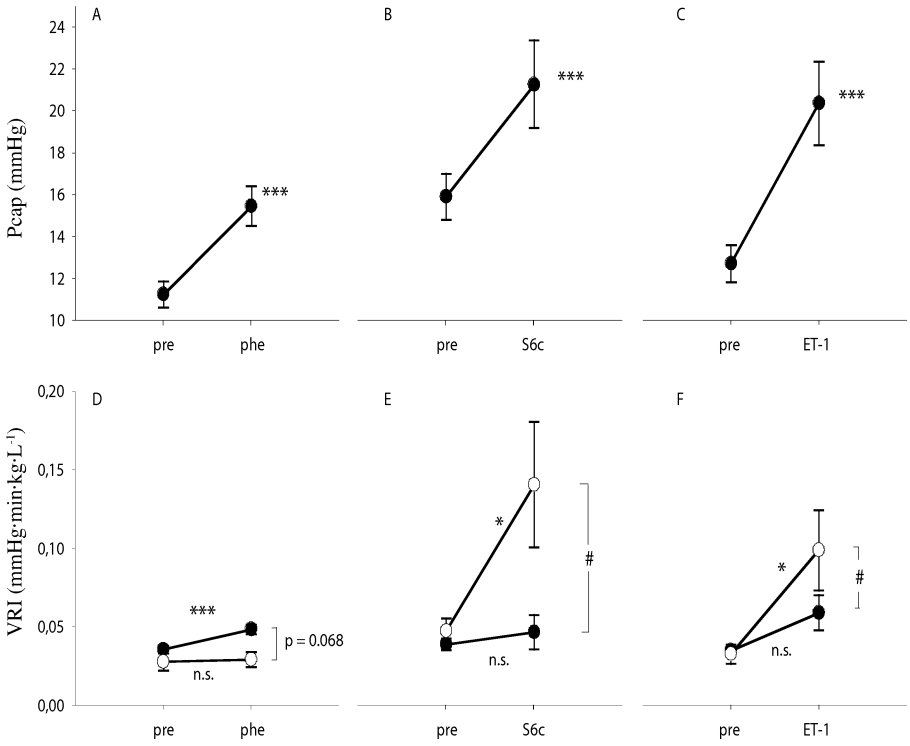

Figure 15. *Pulmonary capillary pressure (Pcap) and pulmonary vascular resistances (VRI) before and during challenges with phenylephrine (PHE), endothelin-1 (ET-1) and sarafotoxin 6c (S6c). Panels A-C: Pcap changes from baseline values (pre) to challenge with PHE (n=10), ET-1 (n=7) and S6c (n=6). Panel D-F: Pulmonary arterial vascular resistance index (PaVRI, filled circles) and pulmonary venous vascular resistance index (PvVRI, open circles). Mean ± SEM. Difference between baseline and intervention denoted by * for p<0.05 and *** for p<0.001 and n.s. for non significant. Differences in time-effect interactions (RM-ANOVA) between PaVRI and PvVRI from basline to challenge denoted by # for p<0.05.*

Of the three substances used *in vitro* ET-1 was the most potent constrictor of both arteries and veins, with a venous predominance. S6c had a profound effect on veins but a negligible effect on the arteries whereas the opposite was observed for phenylephrine. The sarafotoxin-induced venous contraction was not as strong (in absolute values) as the contraction induced by ET-1, possibly indicating that ET_A-receptors might contribute to the venous contraction to some extent (fig.16). The *in vitro* results are consistent with

the *in vivo* findings except for the lack of an increase in pulmonary arterial vascular resistance in response to ET-1 *in vivo*. This discrepancy might be due to the relatively few observations.

