The microvascular endothelial cell in shock

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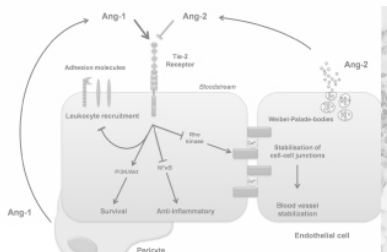
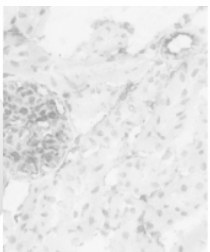
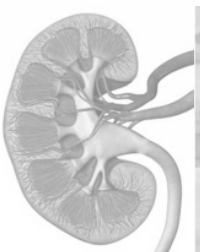
Chapter 1

General introduction & Aim of the thesis




This thesis focuses on endothelial behavior in critical illness. The research questions originated at the bedside of the critically ill shock patient, were researched in the laboratory, and eventually have to end at the bedside of the critically ill shock patients. This introduction was written to introduce the basic researcher to the critically ill shock patient and to introduce the practicing intensivist to the endothelial biomedicine research field. Interested readers from either field are referred to excellent text books on endothelial biomedicine[1] and critical care[2] for more in depth reviews on both topics.

1.1 General introduction

1.1.1 History of critically ill patients and the ICU

'*A sharp nose, hollow eyes, collapsed temples; the ears cold, contracted, and their lobes turned out: the skin about the forehead being rough, distended, and parched; the colour of the whole face being green, black, livid, or lead-coloured. ……. it is to be known for certain that death is at hand.*'[3]. To Hippocrates, one of the founders of western medicine, the previous sentence was a description of imminent death. For the modern intensivist the first part is an indication of shock. It denotes the start of resuscitation and other active interventions, which, hopefully, will transform imminent death into survivable critical illness.

Critically ill patients are patients that would die without the help of machines and life support measurements. From the description by Hippocrates, it can be concluded that critical illness was a syndrome recognized already by the ancient Greeks. Critical care historically evolved from the recognition that the needs of patients with acute, life-threatening illness or injury could be better treated if they were grouped into specific areas of the hospital, leading to the so called Intensive Care Units (ICUs). ICUs roots can be traced back to the Monitoring Unit of critical patients initiated by nurse Florence Nightingale. During the crimean war, when Britain, France and Turkey were at war with Russia (1854), Nightingale started these monitoring units. Before Nightingale, the lack of critical care and the high rate of infection, resulted in a high mortality rate of hospitalized soldiers, reaching as high as 40% of deaths recorded during the war. Nightingale described her set of nursing rules as a 'critical care protocol'. She stated that '*proper use of fresh air, light, warmth, cleanliness, quiet, and the proper selection and administration of diet*

– *all are at the least expense of vital power to the patient*'[4]. When Nightingale left for the battlefields in Turkey, she took this 'critical care protocol' with her. After introducing the protocol, the mortality rate fell to 2%[5].

In 1947, the polio epidemic raged through Europe and the United States, leaving patients paralysed, and posing a new medical challenge, which resulted in a breakthrough in the treatment of patients dying from respiratory paralysis with mechanical ventilators. In Denmark, manual ventilation was accomplished through a tube placed in the trachea of the polio patients, as these patients with respiratory paralysis required intensive nursing care. During the 1950s, the development of mechanical ventilation led to the organization of respiratory intensive care units in many hospitals[6]. The creation of these first surgical ICUs was a natural consequence of advancement in resuscitation and support of severely ill or traumatised patients. Leaving patients alive that would have died in previous times, critical illness itself can be seen as a iatrogenic disorder[7]. After the start of ICUs being a place where mechanical ventilation is applied to man, further techniques for organ support and replacement were developed. For example, haemodialysis developed by Koch during the Second World War, was adapted for acute kidney injury during the Korean war[8; 9]. From here, critical care has developed, with patients that could benefit from ICU interventions, specialized nurses and doctors, a well defined knowledge domain, and research on all aspects of critical care.

1.1.2 The disease: classification of shock and the definition of multiple organ dysfunction

Shock is classically defined as a systemic reduction in tissue perfusion, resulting in decreased tissue oxygen delivery. Four types of shock are being recognized[10]:

- Hypovolemic shock is a consequence of decreased preload of fluid to the heart due to intravascular volume loss. One of the main causes of this shock is massive bleeding (hemorrhagic shock, HS).
- Distributive (vasodilatory) shock is a consequence of severely decreased vascular resistance. One of the main causes of this type of shock is sepsis (septic shock), in which the host responses to host to infection is deranged.
- Cardiogenic shock is a consequence of cardiac pump failure.
- Obstructive shock is an obstruction to the heart that leads to malfunctioning. It can result from conditions such as tension pneumothorax, cardiac tamponade,

and massive pulmonary embolism.

