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VASCULAR EFFECTS OF ENDOTHELIN IN EXPERIMENTAL LUNG INJURY

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Stockholm 2012

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Published by Karolinska Institutet.
Printed by Larserics Digital Print AB.

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ISBN 978-91-7457-633-7

ABSTRACT

Acute lung injury remains a frequent and life threatening consequence of severe sepsis. This thesis has investigated the role of the endothelin (ET) system in sepsis-induced lung injury, with special reference to its effects on two hallmarks of this syndrome - formation of edema and pulmonary hypertension. This was explored in a porcine endotoxin model of sepsis *in vivo*, as well as *in vitro* using isolated porcine pulmonary vessels.

In paper I we show that endotoxemia *via* ET-dependent mechanisms predominately increases pulmonary downstream resistance and subsequently augment pulmonary capillary hydrostatic pressure. In addition, we demonstrate that ET_B-receptor stimulation constricts pulmonary veins more than arteries *in vitro*. In paper II, we show that endotoxin-exposure induces differentiated effects in isolated pulmonary arteries and veins. In veins, endotoxin increases ET-receptor dependent constriction, whereas the response to α1-adrenergic stimulation, predominantly acting on arteries, is reduced. In addition, we demonstrate that the changes in response to ET-receptor stimulation are not induced by alterations in expression or distribution of ET-receptors in the preparations. In paper III we find that inhalation of a dual ET-receptor antagonist, tezosentan, during porcine endotoxemia potently reduces pulmonary hypertension without causing systemic effects. In paper IV we show that endotoxemia induces a marked degranulation of polymorphonuclear neutrophils with increased plasma levels of the highly permeability and edema promoting heparin-binding protein (HBP/CAP37). Treatment with tezosentan distinctly counteracts this increase and simultaneously reduces pulmonary edema, improves respiratory system compliance and decreases hemoglobin concentration, all suggesting that tezosentan treatment reduces transcapillary fluid passage.

In conclusion, our studies show that endotoxemia increases pulmonary capillary filtration pressure, raises levels of the powerful permeability promoting mediator HBP and induces pulmonary edema. Systemic treatment with a dual ET-receptor antagonist markedly counteracts these changes. In addition, inhaled tezosentan efficiently and selectively reduces pulmonary hypertension during endotoxemia. Taken together, these results show that the ET-system is extensively involved in the pathophysiology of endotoxin-induced lung injury. These findings need to be further elucidated, in other experimental conditions and in humans.

Key words: acute lung injury, sepsis, endothelin, extravascular lung water, pulmonary capillary pressure, pulmonary edema, heparin-binding protein, CAP37, tezosentan, pig, pulmonary circulation

LIST OF ABBREVIATIONS

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BE	Base excess
CI	Cardiac index
DAG	Diacylglycerol
ECE	Endothelin converting enzymes
ET	Endothelin
ET-1	Endothelin-1
ET-1-LI	Endothelin-1-like-immunoreactivity
ET_A-receptor	Endothelin receptor subtype A
ET_B-receptor	Endothelin receptor subtype B
ETRA	Endothelin receptor antagonists
EVLWI	Extravascular lung water index
FiO_2	Fractional inspired oxygen
Grav	Gravimetry
Hb	Hemoglobin concentration
HBP	Heparin-binding protein
IP_3	Inositol triphosphate
MAP	Mean arterial pressure
MPAP	Mean pulmonary arterial pressure
MPO	Myeloperoxidase
NF-kB	Nuclear factor-kB
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PAMP	Pathogen-associated molecular patterns
PAOP	Pulmonary artery occlusion pressure
P_{cap}	Pulmonary capillary pressure
PEEP	Positive end-expiratory pressure
P/F-ratio	Ratio of arterial oxygen tension / fraction of inspired O_2
PHE	Phenylephrine
PMN	Polymorphonuclear neutrophils
PVRI	Pulmonary vascular resistance index
$P_{us}VRI$	Pulmonary up-stream vascular resistance index
$P_{ds}VRI$	Pulmonary down-stream vascular resistance index
RM-ANOVA	Repeated measures-analysis of variance
S6c	Sarafotoxin 6c
SaO_2	Arterial oxygen saturation
SEM	Standard error of the mean
STID	Single thermal indicator dilution
SVRI	Systemic vascular resistance
SvO_2	Mixed venous oxygen saturation
TLR	Toll-like receptor
VILI	Ventilator-induced lung injury

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals as indicated below:

I. Rossi P, Persson B, Boels PJ, Arner A, Weitzberg E, Oldner A.
Endotoxemic pulmonary hypertension is largely mediated by endothelin-induced venous constriction.
Intensive Care Medicine 2008;34:873-80.

II. Persson BP, Boels PJM, Lövdahl C, Rossi P, Arner A, Oldner A.
Endotoxin Induces Differentiated Contractile Responses in Porcine Pulmonary Arteries and Veins.
Journal of Vascular Research 2011;48:206-18.

III. Persson BP, Rossi P, Weitzberg E, Oldner A.
Inhaled Tezosentan Reduces Pulmonary Hypertension in Endotoxin-Induced Lung Injury.
Shock 2009;32:427-34.

IV. Persson BP, Halldorsdottir H, Rossi P, Herwald H, Lindbom L, Weitzberg E, Oldner A.
Heparin-binding protein (HBP/CAP37) - a novel link between endothelin-1 and edema formation in sepsis?
Submitted manuscript

TABLE OF CONTENTS

Introduction ... 1
The injured lung ... 1
Epidemiology ... 1
Pathogenesis and pathophysiology ... 2
Edema formation and resolution ... 4
Treatment of lung injury ... 7
Sepsis ... 7
Definitions, epidemiology and pathophysiology ... 7
Treatment of sepsis ... 10
The endothelin system ... 11
Cellular biosynthesis and release ... 11
Receptors and intracellular signal transduction ... 13
The endothelin-system and the lung ... 15
The endothelin-system and sepsis ... 15
Heparin-binding protein (CAP37/azurocidin) ... 16
Aims of the thesis ... 18
Material and Methods ... 19
Animal anesthesia, preparation and interventions ... 19
Endotoxin ... 19
Tezosentan ... 20
Endothelin receptor agonists and phenylephrine ... 21
Biochemical analyses of plasma ET-1, HBP and myeloperoxidase ... 21
Nebulization ... 22
Assessments of extravascular lung water ... 22
Single Thermal Indicator Dilution Method ... 22
Gravimetrical analysis ... 23
Measurement of pulmonary capillary pressure ... 23
Myographic experiments ... 25
Analysis of ET-receptor protein expression with immunohistochemistry ... 26
Analysis of ET-receptor protein expression using immunoblotting ... 26
Statistical methods ... 27
Summary of results ... 29
Effects of endotoxin ... 29
Systemic effects of dual ET-receptor antagonism during endotoxemia 30
Pulmonary effects of dual ET-receptor antagonism during endotoxemia 31
Effects of inhaled dual ET-receptor antagonism during endotoxemia 31
Challenge with endothelin-1 and sarafotoxin 6c (I and IV) ... 32
Challenge with phenylephrine (I) ... 33
Reactivity of freshly isolated pulmonary vessels (I and II) ... 34
Effects of incubation and endotoxin on vascular reactivity (II) ... 35
Expression of ET-receptors in vascular preparations (II) ... 36
Discussion ... 37
Summary and future perspectives ... 43
Conclusions ... 45
Acknowledgements ... 46

References.. 48
Appendix.. 61

INTRODUCTION

THE INJURED LUNG

In 1967 a syndrome of severe and acute onset respiratory failure was described by Ashbaugh and Petty in the Lancet[1]. The condition was termed adult respiratory distress syndrome[2]. However, since this syndrome occurs at all ages, it was later renamed acute respiratory distress syndrome (ARDS) and its milder form acute lung injury (ALI). These are conditions characterized by a life threatening and acute onset respiratory failure that presents with progressive, severe arterial hypoxemia and dyspnea. Ultimately, oxygen demand exceeds ventilatory capability and the ensuing hypoxemic, hypercarbic, respiratory failure frequently leads to a requirement of endotracheal intubation, mechanical positive pressure ventilation and intensive care.

Since ARDS and ALI are not uniform entities, but a syndrome with diverse clinical etiologies, the definition is based on specific criteria. These have been continuously reviewed in order to improve the precision of the definition[3,4]. In 1994 the American-European Consensus Conference proposed a definition[5], which was widely accepted as a tool for patient characterization and conduction of research trials. ALI and ARDS was defined as a syndrome of inflammation and increased permeability together with three diagnostic criteria: the presence of acute severe hypoxemia (defined as a ratio of arterial oxygen tension over fractional inspired oxygen (PaO_2/FiO_2) < 300 mmHg (40 kPa) for ALI and < 200 mmHg (27 kPa) for ARDS), new and bilateral infiltrates on chest radiography and the absence of raised pulmonary artery occlusion pressure (PAOP). Although this definition is widely used, it has been subject to increasing criticism. As late as in October 2011 new criteria were presented[6], aimed to better distinguish between the different severity levels of this syndrome.

Epidemiology

The estimated incidence and mortality of this syndrome varies significantly between reports, probably due to limitations of the diagnostic criteria and the varying background characteristics of the studied populations. In a US population, reported in 2005, the incidence of ARDS was 59 per 100 000 person-years with an in hospital mortality of near 40 %[7]. A Scandinavian estimate published in 1999 was more conservative, with a reported incidence of ARDS of 13.5 per 100 000 person-years[8]. The 90 day mortality was 41%. These results are in line with a recent prospective

multicenter study from Spain, were the incidence was 7.2 per 100 000 person-years and the hospital mortality 48 %[9]. A substantial proportion of the survivors has a slow recovery, with up to 10 % of patients needing more than one month of mechanical ventilation[10]. Pulmonary function often recovers[11,12], but the overall health may not improve completely, even in previously healthy patients[13]. In recent years a decline in the incidence and mortality of lung injury has been proposed[14,15]. This decrease has been attributed to advances in many aspects of care, such as ventilator and fluid management as well as improvements in diagnosis and treatment of infections.

Pathogenesis and pathophysiology

Several clinical conditions are associated with the acute development of lung injury, with serious infections such as pneumonia and sepsis as the most common predisposing illnesses[7]. Other important causes include aspiration of gastric contents and traumatic hemorrhagic shock or less common disorders such as acute pancreatitis, transfusion-associated lung injury, near-drowning and drug reactions. The causes are often divided into two general pathophysiologic pathways: direct (pulmonary) or indirect (extrapulmonary) injury[16]. Direct injury refers to conditions in which the injurious event directly affects the lungs, such as diffuse pneumonia, aspiration pneumonitis, lung contusion or inhalation of noxious gases. In these situations the severity of the pulmonary injury is highly dependent on the grade of exposure to the noxious mediator. Indirect, extrapulmonary, lung injury refers to conditions were the primary disease is outside the lung, for example after multiple fractures, acute pancreatitis or non-pulmonary sepsis. Comparison of outcome in pulmonary versus extrapulmonary triggers for the lung injury has demonstrated conflicting results, but suggests a trend towards higher mortality in the population with disease of pulmonary origin[17].

The course of lung injury is often divided into two main phases, starting with an early inflammatory response, known as the exudative phase, followed by prolonged healing process, known as the fibroproliferative phase[18,19]. The initial phase of lung injury is characterized by an acute inflammation that causes disruption of the pulmonary endothelial and epithelial barrier with subsequent vascular hyperpermeability. This change in permeability permits the efflux of protein-rich fluid into the extra vascular interstitium, causing formation of interstitial and alveolar edema. The edema, in turn, disturbs gas-exchange, causes mismatch of ventilation/perfusion, reduces pulmonary compliance and increases the work of breathing. The late fibroproliferative phase is

characterized by a progressive organization of the exudates and hyaline membranes with interstitial fibrosis. In the lung, certain pathogenic processes are considered as especially important in the course of events leading to respiratory failure.

Atelectasis

When small airways in the inflamed and edematous lung parenchyma are collapsed or obliterated by stagnant secretions, the entrapped gas distally to the obstruction eventually will be resorbed. In addition, injuries to the alveolar epithelial type II cell reduce surfactant production which leads to impaired pulmonary compliance. Together these events results in collapsed, non-aerated lung parts and increased right-to-left shunting of deoxygenated blood. During the last decades the importance of using ventilator approaches that minimizes formation of atelectasis has been increasingly recognized[20].

Microthrombosis and neutrophil induced lung injury

Post mortem angiographic studies and pathological examinations of ARDS patients have shown that macro- or microscopic thromboembolism and diffuse pulmonary endothelial injury is a frequent finding[21]. Even though lung injury may well develop in patients with severe neutropenia[22], activation of polymorphonuclear neutrophils (PMN) are considered as important events in the pathogenesis of the disease. The concentration of PMN in bronchoalveolar lavage fluid of ARDS patients correlates with severity of disease and predict outcome[23]. Moreover, PMN have been shown to be crucial in various animal models of lung injury as PMN depletion or blocking of chemoattractants ameliorates lung injury[24-26]. During inflammation, endothelial cells exhibit increased activity of the transcription factor nuclear factor-κB (NF-κB), which up regulates the surface expression of adhesion molecules[27]. This mediates adhesion and migration of PMN across the endothelium to the alveolar epithelium. Activated PMN accumulate and release proinflammatory and procoagulant mediators, reactive oxygen species and proteases, which induce a loss of the normal endothelial barrier function. In addition, the release of potent metabolites of arachidonic acid, such as leukotrienes, prostaglandins and thromboxane, induces platelet and leukocyte aggregation and vasoconstriction. Together, these events cause obliterations of pulmonary capillaries with subsequent increments of dead space ventilation.

Ventilator-induced lung injury

Mechanical ventilation may by itself induce injuries, denoted as ventilator-induced lung injury (VILI). Even though the inflamed and fragile lung most likely is more prone to suffer from VILI, it can also be induced in previously healthy lungs. VILI has been proposed to be a continuous phenomenon that manifest depending on the level and duration of the applied harmful stress[28]. The pathogenesis of VILI includes specific events that distress the lung; repeated recruitment and derecruitments of alveoli during the respiratory cycle induce shear stress (atelectotrauma) that not only injures the lung but also induces production of pro-inflammatory mediators that may induce distant harmful effects[29]. In addition, when aerated and less injured parts of the lung-parenchyma are exposed to harmful levels of pressure, it causes over-distension and a further progression of lung injury. In summary, an increasing number of studies has revealed that a ventilation strategy with lower tidal volumes and lower airway pressures is protective to the injured lung[30]. Thus, in the past decades, strategies have been changed from preservation of normal blood-gases to a permissive approach aimed to protect the lung from secondary injuries, termed as lung-protective ventilation strategy[10].