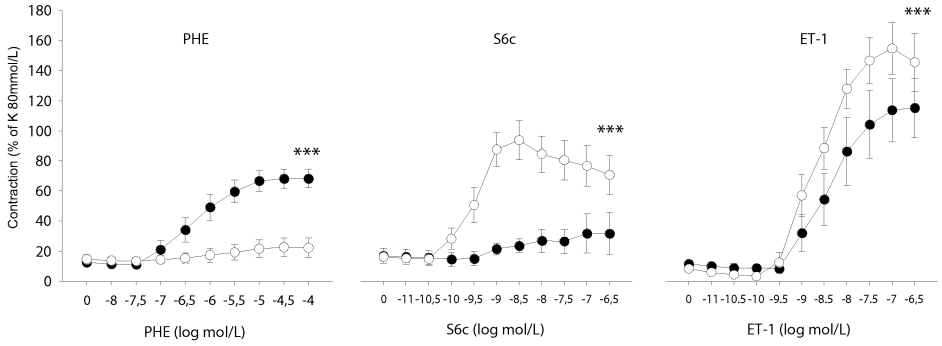

Figure16. *In vitro cumulative concentration response curves in porcine pulmonary arteries (filled circles) and pulmonary veins (open circles) after administration of phenylephrine (PHE, n=8), sarafotoxin 6c (S6c, n=5,) and endothelin-1 (ET-1, n=6, panel). Mean ± SEM. Difference in concentration-effect interaction of arterial and venous contraction was calculated by RM-ANOVA and indicated by *** for p<0.001*

DISCUSSION

A key feature in the pathophysiology of early ALI is edema formation which is clinically manifested as deteriorations in gas exchange and lung mechanics. As of today there are no reliable and repeatable monitoring tools that may help in guiding therapy against edema. Such a method could possibly promote a favorable outcome assuming that edema is a prognostic factor in ALI. Although our knowledge about the underlying mechanisms of ALI has increased substantially, few therapies are directed towards endogenous mediators or cascade systems in this disease.

Edema development is governed by increases in filtration pressure and capillary permeability. ET-1 is an endogenous peptide with inherent pulmonary vascular vasoconstrictive properties in combination with a potential to increase permeability. In addition, ET-1 has pro-inflammatory and mitogenic properties and seems to play an important role in pulmonary fibrosis[36]. It is noteworthy that pulmonary fibrosis is a main characteristic of late lung injury. Moreover, the lung is a major production site of ET-1, both tissue and plasma levels of this peptide are increased in experimental sepsis[61,118]. These facts support further investigations concerning the role of ET-1 in acute lung injury and sepsis.

We have evaluated different methods for measurement of EVLW and their usefulness in endotoxin-induced lung injury. Furthermore, we have investigated the role of the ET system in edema formation, pulmonary function and pulmonary vascular physiology during endotoxemia. Below follows a general discussion in relation to our findings but for a more detailed reading we refer to the individual papers.

EXTRA-VASCULAR LUNG WATER

In the current study gravimetrically determined extra-vascular lung water was found to be in the same range irrespective of duration of anesthesia (paper II,III) indicating experimental stability in terms of edema formation. Extra-vascular lung water increased markedly in endotoxin challenged animals (paper I-III) compared with non-endotoxemic control animals. For summary of extra-vascular lung water findings see fig. 17.

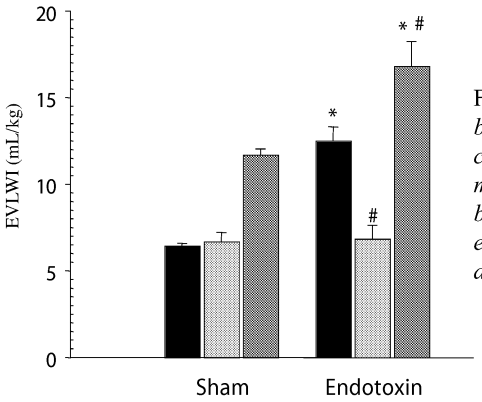

Figure17. *Extra-vascular lung water measured by different methods in sham and endotoxemic conditions. Gravimetry black bars, MDID-method light grey bars, STID method dark grey bars. * denotes differences between sham and endotoxemia with the same method, # denotes difference compared to gravimetry.*