Often the different types of shock are present in one patient[10]. As an example, patients with septic shock often have a hypovolemic component due to vomiting or diarrhoea. Regardless of the type of shock a physiologic continuum of signs and symptoms is present in every patient. Shock begins with an initiating event, such as an infection (e.g., an abdominal infection) or an injury (e.g., traumatic blood loss). This initiating event produces a systemic circulatory abnormality that may progress through several complex stages. The first stage is compensated shock which is characterized by rapid compensation for diminished tissue perfusion by various homeostatic mechanisms. The first clinical sign is tachycardia and peripheral vasoconstriction. The second stage is the shock stage, during which the compensatory mechanisms fall short and signs and symptoms of organ dysfunction appear. These signs include tachycardia, hypotension, oliguria, dyspnoea, altered mental status (restlessness, fear of dying), metabolic acidosis, and a cool and clammy skin. The third stage is characterised by organ dysfunction, in which progressive organ dysfunction leads to organ damage and, if not adequately treated, death[10].

1.1.3 Therapy of shock

Patients with all kinds of shock are treated in ICUs. Therapy for haemorrhagic shock seems rather simple - asking the surgeon to `plug the hole' and adding the lost circulating blood volume. The hemorrhagic shock related mortality and morbidity in ICUs has hence shifted away from the early deaths of massive bleeding in the last century towards patients with multiple failing organs. Advancement in shock resuscitation has furthermore occurred during military conflict because of the large number of patients during war. Shock resuscitation is an intervention that has changed the epidemiology of deaths from hemorrhagic shock[9]. Initially patients did not receive any resuscitation and died at the battlefield. In later years patient survived because of initial resuscitation, but they developed single organ failure, with acute renal failure being the first major problem. In later days the acute respiratory distress syndrome (ARDS) became manifest. With the introduction of intensive care units these single failing organs were supported with renal replacement therapy and mechanical ventilators. With support for the lung and the kidney patients did not die of single organ failure anymore but developed

multiple failing organs. Although the mortality of multiple organ failure is decreasing, it remains the major cause of prolonged ICU stay[9].

Therapy for septic shock seems more difficult, as the normal host response to infection is complex. Severe sepsis is defined as sepsis with more than one sign of organ failure[11]. Septic shock is the most severe spectrum of this continuum being defined by severe sepsis with refractory hypotension. While severity increases from sepsis to severe septic shock, the mortality also increases from 15% to 45%[12]. The host responds in such a way that it localizes bacterial invasion and starts repair of injured tissue. This inflammatory process is normally accompanied by activation of immune cells, production of factors that become systemically available, endothelial cell activation and many other processes. Sepsis arises when the inflammatory response to infection becomes generalized and extends to involve normal tissues remote from the initial site of injury or infection. As this thesis mainly focuses on hemorrhagic shock and septic shock, the other causes of shock are not further described.

1.1.4 MODS: theories and therapies

The concomitant failure of multiple organ systems after shock was first described in 1969 by Skillmann et al[13]. Arthur Baue placed this failure of multiple organs in its context in an editorial in which he wrote: '*However, it seems that the major limiting factor after injury in patients who do not have brain injury is not so much a system, but rather a combination of events that can best be called multiple systems failure, progressive systems failure, or sequential systems failure'*. Although not a well defined syndrome, it involves progressive failure of many or all systems after an overwhelming injury or surgery. Nowadays there are no direct therapies to reverse multiple organ dysfunction syndrome, and organ support is initiated to borrow time for the body to recover[14].

Patients admitted to the ICU with multi-organ dysfunction syndrome (MODS) have a high mortality. Surprisingly, this high mortality does not primarily seem to depend on whether the underlying condition is septic shock, hemorrhagic shock or another insult[15]. After treating the underlying condition, therapy is often focused on maintaining physiological parameters such as blood pressure, oxygenation and acid-base status, glucose and electrolyte levels within certain boundaries by the application of vasopressors, dialysis, and mechanical ventilation. These measures are not intended to

treat the condition underlying MODS, so they should be viewed as supportive. Optimizing these supportive therapies can therefore only prevent mortality due to complications and provide the necessary time for recuperation.