Pulmonary hypertension

Pulmonary hypertension is commonly recognized as a characteristic feature of lung injury[31]. In the early phase, pulmonary vasoconstriction in response to proinflammatory mediators, capillary obliterations and edema increase pulmonary vascular resistance (PVR) even following correction for the severity of the associated hypoxemia[32]. However, hypoxic vasoconstriction of precapillary arteries significantly augments PVR further. The resulting pulmonary hypertension increases right-ventricle outflow pressure that may reduce right ventricular function. In the later phases of lung injury, a fibrocellular intimal proliferation occurs, involving predominantly small muscular arteries, but also veins and lymphatics which may contribute to edema formation[21].

Edema formation and resolution

Formation of edema is considered as a hallmark of sepsis-induced lung injury[19,33]. The direction of fluid movement across a capillary membrane is determined by the properties of the membrane as well as the balance of the intra and extra-capillary

pressures. This is described by the classical but yet highly valid *Starling equation of fluid filtration*[34]:

$$Jv = S \times Lp\left(P_{cap} - P_{IF} - \sigma\left(\pi_{cap} - \pi_{IF}\right)\right)$$

In the Starling equation, fluid flux (J_v, $cm^3\ s^{-1}$) across a capillary membrane with a surface S (cm^2) and a hydraulic permeability L_p ($cm \cdot s^{-1} \cdot cmH_2O^{-1}$) is determined by the balance between the intra capillary hydrostatic (P_{cap}) and oncotic (π_{cap}) pressure as well as the extra capillary intestinal fluid hydrostatic (P_{IF}) and oncotic (π_{IF}) pressures. The reflection coefficient (σ) defines the endothelial permeability to protein (values from 0 to 1; 0 when the capillary wall is fully permeable to proteins and 1 when the wall is impermeable to proteins). The hydraulic permeability varies between different vessel beds and is also highly affected by hormonal and immunological stimuli. Physiologically, due to the recoil properties of the layer of surfactant at the air-liquid interface of the alveolar space, P_{IF} is slightly subatmospheric. Hence, the transmural filtration pressure (P_{cap}– P_{IF}) is positive and only partly counteracted by the net oncotic pressure. Therefore, there is a continuous filtration of fluid across the capillary endothelium to the interstitial space.

Hydrostatic edema

If the pulmonary capillary hydrostatic pressure (*i.e.* the pulmonary capillary pressure, P_{cap}) is increased, the filtration of fluid increases to a point where the edema counteracting mechanisms are exceeded and a hydrostatic edema is formed. P_{cap} is a crucial determinant of filtration of plasma water across the capillary membrane and dependent on the mean pulmonary arterial pressure, the blood flow through the pulmonary circulation (cardiac output) and the pulmonary vascular resistance. However, the distribution of the pulmonary vascular resistance from precapillary (upstream, *i.e.* arterial), to the post capillary (downstream, *i.e.* venous) compartments varies and depending on the site where the constrictor effect dominates, pulmonary vascular constrictors may affect P_{cap} differentially. Thus, given an identical overall increase in pulmonary resistance, a constrictor with a predominantly venous site of action will generate a relatively higher capillary pressure with potential subsequent edema formation as compared with a constrictor with arterial predominance. Direct measurements of P_{cap} has not been made in human lungs but are estimated to be 5-10 mmHg during physiologic conditions[35,36]. In intact animals the critical capillary

pressure at which fluid filtration exceeds edema counteracting mechanisms has been shown to be approximately 20 mmHg when oncotic pressure and permeability are normal[37-38].

Inflammatory edema

The endothelium is a dynamic and highly active barrier that selectively regulates efflux of plasma fluid, macromolecules and leucocytes to the surrounding tissues[39]. In the early stage of inflammation, several inflammatory mediators, such as thrombin, bradykinin, leukotrienes and histamine, each may induce transient vascular leakage. At a later stage activation of the endothelium induces actin–myosin interactions with cytoskeletal disarrangements, causing loss of junctional integrity with formation intercellular gaps. This enhances endothelial permeability and causes vascular leakage, in particular in postcapillary venules. In addition, changes in intra- and extravascular vascular oncotic pressures are important for edema formation during inflammatory states. Plasma albumin accounts for 65% of π_{IF}. However, as albumin also crosses the vascular barrier and may enter the interstitial tissue, it also serves as the main interstitial oncotic agent[39].

Edema counteracting mechanism

In the lung, were edema is prone to interfere with gas-exchange, important edema counteracting mechanisms are present[40]. Normally, type I and type II alveolar epithelial cells form tight junctions with each other, selectively regulating the epithelial barrier. The primary mechanism that drives fluid reabsorption from the alveolar space into the interstitium and the pulmonary circulation is active transepithelial sodium ion transporting mechanisms[41]. This transport is thought to be primarily a function of alveolar type II cells[42]. Sodium ions enter the epithelial cells via amiloride-sensitive Na^+ ion channels located on the apical membrane and subsequently "pumped" out at the basolateral side by the activity of the Na^+/K^+-ATPase. This transepithelial sodium ion transport creates osmotic forces which causes water to passively move from the air spaces to the alveolar interstitium *via* aquaporins to be subsequently removed via the lymphatic or capillary system.

Injuries to the epithelium disrupt the barrier and might also impair normal alveolar fluid clearance via down regulation of Na^+ channels and Na^+/K^+ ATPase pumps[40,43]. The mechanisms behind these changes are unclear but it has been shown that alveolar

edema fluid from ALI patients down regulates sodium ion transporting mechanisms and the expression of the responsible genes in cultured human alveolar epithelial type II cells[44]. In addition, it has been shown that alveolar fluid clearance is impaired in the majority of patients with lung injury and that high alveolar fluid clearance was associated with improved clinical outcomes, emphasizing the importance of alveolar fluid resolution[45].

The pulmonary lymphatic system constitutes of small contractile vessels that drive excess fluid toward regional lymph nodes in the pulmonary hilum, aided by breathing movements and valves. The lymphatics can increase their edema clearance rate several-fold, but only up to a critical value. Formation of pleural fluid is also considered as a protective mechanism against pulmonary edema formation[46].

Treatment of lung injury

Since the early descriptions of ARDS and ALI, considerable basic and clinical research have been conducted aimed to find specific therapies, most of them proved futile[47]. However, despite the lack of specific therapies, mortality is suggested to be declining[15]. This is probably due to increased knowledge on the importance of early diagnosis and goal-directed treatments of underlying conditions as well as general improvements in intensive care. In addition, the increased awareness on VILI has contributed to improvements in ventilator strategies. As formation of edema is a significant part in the pathogenesis optimal fluid management is central. In 2006 a controlled trial showed that the use of a conservative fluid-management protocol improved lung function and shortened the duration of mechanical ventilation as well as intensive care, without increasing non-pulmonary organ failure[48].

SEPSIS

Definitions, epidemiology and pathophysiology

Sepsis is considered as the systemic inflammatory response to infection[49]. However, the definition of sepsis has been changing over time. In 1992 a consensus definition was presented by Bone *et al*[50]. Sepsis was here defined as a systemic inflammatory response to a confirmed or suspected infection; severe sepsis as sepsis with organ dysfunction; and septic shock as sepsis with persistent hypotension despite adequate fluid resuscitation. Systemic inflammatory response syndrome is a criterion based description of widespread inflammation; the underlying condition may be infection but

severe systemic inflammation can also be a seen in patients suffering from tissue damage after traumatic injuries, major surgery, burns, acute pancreatitis or inflammatory disease.

The incidence and mortality of severe sepsis varies between studies and over time[51]. In reports from Scandinavia, the incidence of severe sepsis varies from 0.38 to 1.49 per 1000 person-years[52-54]. The incidence of severe sepsis has been reported to increase [55]. Several factors have been suggested to contribute to this; an ageing population, often living with chronic diseases, an increasing use of invasive procedures in modern medicine and a growing number of conditions treated with immunosuppressive drugs. Despite intense efforts to reduce mortality of severe sepsis it remains high, with hospital mortality rates between 20 and 30% in reports from Sweden and Finland[52,53]. Patient related factors that are associated with increased mortality in severe sepsis include advanced age, severity of illness and comorbidities such as kidney failure.

During the recent decade, the theory proposing that the cause of sepsis is the body's own uncontrolled inflammatory response to infection[56], has been partly questioned[57]. The pathophysiology of the sepsis syndrome is heterogeneous and the dominating pathological mechanisms in a patient may vary over the time as well as between patients. In sepsis several major pathways are disturbed, including the immune/inflammatory cascades, procoagulant/antifibrinolytic pathways, cellular metabolism and vascular permeability[33]. This results in an early hyperinflammatory, procoagulant state, with a concomitant or subsequent prolonged condition with immune dysfunction *(Figure 1.)*[58].

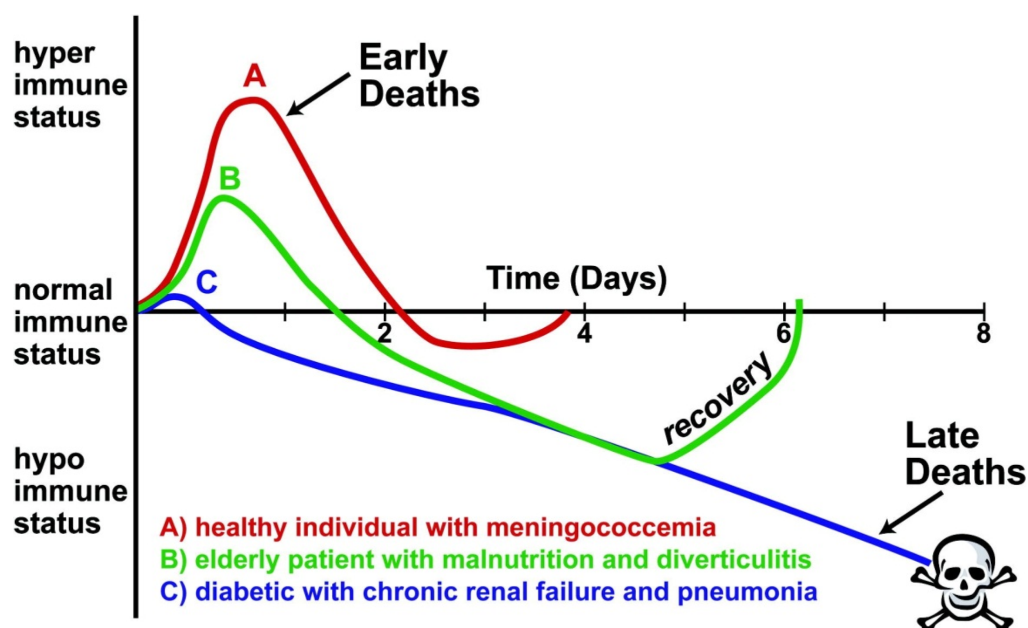

Figure. 1. Illustration of the immunoinflammatory response of three hypothetical patients with sepsis. The individual immune response in sepsis is determined by many factors, including pathogen characteristics, size of the bacterial inoculum and patient comorbidities. The initial immune response is hyperinflammatory, but the response rapidly progresses to hypoinflammatory. In the healthy individual who experiences meningococcemia, there is a powerful, detrimental, hyperinflammatory response. In this situation antiinflammatory treatments might improve survival. In the elderly patient with malnutrition who experiences intraabdominal infection, the initial inflammatory response is limited, and if the infection persists, a prolonged hypoinflammatory response develops. In the patient with diabetes, chronic renal failure, and pneumonia, the initial response is blunted, and there is a prolonged depression of immune function. Reproduced with permission from Skrupky et al: Advances in the Management of Sepsis and the Understanding of Key Immunologic Defects. Anesthesiology 2011; Vol.115 - 6 - p 1349–1362

Endotoxin and Toll-like receptors

Endotoxin is a lipopolysaccharide *(LPS, Figure 2.)* component of the outer cellular membrane of Gram-negative bacteria. Endotoxin consists of three parts, an outer variable polysaccharide O side chain (the O-antigen), a core region and a highly conserved lipid A component. All components of LPS are required for the virulence of Gram-negative bacteria but only the lipid A is essential for the integrity of the cell wall[59]. Variation within the length of the O-antigen can change the phenotypic appearance (*i.e*. smooth or rough) of the bacterium as well as the bioactive response of the host to the bacterium itself[60]. The primary active component of LPS, the lipid A residue, is a normally embedded in the cell membrane, but released when bacteria are either multiplying or being destroyed. In the infected tissues, lipid A is opsonized by a lipopolysaccharide-binding protein forming a complex, which in turn is bound by the receptor CD14 on the surface of host cells.

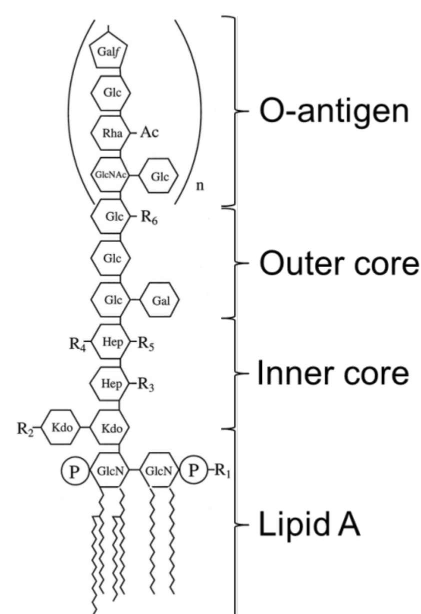

Figure 2. Schematic illustration of the lipopolysaccharide molecule with its three domains; the O-antigen that is comprised of a repeating oligosaccharide, the core - a nonrepeating oligosaccharide, and lipid A - a hydrophobic anchor.

This complex induces a transmembrane signal mediated through activation of the Toll-like receptor 4 (TLR4), first described by Beutler *et al* in 1998[61] (awarded with the Nobel Prize in Physiology or Medicine 2011). The TLR4-pathway is of vital importance for the host reaction to LPS; disruption of the TLR4-pathway in animals totally abolishes the reaction to LPS but makes them highly susceptible to Gram-negative infections[62]. TRL4 is expressed in several tissues, but the detrimental effect of LPS is considered primarily to be mediated via activation of macrophages[63]. However, it has also been shown that endothelial TLR4 can act as an important sentinel for circulating Gram-negative bacteria[64].

TLRs are transmembrane proteins with three sections: an extracellular domain that mediate the recognition of so called pathogen-associated molecular patterns (PAMPs), a transmembrane domain and an intracellular Toll–interleukin 1 receptor domain which is required for downstream signal transduction[65]. After binding the TLR4-LPS complex is internalized and triggers signal transduction which leads to an activation of the transcription factor family NF-κB and mitogen-activated protein kinases. They, in turn induce production of inflammatory cytokines and enzymes, which during sepsis sets a massive proinflammatory response in motion with activation of complement and coagulation cascades[65].