Double indicator dilution techniques

In paper I we evaluated the modified molecular double indicator dilution technique[108] for the determination of EVLW compared with gravimetry under endotoxemic conditions. Despite improved technological properties compared with earlier molecular methodologies the system had an unacceptable accuracy during endotoxemic conditions. In contrast, Wallin *et al.* showed that this MDID method correlated well with gravimetrically determined EVLW in oleic acid-induced ALI in awake goats[200]. Endotoxin-induced ALI produces large changes in pulmonary perfusion as indicated by the changes in venous admixture and dead space in paper I. These changes probably explain the inability of the MDID method to detect the increased EVLW induced by endotoxin. This is consistent with a violation of the acknowledged prerequisite of perfusion to all lung tissue of indicator dilution techniques. In contrast, the thermo-dye technique has been reported to be less sensitive to such alterations although Schreiber *et al.* recently showed that the thermo-dye technique may also be sensitive to larger changes in regional perfusion, such as temporal ligation of pulmonary arterial branches[175].

The MDID technique, despite technological improvement does not seem to be suitable for EVLW measurements in conditions with large perfusion heterogeneities like sepsis-induced ALI. According to the literature the more invasive thermo-dye method might be more accurate during endotoxemic conditions but its abilities to discriminate between changes in flow and volume of distribution have to be elucidated more thoroughly[107,200].

Single indicator technique

The single thermal indicator dilution technique systematically over-estimated EVLW compared with the gravimetrical method, both during sham conditions and endotoxemia (fig 17). Moreover, experimentally-induced changes in regional perfusion and ventilation markedly affected the measurements.

The method is based on the assumption of a linear relationship between intra-thoracic blood volume (ITBV) and global end-diastolic volume (GEDV) and on the

measurements of flow, mean transit time and down-slope time. The linearity in the relationship between ITBV and GEDV was originally postulated by Pfeiffer *et al.* although only reported in abstract form[154]. The single indicator dilution system uses the ITBV-GEDV relationship found in patients by Sakka *et al.*[170]. Interestingly this differs from that originally reported by Pfeiffer *et al*. Subsequently a handful of reports, including paper II in this thesis, have reported different equations for this linearity (table 4). By adapting the ITBV-GEDV equation to the experimental model used in paper II (ITBV = 1.52GEDV+ 49.7) the accuracy of the STID method improved.

Table 4. Different ITBV to GEDV relationships reported in the literature
ITBV = a·GEDV+b

a	*b*	Species	Insult	Author
1.16	86	Human	-	Pfeiffer [154]
1.30	0	Pig	-	Pfeiffer [154]
1.25	-28.4	Human	Various	Sakka [170]
1.73	-7.7	Pig	Oleic acid	Neuman [139]
1.34	0	Sheep	-	Kirov [96]
1.10	99.5	Pig	Oleic acid and hemorrhage	Nirmalan[141]
1.21	99	Pig	Control	Nirmalan [142]
1.45	0.6	Pig	Hemorrhagic shock	Nirmalan [142]
1.52	49,7	Pig	Endotoxin	Rossi(paper II)

Thus, two different equations have been reported in humans, together with six different equations in pigs during different conditions, implying a dependency of the actual pathophysiological condition[139,154]. Nirmalan *et al*. actually found three different relationships in pigs during control conditions, hemorrhagic shock and a combination of oleic acid insult and relative hypovolemia. [141,142].

In addition to the above mentioned methodological aspects, the ability of the thermo-dilution technique to accurately determine mean transit time has been questioned due to hampered thermistor responsiveness caused by its embedding in the catheter[200]. Regarding the measurements of flow, i.e. cardiac output, this limitation is compensated for by the empirical computation constant[89]. Determination of mean transit time is dependent of the shape of the dilution curve which also is affected (i.e. falsely prolonged decay) by catheter mounting of the thermistor. This however, is not compensated for by the computation constant. Furthermore, the shape of the decay of the indicator concentration curve is crucial for the determination of the down slope time used for the calculation of EVLW. The use of thermal indicator instead of dye, which was used in the original work, interferes to a larger extent with the assumptions postulated by Newman *et al*. due to thermal inertia of catheters and loss of indicator to the surrounding tissue. Hence, the down slope time and the intra-thoracic blood volume both crucial to EVLW calculation with the single indicator principle, can be influenced in a yet uncontrolled way.