Endotoxin is considered an important constituent of the pathophysiology of Gram-negative bacterial sepsis. Clinical studies in patients with severe sepsis have shown that patients with increased concentrations of endotoxin at study entry had notably higher mortality than those without measurable endotoxin levels[66]. Moreover, endotoxin-clearing therapies using polymyxin B-hemoperfusion has been reported to improve hemodynamic-stability in patients with abdominal sepsis[67]. Humans given very low concentrations of endotoxin show signs and symptoms of clinical sepsis[68]. In addition, in an anecdotic report, self-administration of a massive dose of endotoxin in one patient, resulted in a sepsis-like hyperdynamic shock with disseminated intravascular coagulation and multiple organ dysfunction[69].

Treatment of sepsis

Due to the considerable morbidity and mortality of sepsis, substantial effort has been put into research to find effective therapies. Most of these therapies have been proved futile once tried in the clinical setting[70,71]. The reasons for the lack of effective anti-mediator treatments have been suggested to relate to the vast heterogeneousity of sepsis

patients with probable differences in immune response, both among patients and over time in the same patient[72]. However, during the last decade less elaborate approaches using early and adequate antibiotic treatment, prompt control of the sources of infection and goal directed fluid therapy has been shown to reduce mortality in sepsis[73,74].

THE ENDOTHELIN SYSTEM

In 1985, Hickey *et al* reported that isolated arteries contracted in response to exposure to the medium of cultured endothelial cells[75]. The active substance, at that present suggested to be a peptide but otherwise was of unknown nature, was later denoted "endothelium-derived contractile factor" (EDCF) in contrast to the "endothelium-derived relaxing factor" (EDRF) discovered by Furchgott *et al* in 1980[76] and later found to be NO[77]. In 1988, Yanagisawa *et al* isolated and characterized an endogenous and highly vasoconstrictive peptide found in the culture medium of porcine aortic endothelial cells[78]. The peptide was named endothelin-1 (ET-1, *Figure 3.*). In a series of experiments this group showed that ET-1 was produced by the endothelium and exerted its potent constrictive (1000 time more potent than noradrenaline[79]) effects by stimulating the underlying smooth muscle cells. In the following decades the research field regarding the endothelin system has expanded dramatically and endothelin has been shown to be involved not only in cardiovascular physiology and disease[80], but also in other pathological conditions such as sepsis[81,81], diabetes[82], pain[83] and malignancy[84].

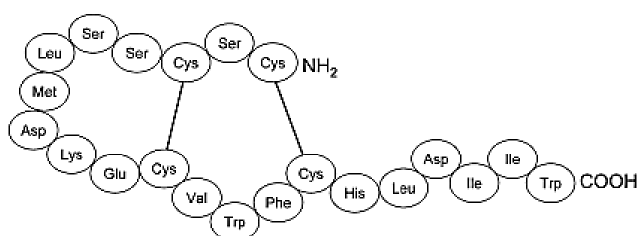

Figure 3. Schematic structure of the human and porcine endothelin-1 molecule.

Cellular biosynthesis and release

In humans the endothelin family consists of three closely related peptides, denoted ET-1, ET-2 and ET-3[85]. In addition to these, a group of venoms isolated from the Israeli Burrowing asp *(Atractaspis endgaddensis),* called sarafotoxins, have been identified as members of this family[86]. The three human ETs are all 21 amino acids in lengths, but encoded by different genes located on chromosomes 6, 1 and 20 respectively. ET-1 is recognized as the major isoform of relevance in human cardiovascular

pathophysiology[87] and is therefore the focus of this thesis. ET-2 is primarily produced within the kidney and the intestine, whereas ET-3 is predominately found in the central nervous system; however the roles of ET-2 and ET-3 are largely unclear.

ET-1 is mainly produced and released by the vascular endothelial cells[88], but production has also been described in several other tissues including lung, kidney, heart, leucocytes and brain[89-92]. ET-1 is generated in a two-step proteolytic process from a large (human 212, porcine 203 amino acid residues[93]) precursor peptide, preproET-1[84]. PreproET-1 is cleaved by endopeptidases to a 39 amino acid peptide denoted big-ET-1, which possesses weak vasoconstrictive properties. Big-ET-1 is further converted by a family of intracellular membrane bound zinc metalloproteases, endothelin converting enzymes, to ET-1 which forms a loop closed by two disulfur bonds. The structure of human and porcine ET-1 is identical. From the endothelium there is a continuous and constitutive release of ET-1 which there by contributes to regulation of basal vasomotor tone[94,95]. However, the peptide is also stored in granules in the endothelial cells. Synthesis and release of ET-1 is stimulated by to various physiological and pharmacological stimuli[96] such as catecholamines[78], angiotensin-II[97], cytokines[98], insulin[99], thrombin[100], tumor necrosis factor-α[101], hypoxia[102], acidosis and ET-1 itself[103] *(Figure 4.)*. Factors that inhibit production and secretion of ET-1 include NO[104], heparin[105], prostacyclin[106] and vascular shear stress[107]. ET-1 is considered foremost to act in a paracrine/autocrine fashion since the majority of its production is released by the endothelial cells abluminally toward the underlying interstitial space and the smooth muscle cells[108]. A minor portion is detected in the plasma, suggesting that elevated circulating levels principally represent an overflow of tissue ET-1 and only can been seen as an approximation of true ET-1 activity. However, in pathological conditions with high circulating levels of ET-1, it may act as an endocrine factor[109]. Plasma clearance of circulating ET-1 is rapid with a biological half-time of 1-2 minutes[110]. The pulmonary circulation acts as a major clearance site of ET-1[111], with a near 50% single-pass extraction of plasma ET-1 in the pulmonary circulation of normal human subjects[112]. However, as the lungs produce significant amounts of ET-1, there is no or minimal arterio-venous ET-1-gradient across the pulmonary circulation. Even though half-time of ET-1 is short, the physiological response, such as vasoconstriction, is sustained and lasting 1-2 hours[113].

Receptors and intracellular signal transduction

The effects of ET are mediated via three main subtypes of ET-receptors, denoted ET_A, ET_B and ET_C. ET_C-receptors have not been found in mammalian tissues. ET_A and ET_B receptors share about 60 % amino acid homology but are encoded by separate genes located on chromosome 4 and 13, respectively. The ET_A-receptor has 10 times more binding affinity for ET-1 and ET-2 than ET-3[87], while the ET_B-receptor has equal affinity to all the three ET-peptides. The ET_A-receptor is predominantly expressed on vascular smooth muscle cells, mediating a powerful and long lasting contraction as well as cell proliferation. The role of the ET_B-receptor is more complex and dependent on their localization. ET_B-receptors are primarily located on the endothelium, mediating vasodilatation through the release of NO and prostacyclin[114,115]. In addition, endothelial ET_B-receptors clear ET-1 from the circulation via endocytosis of the receptor-ligand complex with subsequent degradation in lysosomes[116]. However, ET_B-receptors are also situated on smooth muscle cells and mediate contraction. Under physiological conditions the dominating effect of ET-1 is considered to be an ET_A-receptor mediated vasoconstriction, partially balanced by ET_B-receptor mediated vasodilation through the release of NO. The effects of ET-1 on various vascular beds depend on the local receptor subtype distribution, which not are uniform and may also change during pathological conditions[117,118].

Genetic knockout experiments have revealed that the ET-system is highly involved in normal embryonic development. Mice lacking ET-1, ECE-1 or the ET_A-receptor receptor die at birth due to mechanical asphyxia resulting from severe malformation in structures derived from the mandibular arch, arches of lower jaw and throat structures[119-121]. Mutations in the gene encoding the ET_B-receptor produce congenital pigment abnormalities and aganglionic megacolon, with neonatal lethality around 3 weeks in mice, suggesting that the ET_B-receptor plays an important role in the normal development of the neural crest[122]. In addition, the ET_B-receptor has been suggested to be involved in mediation of inflammatory pain and cutaneous inflammatory responses in mice[123]. In humans, mutations in the ET_B-receptor gene are associated with a variant of the human genetic disease Hirschsprung syndrome[124].

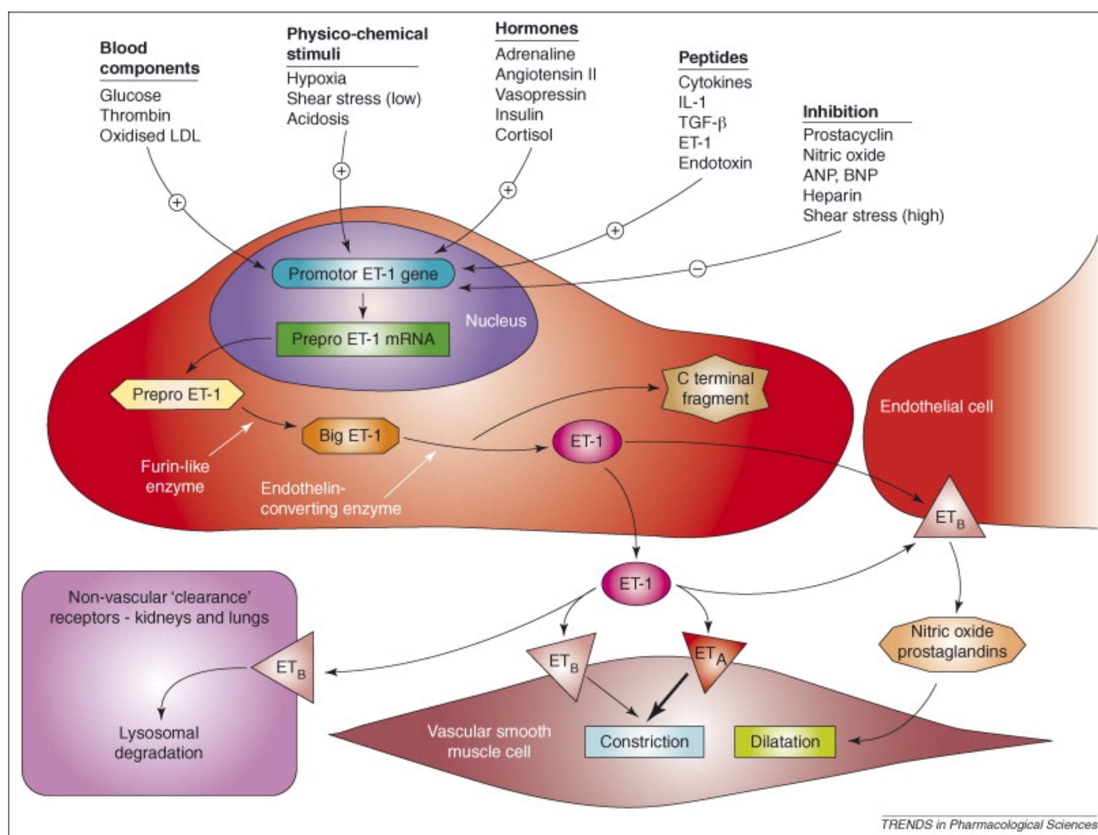

Figure 4. Pathways of endothelin-1 (ET-1) synthesis and sites of action. The synthesis and release of ET-1 from the endothelium is influenced by various factors of different nature. Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; ET_A, ET_B endothelin receptor A and B; IL-1, interleukin-1; LDL, low density lipoprotein; TGF-β, transforming growth factor β. For details, see text. Reproduced with permission from Dhaun et al. Trends Pharmacol Sci. 2007 Nov;28(11).

Both endothelin-receptors belong to the G protein-coupled receptor superfamily. ET-1 binding to constrictive ET_A and ET_B-receptors results in activation of phospholipase C which hydrolyses phosphatidyl inositol to form the two second messengers' inositol triphosphate (IP_3) and diacylglycerol (DAG)[125]. IP_3 and DAG activates calcium-channels causing release of Ca^{2+} from the sarcoplasmic reticulum and Ca^{2+} inflow from the extracellular space. $Ca2^+$ activates myofilaments and induces smooth muscle contraction. In addition, ET-1 activates nuclear signal transduction cascades that induce gene transcription, mediating the mitogenic and hypertrophic effects of ET-1. ET-1 stimulates tyrosine kinase activity leading to induction of growth promoting proto-oncogenes and mitogen-activated protein kinases. ET-1 may also activate phospholipase A2 inducing release of thromboxane A2 and prostaglandins from arachidonic acid[126].

The endothelin-system and the lung

In peripheral human lung tissue ET_A:ET_B receptor ratio is approximately 30:70, however the distribution within the parenchyma is not uniform[127]. Generally, ET-receptors are predominantly found on smooth muscle cells and in alveolar walls[128]. In the human pulmonary circulation, ET_A is dominating in larger arteries with increasing ET_B proportion in more distal arteries[117]. The ET_B receptor is the predominant receptor subtype present in proximal bronchial smooth muscle[129].

ET-1 levels has been shown to be elevated in plasma[130-132] as well as in the epithelial lining fluid of patients with ARDS and to correlate with severity of lung injury[133]. In addition, ET-1 and albumin concentration in epithelial lining fluid correlated, suggesting increased endothelial and epithelial permeability. ET-1 has been show to increase vascular permeability of the bronchial vascular bed[134] and plasma levels of ET-1 has been shown to correlate with extravascular lung water in patients with septic shock and acute lung injury[135]. In addition, endothelin receptor antagonists has been suggested to possess antifibrotic properties in pulmonary fibrosis[136].

The endothelin-system and sepsis

Several investigators have shown increased levels of ET-1 in patients with sepsis and septic shock[109,137-141]. The level of ET-1 in septic shock is much higher than that seen in cardiogenic shock[142] and higher than expected for the degree of hypotension[143] which implies that the elevation of ET-1 cannot be explained by the hypotension in itself. The ET-system is considered to be involved in several of the pathogenic processes of sepsis. Besides possessing powerful vasoconstricting properties, ET-1 activates leucocytes, increases leukocyte adhesion and promotes production of proinflammatory mediators[144]. In experimental sepsis, ETRA has been show to improve cardiac, pulmonary and renal functions as well as splanchnic microvascular perfusion [145-151].

Endothelin-receptor antagonists and their clinical use

Endothelin receptor antagonists (ETRA) have played a key role in the exploration of the physiology and pathophysiology of the endothelin system. ETRA are classified both by their pharmacodynamics (selectivity) and their structural characteristics (peptide/non-peptide). Selective ETRA are compounds that exhibit at least 100-fold greater affinity for one of the ET-receptor subtype than the other[94]. The first developed ETRA were small peptides (denoted BQ-123[152] and BQ-788[153]). However, the peptidic

nature of the compounds restricted their use to mainly mechanistic studies, often in small animals. Bosentan (Ro 47–0203), a non-selective/dual ETRA, non-peptidic ETRA, was the first ETRA to find clinical use[154]. The number of ETRA has expanded and there are now several non-peptidic ETRA engaged in numerous clinical trials targeting the ET-pathway for patients suffering from a range of disorders, including malignancy, pain, atherosclerosis and myocardial infarction[155,156]. At present the major clinical field for ETRA treatment is chronic pulmonary hypertension where these drugs have been show to improve hemodynamics, counteract right ventricular hypertrophy and to improve pulmonary arterial remodeling[157].

HEPARIN-BINDING PROTEIN (CAP37/AZUROCIDIN)

Heparin-binding protein (HBP) is a PMN-derived granule protein that is considered as a key mediator in PMN induced hyperpermeability[158]. In 1984 Shafer *et al* isolated a protein with anti-microbial properties from the granules of human PMNs[159]. Due to its size and charge the protein was named cationic antimicrobial protein 37 (CAP37). Later, azurocidin was identified as a protein with bactericidal properties stored in azurophilic (primary) granule of PMN[160]. Flodgaard *et al* isolated a protein from human and porcine PMNs with the ability to bind heparin, hence the name heparin-binding protein[161]. Sequencing confirmed that CAP37, azurocidin and HBP are the same protein and revealed the proteins resemblance to other members of the serprocidin family[162]. These are serine proteases which have a conserved catalytic active site consisting of histidine-57, aspartate-102 and serine-195. In the HBP molecule two amino acid residues has been replaced, rendering an enzymatically inactive protein. However, instead of proteolytic activity, HBP possesses several other important properties. Initially most interest was directed to HBPs broad antimicrobial effects but later more attention has been focused on HBPs function as a paramount mediator of PMNs immune-modulating and permeability increasing effects[158].

HBP is contained within two different granule subsets, azurophilic/primary granules and secretory vesicles[163]. Primary granules have a lower propensity for release and are considered to primarily be secreted and acting in the extra vascular space at the site of inflammation. The other granule subset containing HBP, the secretory vesicles, are instead localized close to the PMN cell surface and are promptly mobilized and secreted already in the bloodstream upon stimulation.

HBP is the only granule protein that is secreted from secretory vesicle[163], which allows an almost instant permeability change of the endothelium upon neutrophil activation. This exclusive intracellular localization might be one aspect of HBPs function as a vital mediator in PMN induced in permeability changes[158]. Recently HBP has gained attention as a biomarker to predict circulatory failure in febrile patients with suspected infection[164]. The notion that high plasma concentrations of HBP are associated with circulatory instability in severe infections, suggests that this protein might be an important mediator of vascular leakage and subsequent hypovolemia in sepsis. However, the exact mechanisms by which HBP activates signaling pathways in the endothelial cells that causes reversible cytoskeletal changes remain elusive. HBP has a strong dipole moment due to the concentration of positively charged amino acid residues on one side of the molecule[165]. It has been suggested that HBP induces endothelial permeability changes through an interaction between the positive patch of HBP and the negatively charged proteoglycans on the endothelium[166]. In addition, deposited HBP induces synthesis of cell adhesion molecules on the endothelial cells[167]. Together, these events cause disruption of the endothelial barrier with subsequent leakage of plasma fluid and facilitation of leukocyte rolling and migration through the vessel wall[158]. Moreover, HBP potently attracts and activates monocytes as well as potentiates endotoxin-induced production of proinflammatory cytokines[168,169].

AIMS OF THE THESIS

The overall aim of this thesis was to investigate the role of the endothelin system in experimental lung injury with focus on vascular responses and edema formation. The specific aims were:

- To study the effects of ET-receptor stimulation on pulmonary arteries and veins *in vitro* and on pulmonary capillary pressure and pulmonary up- and downstream vascular resistances *in vivo.*

- To determine the effects of endotoxin on pulmonary capillary pressure and pulmonary up- and downstream vascular resistances *in vivo.*

- To investigate the effect of endotoxin on contractile and relaxing responses in isolated pulmonary arteries and veins.

- To study the effects of inhaled and systemically administered dual ET-receptor antagonist on pulmonary and systemic parameters during endotoxemia.

- To investigate the effects of a graded ET-1 challenge in non-endotoxemic pigs and dual ET-receptor antagonism during endotoxemia on plasma levels of heparin-binding protein and formation of extravascular lung water.

MATERIAL AND METHODS

For detailed descriptions of the used materials and methods, the reader is referred to the respective publications of this thesis.

ANIMAL ANESTHESIA, PREPARATION AND INTERVENTIONS

Domestic landrace pigs (*Sus scrofa domesticus*) of both genders with weights 26-36 kg and age 60-120 days were used in all three *in vivo* studies. The animals were fasted over night with free access to water. In paper I the animals were pre-medicated with intramuscular injections of ketamine 20 $mg \cdot kg^{-1}$ and atropine 25 $\mu g \cdot kg^{-1}$, in paper III and IV with intramuscular injections of medetomidin 40 $\mu g \cdot kg^{-1}$, zolazepam 1 $mg \cdot kg^{-1}$ and atropine 25 $\mu g \cdot kg^{-1}$. Anesthesia was induced with pentobarbital 12 $mg \cdot kg^{-1}$ intravenously and maintained thereafter by continuous infusions of ketamine 20 $mg \cdot kg^{-1} \cdot h^{-1}$, midazolam 0.4 $mg \cdot kg^{-1} \cdot h^{-1}$ and fentanyl 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (paper I), ketamine 20 $mg \cdot kg^{-1} \cdot h^{-1}$, midazolam 0.6 $mg \cdot kg^{-1} \cdot h^{-1}$, fentanyl 20 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and cisatrakurium 0.125 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (paper III) and ketamine 8 $mg \cdot kg^{-1} \cdot h^{-1}$, midazolam 0.5 $mg \cdot kg^{-1} \cdot h^{-1}$ and fentanyl 18 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (IV). Single bolus doses of pancuronium 0.25 $mg \cdot kg^{-1}$ were given prior to tracheotomy and if necessary an additional dose was administered during cautery in order to facilitate surgical preparation. Anesthetic level was evaluated before the administration of muscle relaxants by painful stimuli to the fore hoof with a forceps. Continuous infusions of isotonic saline at a rate of 20 (I) or 30 (III and IV) $mL \cdot kg^{-1} \cdot h^{-1}$ were administered throughout the experiments. Body temperature was maintained at 38-39°C by heating pads. The animals were placed in supine position, tracheotomized and ventilated using volume-controlled ventilation with a respiratory frequency of 18 min^{-1}, tidal volumes adjusted to achieve normoventilation at baseline and a positive end-expiratory pressure of 5 cmH_2O. After surgical preparation the animals were allowed to stabilize for 30 minutes before the endotoxin infusion was started. At the end of the experiments the animals were euthanized by a lethal dose of pentobarbital in ethanol.

Endotoxin

In the *in vivo* experiments (paper I, III and IV), *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich Sweden AB, Stockholm, Sweden) lipopolysaccharide was used. In paper I, the endotoxin infusion rate was increased during the initial phase and titrated to

obtain an increase in mean pulmonary arterial pressure above 30 mmHg (mean rate 0.2 $\mu g \cdot kg^{-1}\ h^{-1}$, range 0.16–0.5). In paper III and IV, the animals received an infusion beginning at 0.05 $\mu g \cdot kg^{-1} \cdot h^{-1}$ then gradually increased to reach 0.25 $\mu g \cdot kg^{-1} \cdot h^{-1}$ within 30-45 min. The infusions were continued during the whole experiment until termination of the protocols after 5 h.

In paper II, the vessels were incubated in medium containing endotoxin from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich Sweden AB, Stockholm, Sweden) at a concentration of 10 $mg \cdot mL^{-1}$.

Tezosentan

Tezosentan *(Figure 5.)* belong to the sulfonamide class of ET-receptor antagonists. It is a competitive, potent and highly water-soluble ET-receptor antagonist designed for parenteral use[170]. Tezosentan has an affinity to ET_A and ET_B- receptors respectively of 30:1 and is thus considered as an unselective, dual antagonist. The distribution half-life of tezosentan in humans is 0.1 hours and the elimination half-life 3 hours. The primary elimination mechanism is biliary excretion of unchanged compound, even though some metabolism occurs by hydroxylation[171]. The effect of tezosentan metabolites is low and no accumulation has been reported.

When used in humans, the main side effect is headache, suggested to be due to cerebral vasodilation. Tezosentan has been studied in phase III clinical trials aimed to treat acute congestive heart failure[172,173]. In these studies no favorable effect of treatment were found and the main side effect was systemic hypotension.

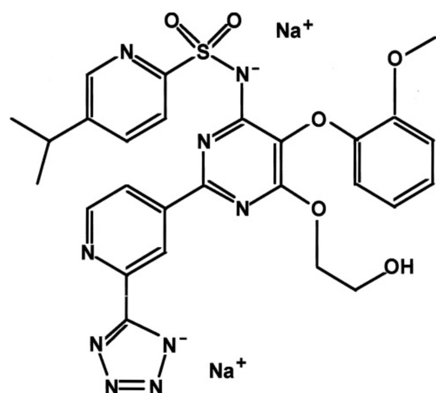

Figure 5. The molecular formula of the dual endothelin receptor antagonist tezosentan.

In the publications of this thesis, tezosentan (Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) was dissolved in saline on the day of the experiment. In paper I tezosentan was administrated as a bolus of 1 $mg \cdot kg^{-1}$ over 10 minutes followed by a continuous

infusion at 1 $mg \cdot kg^{-1} \cdot h^{-1}$ for one hour. In paper III tezosentan was administered as a bolus of 0.5 $mg \cdot kg^{-1}$ i.v. or inhaled or 0.05 $mg \cdot kg^{-1}$ inhaled, all during 20 minutes. In paper IV tezosentan was administrated as a bolus of 1 $mg \cdot kg^{-1}$ over 10 minutes followed by a continuous infusion at 1 $mg \cdot kg^{-1} \cdot h^{-1}$ for three hours.

In paper III plasma concentrations of tezosentan were analyzed by reversed-phase ultra performance liquid chromatography and tandem mass spectrometry after protein precipitation with acetonitrile, an method slightly modified from original description of the analysis described by van Giersberger *et al*[174].

Endothelin receptor agonists and phenylephrine

Synthetic ET-1 (American Peptide Co., Sunnyvale, CA, USA) was used in paper I, II and IV. In paper I, the average dose used was 35 $\rho mol \cdot kg^{-1} \cdot min^{-1}$ (range 15- 65); due to variability in response the dosage was adjusted in order to get significant, but not detrimental increases in pulmonary vascular resistance. In paper IV, an intravenous infusion of ET-1 was started at 10 $\rho mol \cdot kg^{-1} \cdot min^{-1}$ and increased to 20 $\rho mol \cdot kg^{-1} \cdot min^{-1}$ after 90 min.

Sarafotoxin 6c (S6c, American Peptide Co., Sunnyvale, CA, USA) was used in paper I and II. In paper I the average infusion rate was approximately 30 $\rho mol \cdot kg^{-1} \cdot min^{-1}$.

In the myographic experiments (paper I and II), concentration-response relationships for ET-1 and S6c was recorded using concentrations between 10^{-11} and $10^{-6,5}$ M.

Phenylephrine (Apoteksbolaget AB, Stockholm, Sweden), a selective and synthetic α_1-adrenergic agonist was used in the myographic experiments as well as a reference substance in paper I. The infusion rate *in vivo* was 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$, a dose within the range of clinical use.

Biochemical analyses of plasma ET-1, HBP and myeloperoxidase

In paper III and IV, arterial plasma levels of ET-1 immunoreactivity were analyzed with radioimmunoassay as described previously[175]. Arterial plasma levels of HBP were analyzed with an enzyme-linked immunosorbent assay[163]. Briefly, multi-well plates were coated with a monoclonal antibody against porcine HBP diluted in a buffer solution. Plates were washed with phosphate-buffered saline containing Tween 20 and were thereafter blocked with washing buffer containing bovine serum albumin (incubation buffer) for 30 minutes at 37°C. This was followed by incubation with samples containing porcine plasma HBP or a serial dilution of a HBP standard (1.95-

250 ng·mL^{-1}) which were added to the incubation buffer for 1 h at 37°C. After a washing step, the plates were incubated with a polyclonal antibody against porcine HBP. Bound antibody was detected by a horseradish peroxidase-labeled secondary antibody against rabbit-IgG and a chromogenic substrate solution. Each incubation step was followed by a washing step. Arterial plasma levels of myeloperoxidase (MPO) were analyzed with enzyme-linked immunosorbent assay for porcine myeloperoxidase (Uscn Life Science Inc., Wuhan, China) in accordance with instructions from the manufacturer.

Nebulization

In paper III a standard ultrasonic nebulizer (Servo Ultra Nebulizer 345, Maquet, Solna, Sweden) was used. The nebulizer provides aerosol particles with a mean mass aerodynamic diameter of 4.7 µm, the lower tenth percentile at 2.4 µm and the higher at 8.6 µm (data from manufacturer, Maquet, Solna, Sweden). The administered volume of 4 ml was completely nebulized after 20 minutes.

ASSESSMENTS OF EXTRAVASCULAR LUNG WATER

As formation of edema is a major issue in development of lung injury, measurements of extravascular lung water (EVLW) are considered as an potentially valuable parameter in pulmonary dysfunction[176]. Accordingly, significant efforts have been made aimed to develop methods which would provide accurate bedside measurement. However, so far no method has gained widespread acceptance, mainly due to uncertainty regarding the accuracy of bedside EVLW measurements in patients with heterogeneous lung perfusion[177].

Single Thermal Indicator Dilution Method

The commercially available single thermal indicator dilution system (PiCCO®, Pulsion Medical Systems AG, Munich, Germany) was used. A thermistor-tipped arterial catheter placed in the lower abdominal aorta through a cut down of the femoral artery. The single thermal indicator dilution (STID) technique has been described previously[178] and been validated in experimental settings[179-181]. Each EVLW measurement was determined by the average of three injections of 10 mL of ice-cold saline to a central vein. EVLW was presented as indexed to body weight ($EVLWI_{STID}$).

Gravimetrical analysis

The gravimetrical method is considered as the golden standard of EVLW assessments. However, the gravimetrical method has a major drawback as it demands extraction of the lungs and therefor only provides a single value at the end of the experiment. The method was performed in accordance with the original procedure described by Pearce[182] with the modification in ultracentrifugation according to Selinger[183]. After termination of the protocol a sternotomy was performed and both lung hili was located. A large volume sample of blood was drawn from the central line for determination of hemoglobin concentration, hematocrit and wet to dry weights of the blood. Thereafter the animals were euthanized followed by immediate clamping and removal of the lungs. After passive drainage of blood, the lungs were weighed and homogenized with an equal amount of deionized water. Half of the homogenate was used to determine its wet to dry weight and the other half was centrifuged at 30 000 g for 1 h at 4°C. The supernatant was separated, and its hemoglobin concentration and wet to dry weight were determined. The dry weights of the blood, homogenate, and supernatant were determined after 72 hours of incubation in a heat chamber at 85°C. EVLW was then calculated and indexed to body weight ($EVLWI_{Grav}$, for equations see Appendix).