Intra-cardiac volume and pulmonary tissue volume varies with gender, age and somatotype (i.e. physique) as well as with the degree of physical training[3,73,92,119,172]. Considering this, generalizing the empirically established linear relationship initially derived from 57 critical ill patients, for the determination of ITBV and subsequent calculation of EVLW in all patients, could be questioned.

Our results that the default relationship of the device overestimated EVLW is in line with the findings of Katznelson *et al.*[91]. In addition, Kirov *et al.* still found a slight overestimation despite the use of an adapted GEDV to ITBV relationship[96].

When summarizing the performance of the STID method it is obvious that it is potentially influenced by changes in species, subject physique and the pathological condition under which it is used. Nevertheless, the method was able to detect a relative increase in EVLW during the course of endotoxemia suggesting that it may be useful as a trend indicator.

PULMONARY CAPILLARY PRESSURE

Edema formation in ALI is likely a combined effect of increased capillary filtration pressure and permeability. In intact lungs edema formation has been shown to increase linearly at a Pcap above 28 mmHg[65] which is consistent with the pressure at which, structural changes in the blood-gas barrier have been observed[211]. In paper IV we report that endotoxemia generated a mean Pcap of 28 mmHg and 26 mmHg in paper III (estimated by the Gaar equation). These levels of Pcap would not necessarily lead to edema formation in intact lungs but the extent of edema formation reported in paper III was probably a synergistic effect between increased Pcap and increased permeability induced by endotoxin. Since Pcap was estimated with the Gaar equation in these experiments it is plausible that the actual Pcap might have been even higher (see below).

Pulmonary arterial occlusion pressure alone is a poor surrogate for the filtration pressure[150]. Different PAOP-based estimates of the filtration pressure like the Gaar equation[65] and the transpulmonary pressure gradient (i.e pulmonary diastolic pressure-PAOP) has been suggested in the literature. However, Fang *et al.* showed that there may be dissociation between PAOP and left atrial pressure during endotoxemia-induced pulmonary hypertension[54]. They suggested that this dissociation was due to Starling resistor forces rather than venoconstriction. Independent of underlying mechanisms this may invalidate the assumption of a fixed relationship between PAOP and pulmonary capillary pressure as assumed in the Gaar equation[66]. This dissociation has also been suggested in ALI patients, making measurement of Pcap necessary for estimations of the pulmonary capillary filtration pressure[110,143].

The notion that PAOP might overestimate left arterial pressure would lead to an underestimation of the pulmonary venous resistance, and in that case both the effects of endotoxin and the effects of ET receptor antagonism found in paper IV would actually be greater. However, unpublished data from our group shows good agreement of PAOP and left ventricular end-diastolic pressure in endotoxemia suggesting that the findings in paper IV are relevant.

The question whether the time constant for determination of Pcap may vary with the state of pulmonary circulation has been raised[185]. This is a potential source of error when using the current occlusion method. However, Pellet *et al.* found the 175ms time constant to be suitable both in states of predominantly venous and arterial pulmonary vasoconstriction[150]. It has further been argued that in the clinical setting this might be of minor importance[66,191]. Moreover, our finding that endotoxemia induced a predominant increase in pulmonary venous resistance is in line with findings both in the experimental setting[54] and in patients with ALI[79,193]. These reports taken together with our *in vitro* findings lend further support to the conclusion that ET receptor antagonism actually reduces endotoxin-induced increases in pulmonary venous resistance.

The relevance of Pcap in monitoring treatment of ALI has this far only been addressed to a limited extent. Nunes *et al.* reported that Pcap tended to decline during the course of ARDS and they only identified one of nine patients as having a Pcap above 20 mmHg at the time of diagnosis[143]. In contrast, Collee *et al.* reported a positive correlation between Pcap and the severity of ALI although they did not report the timing of the measurement in relation to the course of the disease[37]. The effects of pulmonary vasodilators on Pcap have been reported in small interventional clinical studies with inhaled nitric oxide, prostaglandin E1, nitroglycerine and prostacyclin,[17,160,161]. In general only modest reductions of Pcap were noted with these substances.

Considering the central role of pulmonary capillary pressure in edema formation it could possibly be important to reduce an increased capillary pressure in ALI. However, further characterization of the dynamics of pulmonary capillary pressure development during the course of clinical ALI is necessary in order to identify proper timing of such interventions.

INTERVENTION IN THE ENDOTHELIN SYSTEM

In paper III we found that intervention with the dual ET receptor antagonist tezosentan improved pulmonary function in endotoxin-induced ALI. Paramount effects on pulmonary hypertension and the formation of extra-vascular lung water as well as improved gas exchange and pulmonary mechanics were noted. These beneficial effects were obtained without a significant reduction in systemic arterial pressure. Table 5 summarizes the effects of tezosentan intervention.

Using vasodilating agents in the setting of circulatory shock has the potential to aggravate systemic hypotension. However, the use of ET receptor antagonists during ALI has not been reported to excessively aggravate hypotension in other studies[41,70,84,205]. In paper IV we noted a small decrease in systemic arterial pressure in response to tezosentan compared with the findings in paper III where the animals were subjected to a more aggressive volume load.

Table 5. Effects induced by dual endothelin receptor antagonism during endotoxemia

Extra-vascular lung water	↓	Arterial partial pressure of oxygen	↑
Pulmonary capillary pressure	↓	Mixed venous hemoglobin oxygen saturation	↑
Pulmonary venous resistance	↓	Arterial hemoglobin oxygen saturation	↑
Venous admixture	↓	Cardiac index	↑
P/F ratio	↑	pH of arterial blood	↑
Physiological dead space	↓	Mean arterial pressure	→
Pulmonary compliance	↑	Pulmonary arterial occlusion pressure	→
Mean pulmonary arterial pressure	↓	Heart rate	→

Another potential deleterious effect of systemically administered vasodilators is aggravated ventilation-perfusion mis-match by increasing intra-pulmonary shunting i.e. reducing protective mechanisms like pulmonary hypoxic vasoconstriction. Earlier, ET-1 has been proposed to be a major player in hypoxic pulmonary vasoconstriction[82]. More recently though, hypoxic pulmonary vasoconstriction has been found to be regulated by oxygen sensing mechanisms in the pulmonary arterial vascular smooth muscle cells rather than mediated by ET-1, although this peptide might modulate the response over time[128]. ET-1 is still however, considered important in chronic hypoxemic conditions and the development of secondary pulmonary hypertension. The improvement of ventilation-perfusion matching by tezosentan indicates that hypoxic pulmonary vasoconstriction probably was preserved and that ET-1 was not involved to a major degree in this protective mechanism. Furthermore, the tezosentan-induced increase in cardiac output probably also explains the actual reductions in venous admixture and physiological dead space.

The beneficial changes in respiratory mechanics induced by ET receptor antagonism as indicated in paper III by improved compliance is in line with the reduction in peak inspiratory pressure in response to tezosentan reported by Wang *et al.*[205]. Furthermore, Kuklin *et al.* demonstrated improved compliance in isolated lungs from tezosentan-treated rats, subjected to cecal ligation and puncture[100]. The changes in compliance are probably due to reduced extra-vascular lung water as observed in paper III. The EVLW-reducing effects of tezosentan is further supported by the similar findings reported by Kirov *et al.* in endotoxemic awake sheep[95], using the thermo-dye technique for EVLW determination.