MEASUREMENT OF PULMONARY CAPILLARY PRESSURE

As mentioned before, even though P_{cap} is of significant interest due to its impact on edema formation is it not possible to directly measure P_{cap} in patients or intact animals. Therefore the arterial occlusion technique has been developed[184]. According to this method, the pulmonary circulation may be described as an electric circuit where the pulmonary vascular resistance is represented by two resistors in series, one pre- and one post capillary. The capillary blood volume, which composes a major portion of the total pulmonary blood volume, is represented by a capacitor at the capillary level. When the circuit is broken precapillary (*i.e.* the artery is occluded by the balloon in systole) the current first drops rapidly to reach the level of the current in the capacitor, then more slowly when the capacitor is discharged to finally reach the pulmonary venous pressure.

The pulmonary arterial occlusion method for determination of Pcap is based on assessment of the shape of the pulmonary artery pressure decay curve during balloon occlusion using a pulmonary artery catheter with an inflatable balloon. The occlusion should be performed in the systolic upstroke, during an expiratory hold to avoid the

effect of changes in intrathoracic pressure and lung volume on the pressure curve. After occlusion, there is a rapid decline in the pressure distally to the balloon, followed by a slower pressure decrement approaching the pulmonary artery occlusion pressure *(Figure 6.)*. When a straight line is drawn tangent to the rapid component pulmonary capillary pressure can be estimated as the inflection at which the pressure transient begins to deviate from the rapid portion of the pressure tracing. The use of a time constant and a bi-exponential curve fitting has been suggested to improve the accuracy of the measurement[185].

In our experiments, ordinary pulmonary artery catheters (Edwards Life Science, St. Ana, CA, USA) were modified in order to allow simultaneous measurements of pressures at the tip and two centimeters proximal of the balloon. The recordings were sampled at 1000 Hz with a digital data acquisition system (Acqknowledge, Biopac Systems, Santa Barbara, CA, USA). The measurements were performed in an extended expiratory pause and repeated balloon occlusions were performed in sequence at each point of registration in order to obtain adequate curves with a distinct point of occlusion in systole. The tracing of the pressure decay from the time of occlusion and 4000 ms was fitted to a biexponential function in order to determine the P_{cap} at 175 ms, all according to description by Pellet *et al*[185]. Only curves with an accurate systolic occlusion point and without significant artifacts were used. Pcap was used to calculate pulmonary up- and downstream vascular resistance index using standard formulas (presented in the Appendix).

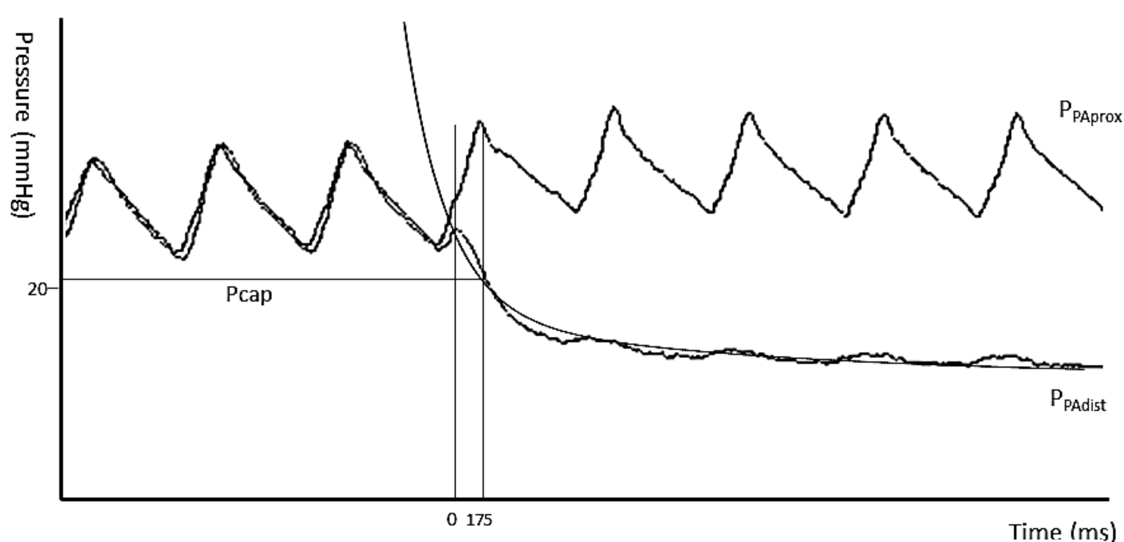

Figure 6. Representative pressure tracing recorded during porcine endotoxemia with a modified pulmonary artery catheter. Pressure tracing proximally (P_{PAprox}) or distally (P_{PAdist}) to the occluding balloon. 0 ms denotes time of arterial occlusion. 175 ms denotes time constant for determination of pulmonary capillary pressure (Pcap) from the bi-exponentially fitted curve.

MYOGRAPHIC EXPERIMENTS

All myographic experiments were conducted on vessels isolated form porcine lungs collected at regional abattoirs instantly after killing of the animals. The lungs were rinsed with a cold transport buffer solution and transported on ice to the laboratory within 1 h. Arterial and venous rings (internal circumference 2.6–15.2 mm, segment length 1.1–4.3 mm) were dissected under sterile conditions and subjected to immediate myographic experiments or after 24 h. After dissection, the vessel rings were placed in sterile Dulbecco's Modified Eagle's Medium supplemented with antibiotics and L–glutamine until further experiments. The arterial and venous vessel rings were mounted on two parallel stainless steel pins in organ baths filled with Krebs-Ringer physiological salt solution continuously gassed with 95% O_2 / 5% CO_2 at a temperature of 37 ° C. One pin was connected to a force transducer and the other to a micrometer screw for length adjustment. Force data were collected with a computerized A/D converter. The vessels were stretched to the optimal circumference for maximal isometric force development [high K^+ (80 or 125 mM)-induced contraction considered maximal]. This length was defined as the length (that is, circumference) at which the high potassium-induced contraction was maximal. In previous studies, this length was found to correlate with the initial slack circumference allowing an estimate of optimal length based on the slack circumference of the vessels[186]. After stretching and equilibration, the viability of the preparations was tested by measuring the contractile response to high K^+. Preparations not contracting to K^+ were excluded from the study. To assess endothelial function all preparations were challenged with U46619 (stable thromboxane A 2 analogue). After the contraction to this agonist had stabilized (approx. 5 min), bradykinin (arteries) or acetylcholine (veins) were added. Failure to relax to these two agonists (in the freshly isolated vessels) excluded the preparations from further experimentation. Constrictive agonists were added to the baths cumulatively and concentrations-response relationships were recorded. To assess endothelium-independent vasorelaxation, a separate set of precontracted incubated venous rings was exposed to cumulative concentrations (1 nM to 1 mM, log unit steps) of sodium nitroprusside (NO donor) and concentration-response relationships were recorded. The segment lengths of the preparations were recorded at the end of the experiments. Relaxation was calculated as percent of reduction of the active force at the stable plateau level. Contractile responses to high K^+ were expressed as the ratio of force and segment length (active tension, mN/mm) and the other responses are

expressed as percentage of the maximal contraction induced by high K^+. The force (Y) and concentration (X) data were analyzed by fitting a sigmoidal function using nonlinear regression (Prism 4.0; GraphPad Software Inc., La Jolla, CA., USA) using the formula: $Y = E_{max} \cdot X^h / (X^h + EC_{50}^{\ h})$, where E*max* denotes the estimated maximal amplitude of the response, h the steepness of the relationship (Hill coefficient) and EC_{50} the concentration giving half maximal response.

ANALYSIS OF ET-RECEPTOR PROTEIN EXPRESSION WITH IMMUNOHISTOCHEMISTRY

Approximately 10 mm long intact pieces of pulmonary vessels were transferred to 4 % paraformaldehyde for fixation for 24 h immediately after dissection or after 24 h exposure to endotoxin. After washing the vessels were embedded for cryosectioning (8 µm sections). Sections were rehydrated, permeabilized and unspecific binding was blocked. This was followed by incubation with primary antibodies against for the ET_A or ET_B receptors and Cy3-conjugated smooth muscle α-actin. After washing, the sections were incubated with fluorescent secondary antibodies and finally incubated with nuclear stain DRAQ5, rinsed briefly and mounted with fluorescent mounting medium. The mounted sections were refrigerated dark until confocal microscopy analysis.

ANALYSIS OF ET-RECEPTOR PROTEIN EXPRESSION USING IMMUNOBLOTTING

Samples from pulmonary vessels were extracted in a protease inhibitor solution using a glass mortar and pestle for homogenization. When the tissue was completely dissolved, the extract was centrifuged and the supernatant was collected for protein determination. Tissue extracts was mixed with buffer, denatured, separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Ponceau staining was used to evaluate protein loading. Nonspecific binding sites were blocked by incubation with bovine serum albumin. Membranes were incubated with specific antibodies anti-ET_A and ET_B receptor respectively or mouse monoclonal antibody specific for smooth muscle-specific α-actin. After primary antibody incubation, membranes were washed and bands were visualized after incubation with a horseradish peroxidase-conjugated secondary antibody using ECL technique. Equal amounts of protein of the different samples were loaded on each gel and the band ECL intensities were measured using

GelDoc and an image software. Band intensities were expressed as percentage of the intensities of bands from freshly isolated vessels.

STATISTICAL METHODS

Data are presented as mean ± SEM. The statistical calculations were made using Statistica (version 7.0; StatSoft Inc., Tulsa, Oklahoma, USA). A p value of less than 0.05 was considered statistically significant.

Paper I and II

Differences in contractions and relaxations between the *in vitro* preparations and between band intensities from Western blot were analyzed using Student's t test. Concentration-response data were assessed using repeated measures analysis of variance (RM-ANOVA). Student's t-test was used for analysis of within group effects induced by pharmacological challenge *in vivo* and RM-ANOVA was used for detection of effects of endotoxin (T0-T3) and effects of tezosentan treatment (T3-T4). Differences between groups and arterial and venous responses were analyzed with time-effect interactions.

Paper III

Normal distribution of data was analyzed using Kolmogorov-Smirnov test. In case of non-normal distribution, a logarithmic transformation was made to reach normal distribution of data. One-way univariate analysis with RM-ANOVA was used for analyzing changes over time in response to endotoxin from baseline until onset of intervention at 2 h. One-way ANOVA was used for evaluating differences between groups before onset of intervention at 2 h. The treatment effects were evaluated by one-way ANOVA for group differences at each time point from 3 to 5 h, based on differences from the time point of 2 h. This ANOVA was followed by a *post hoc* analysis (Fisher least significant difference [LSD]). Correlation between ET-1-LI and MPAP was analyzed using the Spearman rank test.

Paper IV

Normal distribution of data was analyzed using Kolmogorov-Smirnov test. In case of non-normal distribution a logarithmical transformation was made to reach normal distribution of data. RM-ANOVA was used for analyzing changes over time in

response to endotoxin as well as differences between groups prior to onset of intervention (0-2 h). Effect of treatment was evaluated by two-way RM-ANOVA for group differences from three to five hours, using time point two hours as a covariate. Student´s t-test was used to analyze $EVLWI_{Grav}$. Data from the experiments with ET-challenge were analyzed using Friedman ANOVA followed by planed comparisons between treatment and baseline values.

SUMMARY OF RESULTS

In vivo experiments

Effects of endotoxin

Systemically, endotoxin induced a hypodynamic response with decreased cardiac index (CI), mixed venous saturation (S_VO_2) and increased (paper I and IV) or unchanged (III) systemic vascular resistance index (SVRI). Mean arterial pressure (MAP) was unchanged (I and IV) or reduced (III). In all three *in vivo* studies, endotoxin induced a marked pulmonary arterial hypertension with increases in mean pulmonary arterial pressure (MPAP) and pulmonary vascular resistance index (PVRI). In the two studies (I and III) were P_{cap} was measured, endotoxin induced a dominant increase in pulmonary down-stream resistance (P_{ds}VRI) and an increase in P_{cap} to levels well above those that have been reported to induce edema *(Figure 7.)*. In line with these findings, EVLWI increased during endotoxemia when measured with STID (III and IV). This result was confirmed by gravimetrical analysis of EVLWI (IV). Moreover, endotoxemia induced a deterioration of pulmonary gas-exchange, seen as moderate reductions in P/F-ratio and with simultaneous increases in $PaCO_2$. Concurrently, respiratory system compliance was reduced. In all three studies, the animals developed a metabolic acidosis with decreased pH and BE. In addition, endotoxin administration induced a hemoconcentration seen as significant increases in Hb (III and IV). Arterial plasma levels of ET-1 increased more than two-fold in response to endotoxemia (III and IV). In paper IV endotoxin challenge evoked a marked increase in plasma-HBP *(Figure 8.)*, whereas plasma-MPO was unchanged.

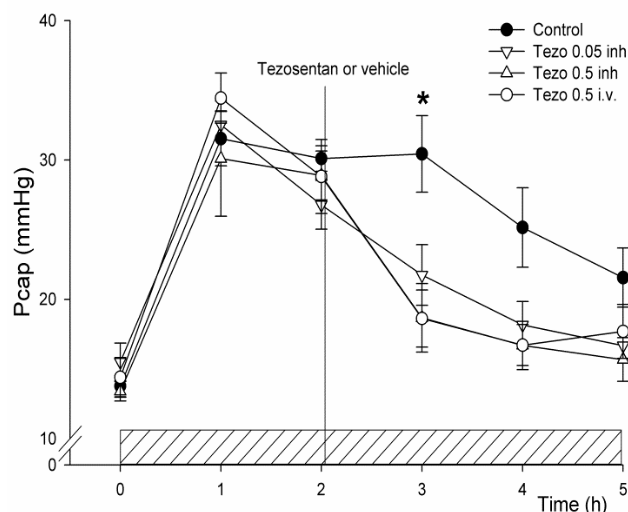

*Figure 7. Effect of inhaled or i.v. tezosentan (Tezo) on pulmonary capillary pressure (P_{cap}) during five hours of endotoxemia. Interventions given during 20 min after two hours. Striped bar illustrate endotoxin infusion time. Mean±SEM. * denotes $p < 0.05$ for inhaled tezosentan 0.5mg/kg versus control.*

Systemic effects of dual ET-receptor antagonism during endotoxemia

In paper I, tezosentan was administered systemically after three hours of endotoxemia as a 1 $mg \cdot kg^{-1}$ bolus and followed by a continuous infusion (1 $mg \cdot kg^{-1} \cdot h^{-1}$). In this setting tezosentan increased CI and reduced MAP, whereas no effect was noted on PAOP. In paper III, tezosentan was administered systemically as 0.5 $mg \cdot kg^{-1}$ bolus during 20 min after 2 h of endotoxemia. In this limited dosage, tezosentan did not induce any statistically significant changes in any systemic cardiovascular or biochemical parameters. However, plasma-ET-1 was markedly increased, indicating that a significant portion of ET_B-receptor-mediated clearance of the ET-1 was blocked even by this modest dose of tezosentan.