The theoretical advantages of identifying and lowering an increased Pcap in states of inflammation-induced pulmonary edema are apparent from the Starling principle. The feasibility of measuring Pcap at the bedside has been known since the mid-1980's and have now reemerged in the literature as a potentially important parameter in ALI[66,191]. In paper IV we found that Pcap and pulmonary vascular resistance were elevated in experimental sepsis and that the venous vascular resistance contributed the most to this increase. We could further demonstrate that intervention with tezosentan completely abolished the increases in Pcap and pulmonary venous vascular resistance, suggesting that this might be an important mechanism in explaining the EVLW reducing effects

of tezosentan. This mechanism is also supported by the findings of Kuklin *et al.* who found that Pcap increased in response to endotoxin challenge in awake sheep and that it was reduced by tezosentan intervention. Interestingly, they found that the arterial- and not the venous resistance were reduced by tezosentan which contrasts our findings in paper IV. Kuklin *et al.* studied the animals for 24 hours and the first measurement was obtained after 4 hours of tezosentan administration compared to our observations after one hour of tezosentan administration. Furthermore, they used a different technique for the measurement of Pcap[101]. Considering these findings, the effects of the Pcap-reducing effects of tezosentan on edema formation might be more pronounced in early ALI.

The *in vivo* findings in paper IV that sarafotoxin and ET-1 induced increases in Pcap and pulmonary venous vascular resistance are supported by the venous predominance in contractive responses noted in the *in vitro* experiments. This suggests that the reductions in Pcap and pulmonary venous resistance during intervention with tezosentan might be due to ET_B-receptor antagonism. However, since the veno-constrictive potency of sarafotoxin was somewhat less compared with ET-1 in the *in vitro* experiments, it might imply that the reduction in Pcap and pulmonary venous resistance by tezosentan was due to some degree of ET_A-receptor antagonism as well.

The importance of the endothelin system in endotoxin-induced ALI is supported by altered expression of ET receptors in lung tissue during endotoxemia[61] Moreover, an association between increased alveolar to arterial difference in oxygen partial pressure and ET_B-receptor expression has been reported in animal models of hepato-pulmonary syndrome[112]. Ishimaru *et al* found that endotoxin administration resulted in decreased ET_A - and unchanged ET_B receptor expression in the rat lungs[88]. This indicates that the relative expression of the two receptors change during endotoxemia, possibly favoring ET_B receptor-mediated effects. However, these changes were detected in pulmonary tissue homogenates, and to our knowledge, specific endotoxin-induced changes of ET receptor expression in pulmonary vessels have not been reported so far.

The aforementioned importance of the ET_B receptor during endotoxemia might suggest a beneficial effect of selective ET_B-receptor blockade. However, during endotoxemia such intervention has been reported to be deleterious, probably due to augmented ET_A receptor activity caused by reduced ET_B receptor-mediated clearance of circulating ET-1 as well as by decreased vasodilation from blockade of the endothelial ET_B receptor[203]. Apart from these vascular aspects ET_A receptor activation has been reported to have pro-inflammatory properties[26,27,99].

Tezosentan has been evaluated for the treatment of acute heart failure in phase III trials but the studies were interrupted due to futility[32,145]. Long term treatment with other dual ET receptor antagonists has been reported to induce dose-dependant liver toxicity, whether this applies to tezosentan is not known[133]. The clinical experience with dual ET receptor antagonists indicates that short term treatment of acute conditions like ALI might become a realistic therapeutic option.

FUTURE PERSPECTIVES

The treatment of ALI offers intricate challenges in modern intensive care. It is obvious that ventilation strategies are able to affect outcome but presently there are no therapies directed towards endogenous pathophysiological mechanisms. Although recent data indicate a reduced short term mortality the opposite has been reported regarding a more long term perspective[7]. Therefore, there is a need for further exploration into new therapeutic and diagnostic possibilities. Regarding the latter, Pcap measurement could be useful to better estimate one of the two determinants of edema formation namely filtration pressure. It is far from proven that there is a strong clinical connection between Pcap and edema formation. Nevertheless, Pcap could be used to follow the effect of different vasodilating agents in patients with ALI. In evaluating such agents in relation not only to their pressure reducing effects but also to their anti-edema properties, the choice should possibly fall on substances with documented effects on pulmonary veins. However, Pcap measurements could be questioned since the use of pulmonary artery catheters have been debated and thorough case-by-case consideration has been suggested for its use[163]. However, extracting additional information like the pulmonary capillary pressure might favor their further use. Even if one is able to measure Pcap there is still a need for a reliable method to estimate edema. Since we found significant biases in the MDID and STID methods their use in the clinical situation must be questioned even if the STID method may allow for evaluation of relative changes. A discussion regarding possible improvements in the algorithms on which this method is based would probably be fruitful.