In paper IV a higher tezosentan dose was used, 1 $mg \cdot kg^{-1}$ bolus during 15 min followed by a continuous infusion of tezosentan 1 $mg \cdot kg^{-1} \cdot h^{-1}$ that persisted to the end of the experiment at 5 h. In this setting, tezosentan-treatment increased CI and reduced MAP as well as SVRI. HR was unchanged indicating that tezosentan-treatment caused an increased stroke volume. Moreover, tezosentan attenuated a further reduction of arterial pH and reduced arterial lactate. Due to inhibition of ET_B-receptor-mediated clearance of ET-1, plasma-ET-1 increased more than four-fold in the tezosentan-treated group. Finally, tezosentan markedly counteracted the endotoxin-induced increase in plasma-HBP *(Figure 8.)* and reduced hemoconcentration.

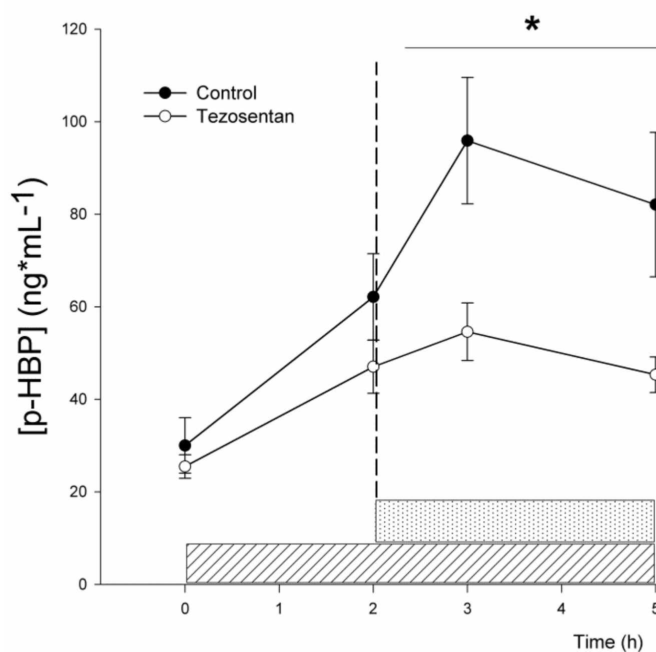

*Figure 8. Effect of i.v. tezosentan on plasma levels of heparin-binding protein (HBP) during five hours of endotoxemia. Striped bar: endotoxin-infusion time, dotted bar tezosentan/vehicle administration time. Mean±SEM. * $p < 0.05$.*

Pulmonary effects of dual ET-receptor antagonism during endotoxemia

Tezosentan markedly reduced MPAP and PVRI (I, III and IV). This was seen with reductions in P_{cap} and P_{ds}VRI (I). In paper III, tezosentan in a lower dose reduced P_{cap} and pulmonary up-stream vascular resistance index (P_{us}VRI) while no statistically significant treatment effects were noted for P_{ds}VRI. $EVLWI_{STID}$ was reduced by tezosentan-treatment (III and IV), a finding that was confirmed by analysis of $EVLWI_{Grav}$ at the end of the experiments in paper IV *(Figure 9.)*. Tezosentan improved respiratory system compliance and increased SaO_2, whereas neither the effects on P/F-ratio nor $PaCO_2$ reached statistical significance (IV). In paper III, where the low dose of tezosentan was used, no effects were seen on gas-exchange variables.

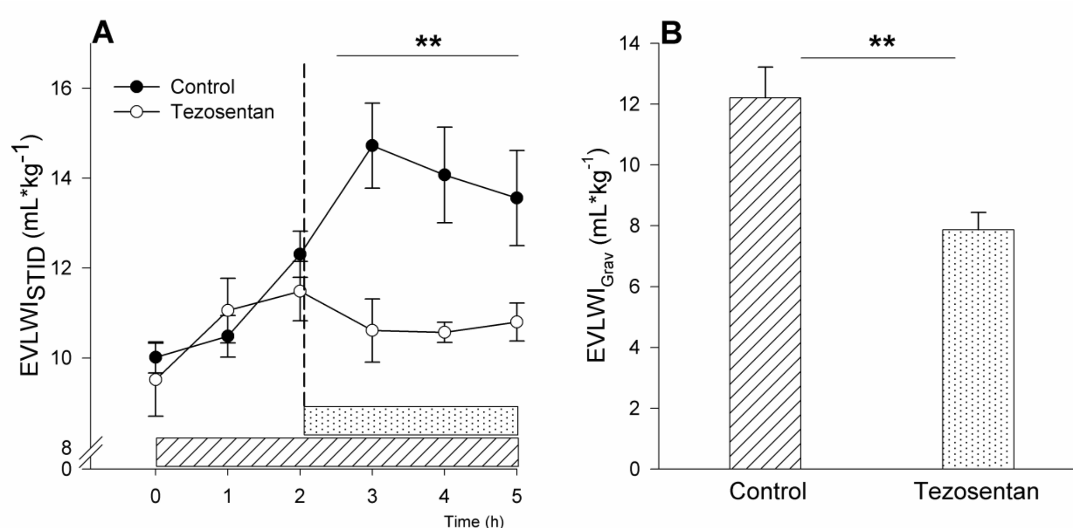

*Figure 9. Effect of i.v. tezosentan on extravascular lung water index (EVLWI) measured with A) single thermal indicator dilution (STID) and B) gravimetry (Grav) during five hours of endotoxemia. Striped bar illustrates endotoxin-infusion time, dotted bar illustrates tezosentan or vehicle infusion time. Mean±SEM. ** p < 0.01.*

Effects of inhaled dual ET-receptor antagonism during endotoxemia

In paper III, the animals were exposed to five hours of endotoxemia. After two hours inhaled tezosentan was administered in two doses (0.05 and 0.5 $mg \cdot kg^{-1}$) during a 20 minute period. This short term administration of a limited dose of tezosentan efficiently counteracted the endotoxin-induced pulmonary hypertension seen as a rapid decline in MPAP *(Figure 10.)* and PVRI. In addition, despite the short duration of the therapy, this effect was sustained over time. Inhaled tezosentan reduced P_{cap} and P_{us}VRI. However, in this setting no statistically significant treatment effect of tezosentan were noted for P_{ds}VRI. Inhaled tezosentan induced a reduction $EVLWI_{STID}$, an effect that not

was sustained over time. Respiratory system compliance was improved by inhaled tezosentan, whereas no effects were noted on gas-exchange variables. In addition, inhaled therapy did not induce any statistically significant differences in MAP nor in any other systemic cardiovascular or biochemical parameters measured.

Inhalation of tezosentan did not significantly affect systemic ET-1 levels compared with controls, suggesting a localized pulmonary effect of the compound. This was also reflected by the plasma concentrations of tezosentan, where inhalation of 0.05 $mg \cdot kg^{-1}$ tezosentan gave almost 200-fold lower plasma level than i.v. treatment.

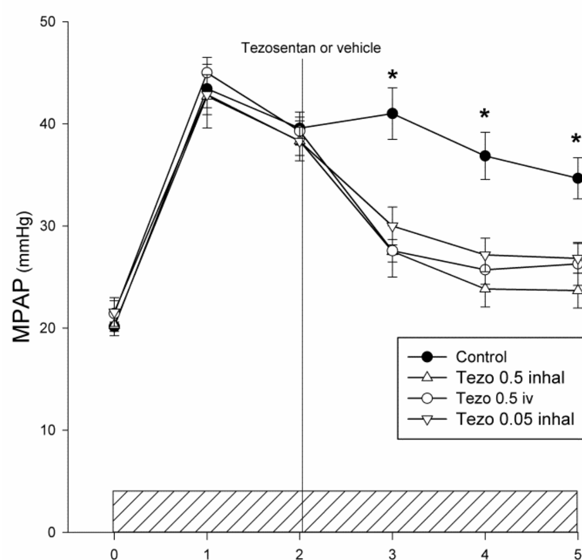

Figure 10. Effect of inhaled or i.v. tezosentan (Tezo) on mean pulmonary artery pressure (MPAP) during five hours of endotoxemia. Intervention given during 20 min after two hours. Striped bar illustrate endotoxin infusion time. Mean±SEM. $p < 0.05$ *for respective interventions versus control.*

Challenge with endothelin-1 and sarafotoxin 6c (I and IV)

Infusion of ET-1 in non-endotoxemic animals reduced CI with simultaneous and marked increases in MAP and MPAP (I and IV). ET-1-challenge increased P_{cap} with a predominant increase in in P_{ds}VRI (I, *Figure 11A.*). This was seen with a concurrent reduction in C_{rs}I and a significant metabolic acidosis. WBC-count and plasma-MPO were not significantly changed in response to ET-1 (IV). However, plasma-HBP increased in a dose-dependent fashion in response to ET-1-challenge, to levels more than three-fold over baseline values *(Figure 12.).* After termination of the ET-1 infusion, plasma-HBP readily tended to decrease. $EVLWI_{Grav}$ was slightly increased by ET-1 when compared to values from non-endotoxemic sham animals in previous reports[147]. However, the interpretation of this result is somewhat difficult as the lungs were extracted 90 min after termination of the challenge.

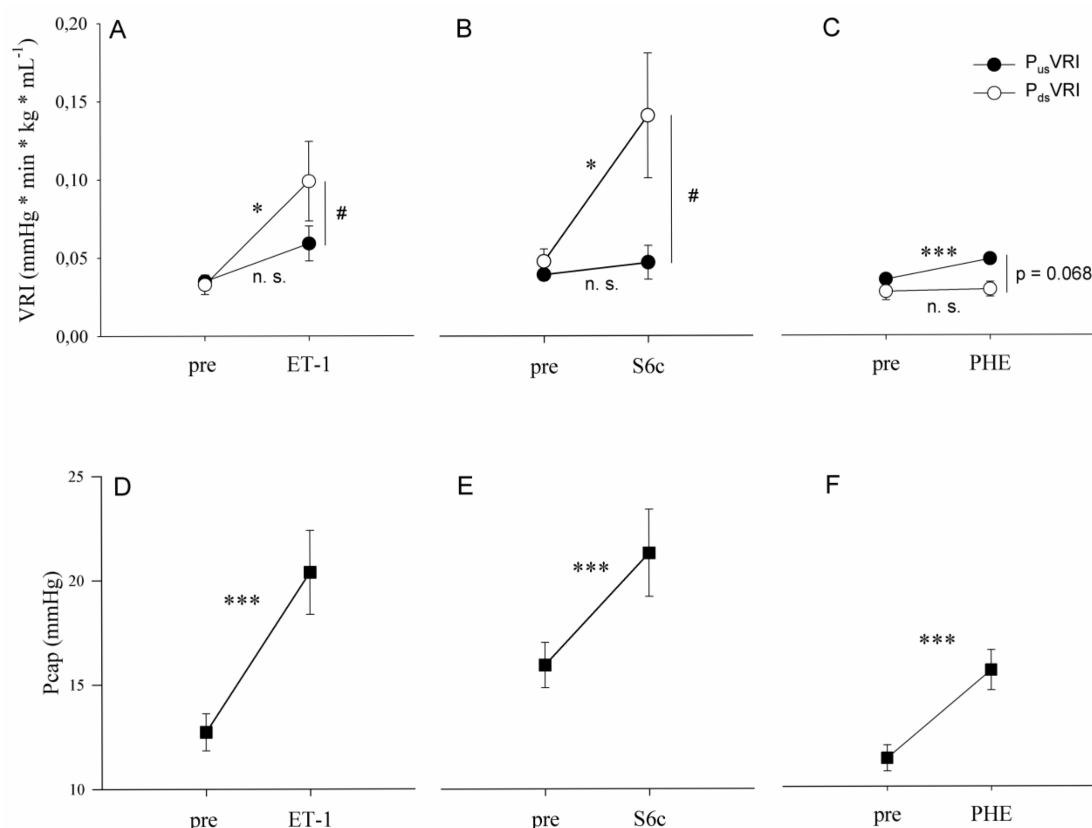

Figure 11. Effect of endothelin-1 (ET-1, n=7), sarafotoxin 6c (S6C, n=6) and phenylephrine (PHE, n=10) infusion on vascular up and downstream resistances (A-C) and pulmonary capillary pressure (Pcap, D-F). Pulmonary upstream resistance (P_{us}VRI, filled circles) and pulmonary downstream resistance (P_{ds}VRI, open circles). Mean±SEM. p <0.05, *** p < 0.001 versus baseline value.*

ET_B receptor stimulation by S6c led to significant increase in P_{cap} and analysis of vascular resistances showed a marked rise in downstream resistance (I, *Figure 11B.*). Finally, MAP and MPAP were unchanged while SVRI and PVRI were markedly augmented by S6c due to a reduction in CI. No measurements of EVLWI were made after S6c challenge in paper I.

Challenge with phenylephrine (I)

Infusion of phenylephrine (PHE) increased P_{us}VRI but not P_{ds}VRI *(Figure 11C.)*. However, P_{cap} was significantly increased by PHE-infusion *(Figure 11F.)*. These findings are explained by the simultaneous rise in pulmonary arterial occlusion pressure (PAOP) and a more pronounced increase in MPAP than P_{cap} induced by PHE. PHE did not affect CI while MAP and MPAP increased.

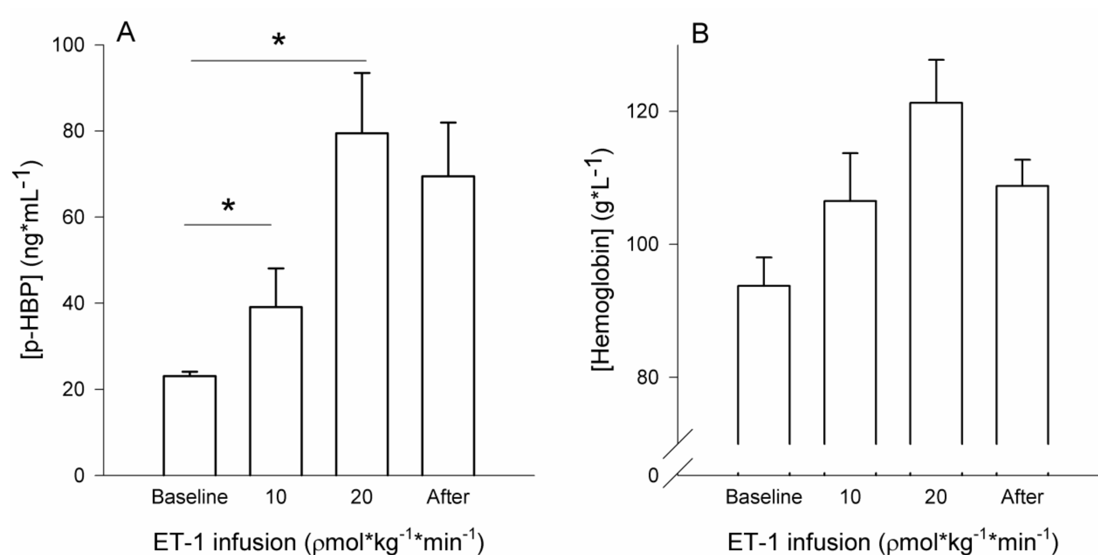

*Figure 12. Effect of graded endothelin-1 (ET-1) infusion on A) plasma levels of heparin-binding protein (p-HBP) and B) hemoglobin concentration. n=4. Mean±SEM. * p < 0.05 versus baseline values.*

In vitro experiments

Reactivity of freshly isolated pulmonary vessels (I and II)

The active wall tension (force per segment length) in response to depolarization with high K^+ solution was similar in the freshly isolated, non-incubated, arteries and veins. No significant difference between arteries and veins was noted in response to the α_2 adrenergic agonist UK14.304, the thromboxane A 2 analogue U46619 or to $PG_{F2\alpha}$ (II). ET-1 evoked responses in both pulmonary arteries and veins with maximal contractions observed at $nmol \cdot L^{-1}$ concentrations. In the experiments of paper I, the venous response to ET-1 was significantly stronger than the arterial as measured by concentration effect interaction and by maximal contraction *(Figure 13A)*. However, in paper II, the response to ET-1 was similar between the arterial and venous preparations. S6c induced a nearly two-fold stronger contraction and was active at lower concentrations in veins than in arteries *(Figure 13B),* a finding that was consistent between the two studies. PHE induced a contraction that was several-fold stronger in arteries than in veins (I and II, *Figure 13C*). In vessels preconstricted with U46619, the β-adrenergic agonist isoproterenol (ISO) induced a greater relaxation in veins compared to arteries (II). The responses to ACH were similar in the freshly isolated arteries and veins (II).

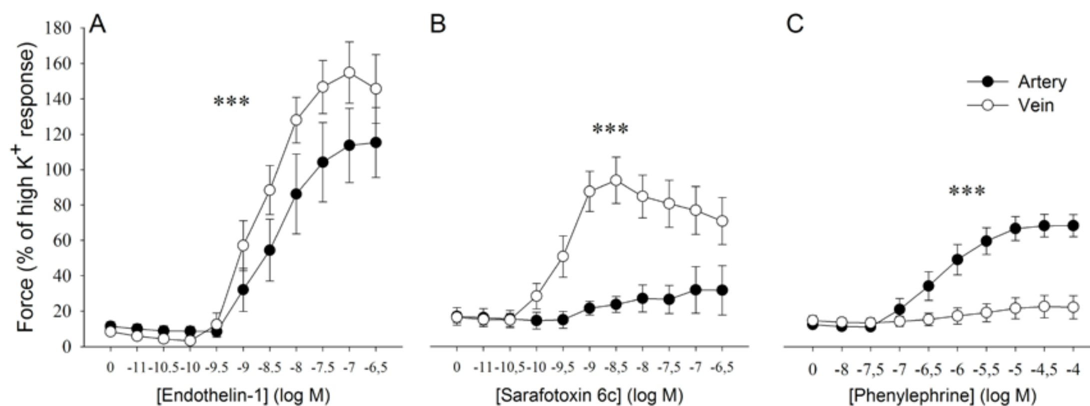

*Figure 13. Cumulative concentration-response relationships for A) endothelin-1, B) sarafotoxin 6c and C) phenylephrine on freshly isolated porcine pulmonary artery (filled circles) and vein (open circles) preparations. Mean±SEM. *** $p < 0.001$.*

Effects of incubation and endotoxin on vascular reactivity (II)

Incubation during 24 h induced only modest and non-significant changes in active force generation of the vessels in response to membrane depolarization by high K^+. In addition, no difference was found in response to the other constrictive substances apart from slightly augmented response to PHE in veins and a tendency towards an increase in response to S6c in arteries after incubation. In veins, incubation moderately attenuated the endothelium-dependent relaxation to ACH, while the artery was not significantly affected. Moreover, the relaxation induced by ISO, studied in arteries and veins and SNP, studied in vein only, was not altered by incubation.

Incubation of the vessels in medium with added endotoxin to a concentration of 10 $mg \cdot mL^{-1}$ during 24 h induced a significant decrease in the high K^+ induced active tension of veins but not in arteries. Moreover, endotoxin also induced a markedly lowered reaction in both arteries and veins in response to PHE *(Figure 14A)*. In veins selectively, the contraction induced by S6c and ET-1 was relatively augmented by endotoxin *(Figure 14B and C)*, whereas the response to the other contractile agonists, except PHE, was unaltered in both preparations. The effects of the relaxing compounds ACH and ISO were not significantly changed by endotoxin, although there was a weak tendency suggesting a modest impairment of the endothelium-dependent relaxation of ACH in veins only. Endotoxin incubation did not change the response to the NO-donor sodium nitroprusside (SNP) in veins.

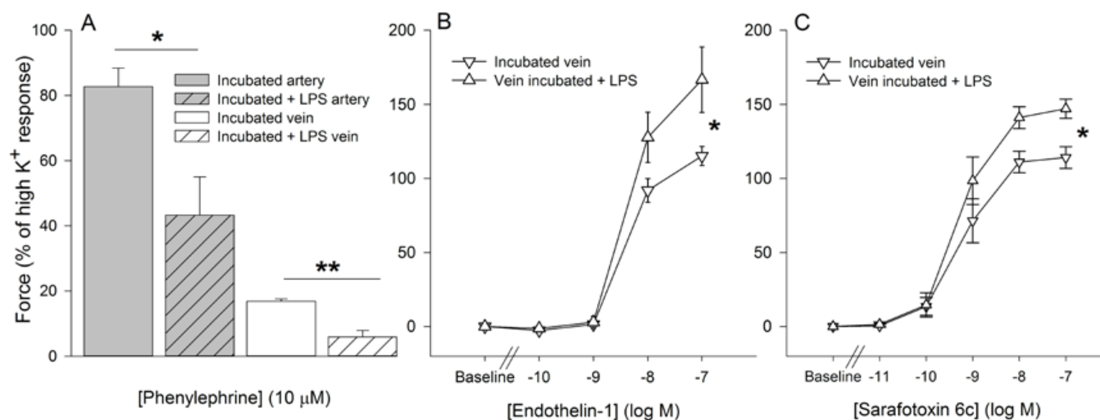

*Figure 14. A) In vitro responses in isolated porcine pulmonary arteries and veins, incubated with or without endotoxin (LPS). A) phenylephrine 10 μM, B and C) cumulative concentration-response relationships for endothelin-1 and sarafotoxin 6c. Mean±SEM. * $p < 0.05$, ** $p < 0.01$.*

Expression of ET-receptors in vascular preparations (II)

Immunohistochemistry was performed to localize the ET_A and ET_B receptors within the vascular tissue and to study the gross morphology of freshly isolated, incubated and endotoxin-incubated preparations. The vascular smooth muscle and the endothelial cell layer showed no signs of disruption or swelling after incubation or endotoxin exposure. Staining against α-actin showed equally intense staining in the smooth muscle cell layer of all preparations. In fresh as well as in incubated vessels, the ET_A receptors were preferentially located in the vascular smooth muscle cells, whereas the ET_B receptors were located in the endothelial as well as the vascular smooth muscle cell layer. No difference in ET-receptor staining intensity or distribution could be detected between freshly isolated arteries and veins or between incubated and endotoxin-exposed vessels. The expression of ET_A and ET_B receptor protein was also investigated using Western blot and compared to α-actin protein levels. In arteries, the levels of both ET_A and ET_B receptor protein was higher compared to the levels in veins, using the same amount of protein loaded on the gel. This difference was not affected by incubation *per se* or by endotoxin. Moreover, no difference in expression of ET-receptor protein or α-actin was detected between freshly isolated, incubated or endotoxin-exposed vessels.

DISCUSSION

The vascular system was once seen as a rather inert transportation system for oxygen and nutrients dissolved in the blood. A new era begun during the 1980´s with the discoveries that showed that the endothelium was a highly active tissue that produced potent mediators of great importance during both health and disease. In sepsis, dysfunction of the vascular system is of vital importance. This thesis aimed to investigate mechanism of vascular dysfunction in a common and severe manifestation of sepsis, acute lung injury, with special reference to effects of the endothelin system.

The porcine endotoxin model of sepsis induced lung injury

A number of different models of lung injury have been described, all with their specific advantages, drawbacks and varying grades of clinical correlate[187]. To investigate one of the most common causes of lung injury, sepsis induced lung injury, the use of animal models is necessary as invasive monitoring, pharmacological interventions and not least the septic event itself, cannot safely or ethically be performed in humans.

Over the years there has been a controversy on which animal model that best resembles the human sepsis syndrome, a debate that is still not resolved[188,189]. Animal sepsis models can be divided into three main categories based on the triggering event: exogenous administration of bacterial toxins, exogenous administration of viable bacteria, or inducing a damage of the animal's natural barrier against bacteria (commonly done by ligating and puncturing of the cecum, allowing fecal peritonitis and bacterial translocation). All these models have their advantages and drawbacks. Bolus infusion of bacterial toxins or live bacteria often induces a powerful hypodynamic shock with high mortality. Models using fecal peritonitis have a strong clinical correlate in human abdominal infection and sepsis; however this development takes time (several hours to days) and the reproducibility is lower than in the other models.

The reproducibility of the response to endotoxin infusion is superior compared with the other models, even though the potency of endotoxin may vary significantly between production-batches. In addition, endotoxin is a significant part of the pathophysiology of Gram-negative sepsis and the responses seen after administration of small doses of endotoxin to human volunteers are similar to those seen in human sepsis[190]. When the aim is to investigate and intervene in basic mechanisms of a biological phenomenon

such as sepsis, reproducibility and homogeneity is central, as great variability may conceal the effect of the intervention and necessitate the use of large numbers of animals. However, when basic findings later are to be transferred into new treatments, the resemblance of the model to the clinical condition becomes increasingly important. Studies using prolonged protocols and animals of high age would increase the resemblance to the clinical setting, but could be problematic due to ethical and financial aspects as well as the reproducibility of the experiments.

The most common animal species that is used in medical science are rodents. They are relatively inexpensive to purchase and maintain and can therefore be used in large numbers. However, in sepsis research, the use of rodents has shortcomings as they are physiologically quite different from humans in terms of immunity, endotoxin-resistance and cardiovascular physiology. The physiology of pigs, especially regarding cardiovascular functions and the ET-system, is markedly more similar to human. In addition, the size of the animals permits extensive surgical preparation, monitoring and repeated blood-samplings - all important in sepsis research.

Effects of systemic dual ET-receptor antagonism during endotoxemia (I, III and IV)

As ET-1 is a powerful vascular constrictor, a striking but anticipated effect of systemic treatment with a dual ET-receptor antagonist was vasodilatation. MAP and SVRI were reduced by tezosentan in both paper I and IV. In paper III, no statistically significant effect of i.v. tezosentan, in a reduced dosage, was noted on MAP. The systemic vasodilatation caused an afterload reduction with ensuing increases in CI and S_vO_2 (IV). The effects on the pulmonary circulation were pronounced as MPAP and PVRI were efficiently and promptly reduced by tezosentan (III and IV). This finding emphasizes the pathophysiological importance of the ET-system in the pulmonary circulation during endotoxemia. Tezosentan reduced P_{cap} to levels less than 20 mmHg, a level that has been reported to be a threshold for edema formation[37-38](I and III). This might be explained by the potent effect on P_{ds}VRI which was reduced to non-endotoxemic levels (I). The changes in P_{us}VRI and P_{ds}VRI induced by endotoxemia were similar to the shifts seen in response to ET-1 and S6c challenge (I). These findings support the notion that endotoxemia-induced changes in pulmonary vascular resistance are mediated, at least partly, by ET-receptor stimulation. In paper III tezosentan i.v. failed to affect the relation between P_{us}VRI and P_{ds}VRI. This discrepancy may in part be explained by the significantly higher dose of tezosentan used in paper I. Moreover, the downstream

predominance seemed to wear off by 5 h, suggesting that this might be a transient phenomenon and that an earlier intervention might induce a more prominent effect in the used endotoxin model.
Tezosentan administered intravenously in higher and prolonged doses has previously been shown to improve oxygenation in endotoxemia[191]. However, in the present experiments only a limited improvement in gas-exchange was achieved by tezosentan treatment. This difference could possibly be attributed to differences in the degree of gas-exchange disturbance compared to the previous study.

Effects of systemic dual ET-receptor antagonism on HBP and EVLW during endotoxemia (IV)

No significant increase of plasma-HBP was seen in response to surgery during the preparation of the animals. In contrast, endotoxin infusion induced a three-fold increase of plasma-HBP after three hours, reflecting a significant activation and degranulation of PMNs. However, as plasma-MPO was unchanged, this degranulation seems to be partly selective. Possibly, this difference could be due to the different granule subsets which contain these proteins. The increase in HBP was similar to what has been shown in patients with septic shock[164]. Simultaneously with the increase in HBP, hemoglobin concentration as well as $EVLWI_{STID}$ increased. This suggests that endotoxin induced a vascular hyperpermeability with substantial leakage of plasma water to extravascular tissue. Systemic tezosentan-treatment counteracted these changes; markedly attenuated endotoxin-induced elevation of plasma-HBP levels, reduced $EVLWI_{STID}$ and hemoglobin concentration. Concurrently, CrsI increased and gas-exchange tended to improve, indicating that pulmonary edema was reduced. After termination of the experiment, gravimetrical analysis of EVLWI confirmed these findings. Together these results suggest that tezosentan, directly or indirectly, reduces plasma-HBP and simultaneously attenuate formation of pulmonary edema. To clarify whether these phenomena are results of indirectly or directly acting mechanisms, experiments using isolated preparations would be of value.

Effects of inhaled dual ET-receptor antagonism during endotoxemia (III)

In paper III, tezosentan was delivered as a bolus inhalation in two different doses, 0.05 and 0.5 $mg \cdot kg^{-1}$. Inhalation increased plasma concentrations of tezosentan in a dose dependent manner, where low-dose inhalation led to plasma concentrations that were

approximately 10-fold lower than those achieved with the higher dose. Furthermore, the concentrations after i.v. treatment were almost 20-fold higher (714 versus 37 $ng \cdot mL^{-1}$) than after inhalational treatment of an equivalent dose. As intra-pulmonary clearance of tezosentan is unlikely, this difference suggests that only a minor portion of the substance reached parts of the lungs were systemic uptake could take place. However, although the amount of inhaled tezosentan that reached the systemic circulation was very low, the biological effects were highly significant, even for the lower inhalation-dose. Both inhaled doses distinctively reduced MPAP and PVRI. In addition, a dose-dependent difference in response was noted for P_{cap} where only the higher inhaled tezosentan dose had significant effects. The effect was marked and P_{cap} was reduced to levels less than the reported threshold for edema formation. $EVLWI_{STID}$ were reduced by both systemic and inhaled tezosentan, but only inhaled treatment counteracted the endotoxin-induced reduction in respiratory compliance. Part of this effect could be due to counteraction of ET-1 mediated bronchoconstriction[192], possibly inhalation may be more efficient in reaching bronchial ET-receptors as compared with systemic treatment.