As mentioned earlier, tezosentan had several highly beneficial effects on endotoxin-induced lung injury in our model. Although, one must be very cautious to extrapolate our findings to potential clinical usefulness of this agent there is a growing body of evidence on the importance of the ET system in endotoxin–induced ALI. Moreover, there is a large number of studies and substantial clinical experience of ET receptor antagonists in other settings. The studies in models of ALI include different species, a variety of insults and time frames but have common findings; reduced pulmonary hypertension, improved gas exchange, reduced edema formation, preserved systemic arterial pressure and increased cardiac index. These findings together with the growing evidence, from *ex vivo* studies, of the potential of ET receptor antagonists to reduce inflammation, permeability, and fibrosis suggest that we might have come to a point where clinical studies could be discussed.

CONCLUSIONS

- Endotoxin infusion generates acute lung injury with significant increases of extra-vascular lung water in the current model.
- The molecular double indicator dilution technique for the determination of extra-vascular lung water is not accurate in the current endotoxemic model.
- The STID method with the default ITBV to GEDV relationship systematically over estimated extra-vascular lung water. However, it might be useful in detecting relative changes over time.
- The ITBV to GEDV relationship used for the calculation of extra-vascular lung water may vary depending on the actual pathophysiological condition under which the STID technique is used and thereby influence the accuracy of the method.
- The STID technique for determination of extra-vascular lung water seem to be influenced by changes in regional changes of pulmonary perfusion and ventilation, but not by general reductions in cardiac output.
- Intervention with a dual ET receptor antagonist during early endotoxin-induced acute lung injury improves gas-exchange and pulmonary matching of ventilation and perfusion.
- Dual ET receptor antagonism during established endotoxin-induced acute lung injury normalizes extra-vascular lung water in the current model.
- Selective ET_B receptor agonism increases pulmonary venous tone both *in vivo* and *in vitro*.
- Dual ET receptor agonism induces pulmonary arterial- and, to a larger extent, venous constriction both *in vivo* and *in vitro*.
- Endotoxin challenge increases pulmonary capillary pressure, pulmonary venous- but not arterial resistance in the current model.
- Intervention with a dual ET receptor antagonist during endotoxin-induced acute lung injury reduce pulmonary capillary pressure as well as pulmonary venous vascular resistance.
- The reported findings suggest that the ET_B receptor may be more important than the ET_A receptor in mediating pulmonary venous constriction.
- The ET system seems highly important in acute lung injury pathophysiology in general, and concerning edema formation in particular.
- This thesis lends further support to the notion that dual ET receptor antagonists may become a future therapeutical option for ALI patients.

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"If people never did silly things,
nothing intelligent would ever get done"
Ludwig Wittgenstein

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APPENDIX

Calculation of gravimetrically determined extra-vascular lung water:

$$Fw_s = \frac{Ww_s - Wd_s}{Ww_s}$$

$$Fw_h = \frac{Ww_h - Wd_h}{Ww_h}$$

$$Qr = Qh \times \frac{Hb_s}{Hb_b} \times \frac{Fw_h}{Fw_s} \times Hct$$

$$Qb = Qr + \left[Qr \left(\frac{1 - Hct}{Hct} \right) \right]$$

$$Fw_b = \frac{Ww_b - Wd_b}{Ww_b}$$

$$EVLW = Qh \times Fw_h - Qb \times Fw_b - Qwt$$

Qlb = weight of removed lungs
Qwt = weight of added water
Ww_b = wet weight of blood
Wd_b = dry weight of blood
Fw_b = fraction water of blood
Ww_h = wet weight homogenate
Wd_h = dry weight homogenate
Fw_h = fraction water of homogenate
Ww_s = wet weight supernatant
Wd_s = dry weight supernatant
Fw_s = fraction water supernatant
Hct = hematocrit
Qb = residual blood content in lungs
Qr = Red cell mass of lung
Qh = total weight of homogenate