There was no significant improvement in gas-exchange parameters by inhaled tezosentan in our endotoxin-model. Other investigators have reported improved oxygenation in porcine, bronchial lavage-induced, lung injury using inhaled selective ET_A-receptor antagonist[193]. However, direct comparisons are problematic as the intervention in that study was started immediately after induction of lung injury and given during one hour. Moreover, endotoxin-induced lung injury induces a more limited and unpredictable degree of gas-exchange disturbance than the lavage induced model. This may have contributed to the lack of effects on oxygenation in response to inhaled tezosentan treatment.

Pharmacological reactivity of freshly isolated pulmonary vessels (I and II)

Myographic experiments on isolated, non-incubated, porcine pulmonary vessels revealed a relatively diversified pattern of responses to pharmacological stimuli. α_1 adrenergic stimulation with PHE induced a more than 5-fold stronger contraction in pulmonary arteries than veins.

ET_B receptor stimulation with S6c had a predominant constrictive effect in pulmonary veins, whereas only a minor response was noted in pulmonary arteries. This finding, which is in line with previous reports[194,195], suggests that ET_B receptor mediated effects

could be a determinant of differential responses to ET in pulmonary arteries and veins. The differential effects of the ET system on the pulmonary vascular tone have previously been investigated in various species, partly with conflicting results. In humans[196] and in other spices[197,198], the pulmonary vein has been suggested to be more sensitive than the artery to ET-1 induced constriction. However, other authors have found no difference[194,199]. Our findings do not give the final answer due to partly conflicting results. In paper I, there was moderate but significant difference between pulmonary arteries and veins, with a predominant constriction in veins in response to ET-1 stimulation. This finding could not be repeated in paper II where we found no difference in response to ET-1 stimulation. The causes of this discrepancy are unclear but differences in vessel size could be one possible explanation.

Effects of endotoxin on vascular reactivity and ET-receptor expression in vitro (II)

The myographic experiment on isolated vascular preparations showed that endotoxin induces differentiated effects on porcine pulmonary arteries and veins. In this setting, endotoxin caused a lowered response to α_1-adrenergic stimulation, with a predominant effect in arteries. Concurrently, endotoxin induces a relative augmentation of the contractile response to both ET-1 and S6c in pulmonary veins, without altering any apparent expression or distribution of ET-receptors. Taken together, these endotoxin-induced changes in arterial and venous contractile responses to the adrenergic and ET pathways can, especially in states with increased sympathetic activity and high ET levels, influence the balance in tone between pulmonary arteries and veins. In turn, these disturbances could lead to an increase in P_{cap} and edema formation, particularly when capillary permeability is increased and intravascular oncotic pressure is reduced.

Effects of ET-receptor subtype stimulation in vivo (I and IV)

Infusion of ET-1 in non-endotoxemic animals induced a marked increase in systemic and pulmonary vascular resistances, reflecting the powerful vasoconstrictive properties of ET-1 (I and IV). Simultaneously P_{cap} was increased with a predominant rise in P_{ds}VRI (I). Measurements of P_{cap} during S6c infusion led to marked increases in P_{ds}VRI. However, as S6c simultaneously decreased CI, P_{cap} was less augmented by S6c than ET-1 (I).

ET-1-challenge increased plasma levels of HBP in a dose-dependent manner, to levels more than three-fold over baseline values. Concurrently, C_{rs}I was reduced and Hb

tended to increase. These results suggest that ET-1 potentially increases both the pulmonary capillary hydrostatic pressure and, *via* HBP and possibly other mediators, the permeability of the pulmonary capillaries. In addition, reports from other authors have suggested that ET-1 also might interfere with alveolar fluid clearance[200,201]. Together, these results suggest that ET-1 can promote edema formation through some quite diverse mechanisms. In our experiments, EVLW was slightly increased (8.2±1.1 $mL \cdot kg^{-1}$) after ET-1-challenge compared to previously reported values (6.6±0.2 $mL \cdot kg^{-1}$) from sham experiments[202]. However, the gravimetrical measurement was performed 90 min after termination of the ET-1 infusion and it cannot be excluded that some reabsorption of EVLW occurred during this time period. Continuous measurements of EVLW with STID-technique might have given additional information but were not performed in these experiments.

ET-1 has previously been reported to increase capillary permeability in isolated lung preparations, changes that required the presence of leukocytes and plasma[203], suggesting that the permeability promoting effect of ET-1 is mediated through leukocyte-dependent factors. In our study, infusion of ET-1 induced a rapid, pronounced and dose-dependent increase in HBP-levels. After termination of the ET-1 infusion, HBP tended to decrease quite rapidly. Our interpretation is that ET-receptor stimulation triggers a reaction which subsequently activates PMNs and promptly induces secretion of HBP. Whether this reaction is directly mediated *via* stimulation of ET-receptors on PMNs, or indirect through other inflammatory mediators, remains to be clarified. Moreover, as ET-1 is a dual ET-receptor agonist these experiments do not answer which ET-receptor subtype that is responsible for the increase in HBP. Preliminary and unpublished data from our laboratory show that also infusion with the selective ET_B-agonist S6c increases plasma levels of HBP. This suggests that the ET_B-receptor is involved in the process leading to HBP release. However, as only the expression of ET_A-receptors has been described in human PMNs[204] indirect mechanisms may well be involved in the interaction between ET-receptor stimulation and HBP-release. To clarify the effect of the two ET-receptors on HBP secretion further, *in vivo* and *in vitro* studies using selective antagonists and agonists are needed.

Effects of α1-adrenergic stimulation in vivo (I)

Infusion of the selective α_1-adrenergic agonist phenylephrine (PHE) increased P_{cap}. However, as PHE also augmented PAOP and relatively increased MPAP more than

P_{cap}, analysis of vascular resistances showed that PHE predominately increased $P_{us}VRI$. Systemically, PHE increased MAP while CI was unchanged. The increase in P_{cap} was significant but P_{cap} did not reach the alleged threshold for hydrostatic edema formation. Measurements of lung water would have been interesting in these experiments but was not performed as edema formation was not of primary interest in that study.

SUMMARY AND FUTURE PERSPECTIVES

In summary, our findings show that endotoxemia predominately increases pulmonary downstream resistance which subsequently augments pulmonary capillary hydrostatic pressure. In addition, endotoxin induces a degranulation of PMN which increases plasma levels of permeability and edema promoting HBP. These endotoxin-induced changes in capillary filtration pressure and levels of permeability promoting mediators increase pulmonary edema. Systemic treatment with tezosentan, a dual ET-receptor antagonist, markedly counteracts these changes and reduces pulmonary edema. In addition, inhaled tezosentan efficiently and selectively reduces pulmonary hypertension during endotoxemia. Taken together, these results show that the endothelin system is extensively involved in the pathophysiology of endotoxin-induced lung injury. These findings need to be further elucidated, in other experimental conditions and in humans.

As numerous large scale trials has failed to translate promising therapeutical interventions from animal sepsis models to the clinical setting, the hunt for new treatments of sepsis has been called "the graveyard of pharmaceutical companies" [205]. The difficulties in transferring effective candidate treatments to the septic patient reflect the complexity of the syndrome and possibly the shortcomings of the often used animal models. However, despite these difficulties, research on the mechanisms of sepsis is highly relevant and new treatments are needed. To increase the probability of successful transfer of novel treatments from animals to patients, candidate therapeutics need to be evaluated in different models of sepsis and when clinically tested, patient selection and timing of treatment is of greatest importance. These factors would undoubtedly be of high significance when it comes to the possible future use of ET-antagonists in sepsis and lung injury. Selection of patients is then crucial but this increase the difficulty with adequate sizing in the studies. In addition, as the course of sepsis is a highly dynamic, proper timing of intervention is of marked importance. Another question is dosing and the way of administration of ET-antagonists. Our results suggest that inhaled therapy permits treatment in reduced dosages, especially if

the primary aim is to reduce pulmonary hypertension. Naturally, the safety of inhaled therapy with ET-antagonists needs to be fully ascertained before trials in patients can be performed.

CONCLUSIONS

- Selective ET_B-receptor stimulation increases pulmonary vascular down-stream resistance and pulmonary capillary pressure *in vivo*.

- Selective ET_B-receptor stimulation constricts isolated pulmonary veins more than arteries *in vitro*.

- Endotoxin-infusion predominantly increases pulmonary down-stream resistance and increases in pulmonary capillary pressure *in vivo*.

- Endotoxin increases the constrictive response to selective ET_B-receptor stimulation in pulmonary veins and reduces the constrictive response to adrenergic α_1 receptor stimulation in pulmonary vessels *in vitro*.

- Endotoxin incubation does not change ET-receptor subtype expression or distribution in isolated pulmonary vessels.

- Systemic dual ET-receptor antagonism reduces pulmonary capillary pressure and pulmonary downstream vascular resistance during endotoxemia.

- Inhaled dual ET-receptor antagonism efficiently and selectively counteracts endotoxin induced pulmonary hypertension.

- Systemic dual ET-receptor antagonism reduces plasma levels of edema promoting heparin-binding protein and formation of extravascular lung water during endotoxemia.

- ET-receptor stimulation increases extravascular lung water and dose-dependently increases plasma levels of edema promoting heparin-binding protein.

ACKNOWLEDGEMENTS

I am deeply grateful for the opportunity to write this thesis. On the way I have met many fascinating people, seen interesting milieus, countries, restaurants and last but definitely not least, learned enormously. In particular, I would like to express my profound gratitude to these people who have made this journey possible:

My tutor, Associate Professor Anders Oldner, for being a great mentor, colleague and near friend. For excellent, and virtually daily, guidance in everything from the latest, must have, smartphone applications, to science, in ups and downs and always with intelligence, humor and total dedication.

Patrik Rossi, tutor and mentor during my residency and chief of ICU, Karolinska University hospital, Huddinge. Thank you for introducing me to experimental science and for sharing your unlimited knowledge and experience in medicine and house renovations as well as your friendship.

Professor Eddie Weitzberg, tutor, for being an impressing innovative and brilliant scientist and especially a really cool guy.

Professor Anders Arner, tutor and head of Section of genetic physiology, KI. I am truly enormously tankful for all support and your enthusiasm that made our collaboration fruitful.

Co-authors, Piet Boels, Professor Lennart Lindbom and Cecilia Lövdahl for innovative and productive teamwork and for showing the many benefits of translational research.

Professor Martine Clozel at Actelion Pharmaceuticals Ltd, Allschwil, Switzerland, for the generous gift of tezosentan.

Professor Lars I. Eriksson, Chair of research at the Department of Anesthesia, Surgical services and Intensive care medicine (ANOPIVA), Karolinska University hospital, Solna. Thank you for your endless enthusiasm and dedication to research and for creating an academic atmosphere that creates ideal conditions for doctoral studies at our section.

Former and present Heads of the ANOPIVA department, Professor Sten Lindahl, Associate Professor Lars Irestedt, Professor Claes Frostell and Eva Bågenholm for promoting an academic approach in the everyday clinical work and for preserving “the Torsten Gordh-spirit” at our department.

Former endothelin/pig researcher, David Konrad, chief of central ICU, ANOPIVA for all encouragement, support and friendship through the years.

Michael Wanecek, also he a legendary endothelin/pig researcher, for sound clinical discussions and great friendship, not at least during travels and gastronomical experiences.

Associate Professor Claes-Roland Martling, Medical director, New Karolinska, Solna, for introducing me to our department and to the World of intensive care medicine. I cannot imagine how my life would look like today if we did not meet back in 1998.

My former boss, Kristina Hambraeus-Jonzon, for your support and interest in my project, and for giving me the opportunity to combine my residency with research.

Johan Peterson, for sharing your vast clinical knowledge and your friendship through the years.

Kirsi Dolk, for your nearly mystical skills in planning the detailed scheme for ANOPIVAs over 100 doctors and for allowing me to focus on this thesis during the recent months.

Margareta Stensdotter, Annika Olsson, Carina Nihlén and Annette Ebberyd for your outstanding laboratory work and never ending expertise in solving everyday lab hitches.

All dear coworkers at ANOPIVA, for treating and nursing the patients instead of me while I had “F-KID”. I am looking forward to see you more often from now on.

My fellow colleagues at KI – Jessica Kåhlin, Malin Ax, Daniel Gustavsson, Karin Eriksson, Eva Christensson, Andreas Wiklund, Malin Jonsson-Fagerlund, Jonas Blixt, Anna Hårdemark-Cedborg, Åsa Konradsson-Geuken, Ingeborg Inacio and Anette Ebberyd (again) for all discussions and laughs during numerous coffee breaks.

All my dear friends from Viksjö, for 30 years of friendship and almost as many years of unpretentious, down to earth, beer drinking.

My parents in-law, Eeva-Liisa and Jouko Pakarinen for all love and support during huge home renovations and long working hours.

My incredible uncle Göran Rydberg, for your humor and warmth.

My dear parents Marianne and Ulf Persson for teaching me that there is much more to life than molecules, pressure-curves and equations.

My lovely kids, Klara, Erik and Axel, for just being you.

My incredible and beloved wife, Mia, writing this thesis would have been impossible without your efforts; I am endlessly grateful for your patience, support and love.

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APPENDIX

Gravimetrical analysis of extravascular lung water according to Pearce *et al:*
Calculation of erythrocyte cell mass of the lung:

$$Qr = Qh \times \left(\frac{Hb_s}{Hb_b}\right) \times \left(\frac{Fw_h}{Fw_s}\right) \times Hct$$

Qr	*erythrocyte cell mass of the lung*
Qh	*weight of lung homogenate*
Hb_s	*hemoglobin concentration of supernatant*
Hb_b	*hemoglobin concentration of whole blood*
Fw_h	*fractional water content of homogenate (= (wet - dry) / wet)*
Fw_s	*fractional water content of supernatant*
Hct	*hematocrit of blood sample*

Residual blood content of the lung is the sum of erythrocyte cell mass and plasma mass:

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

Qb	*residual blood content of the lung*

Extravascular lung water content of the lung:

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

Qwl	*extravascular lung water*
Fw_b	*fractional water content of blood*
Qwt	*weight of water added during homogenization*

Calculations of pulmonary vascular resistances:

$$PVRI = \frac{MPAP - PAOP}{CI}$$

$$PusVRI = \frac{MPAP - Pcap}{CI}$$

$$PdsVRI = \frac{Pcap - PAOP}{CI}$$

PVRI	*pulmonary vascular resistance index*
MPAP	*mean pulmonary artery pressure*
PAOP	*pulmonary artery occlusion pressure*
CI	*cardiac index*
Pcap	*pulmonary capillary pressure*
PusVRI	*pulmonary upstream vascular resistance index*
PdsVRI	*pulmonary downstream vascular resistance index*