The original hypothesis that MODS was caused by tissue hypo-perfusion and oxygen debt has been challenged. In the last 30 years several other theories on the aetiology of MODS have been postulated. Some of these theories are summarized in table 1.1.

These different theories have led to several etiologic and molecule based therapies, especially for sepsis induced MODS[17]. Unfortunately, apart from early, goal-directed therapy, lung-protective ventilation, antibiotics, possibly activated protein C, and treatment with selective gut decontamination[18; 19], no specific therapy has been shown valuable so far in MODS[17]. A major obstacle in treating patients with MODS is that, at present, we still do not understand the precise underlying pathophysiological mechanisms. A wide variety of individually complex systems including immunological status, inflammatory activity, coagulation, cell survival, cell metabolism, paracellular

Table 1.1. Derangements in the host as etiological factors for MODS. Presumed pathology and (clinical) manifestations, adapted from[15].

Pathologic Process	Manifestations
Tissue Hypoxia	Increased lactate
Uncontrolled infection	Nosocomial ICU-infection
Systemic inflammation	Cytokines (TNFα,IL-6,IL-8); Leukocytosis
Immune paralysis	Increased anti-inflammatory cytokines (IL-10)
Endothelial activation	Leukocyte adherence, increased capillary permeability
Macrovascular disturbances	Hypotension
Microvascular disurbances	Decreased microvascular flow
Dysregulated apoptosis	Decreased neutrophil apoptosis
Gut-liver axis	Increased infection, Kupffer cell activation
Mitochondrial dysfunction	Cellular energetics are deranged in sepsis
Production of allarmins	Injured tissues and cells release endogenous danger signals

leakage, and cell motion have been associated with MODS. All of the above mentioned systems represent dynamic processes that are closely interconnected, making a concise description of MODS an almost intractable knot[20-23]. In infection related MODS, enormous efforts have been made to find options to intervene in the cascade of events leading from infection to MODS to death. However, therapies directed at crucial points in the inflammatory and the coagulation cascades have not yet resulted in therapeutic options[21; 22]. Many interventions have been tried but it remains unclear whether the interventions are intrinsically ineffective, or whether the manner in which these drugs have been tested has been inadequate. With new promising therapeutic targets recently entering the stage, a truly translational research approach might lead to increased knowledge on the pathophysiological mechanisms underlying MODS, and help to prevent failure of phase III clinical trials[24]. Opinion leaders in intensive care medicine already suggested to abandon the randomized controlled trial in the intensive care unit[25]. Some aspects of this translational approach are discussed in the **Future Perspectives** chapter of this thesis.

1.1.5 MODS the vasculature and the endothelium

The reason to focus on blood vessels to unravel part of the pathophysiology of MODS can be found in the clinical hallmarks of the often preceding shock. In shock there is hypotension, vascular leakage and leukocyte influx into organs. All these processes are regulated or facilitated by the vasculature: hypotension by diminished vascular contraction, vascular leakage by increased vascular permeability, and leukocyte influx into organs by attraction of the cells by the activated endothelial cells.

The history of the knowledge of the vascular system and the vascular wall is paved with notable names in medicine. The theory of Galen of Pergamon (129-200 A.D.) which describes the vasculature in which veins and arteries were separated, arteries transporting air into the tissues and veins transporting blood, lasted for more than 1,000 years. William Harvey was the first to describe the circulation of the blood from arteries to veins in 1628. Using light microscopy, Malpighi next discovered the existence of capillaries which connected the arterial and the venous part of the circulation. In 1839, Theodor Schwann was the first to describe the cells that line capillaries, in later years called the endothelium. The interested reader is referred to an excellent review on the history of the discovery of the capillary wall by Hwa and Aird[26].

The different parts of the circulation perform different functions in health and disease. The large arteries and veins can be seen as conduit vessels, while the smaller vasculature has more specific functions[27]. Vasomotor tone is mainly regulated by arterioles, whereas permeability is regulated in capillaries and postcapillary venules. Leukocyte transmigration takes place in capillaries and postcapillary venules[28]. In different organs, specific anatomical parts of the circulation have specific functions, as reviewed in[27; 28].

The endothelium forms the inner cellular lining of all blood vessels and is a major regulator of vessel specific functions[29]. Endothelial cells do not act as an inert lining of the blood vessels but as active regulators and can be seen as input-output devices[27]. The endothelium plays an important role in many physiological functions, including in the control of vasomotor tone, leukocyte trafficking, haemostatic balance, regulation of vascular permeability, regulation of angiogenesis (figure 1.1), and innate and adaptive immunity.

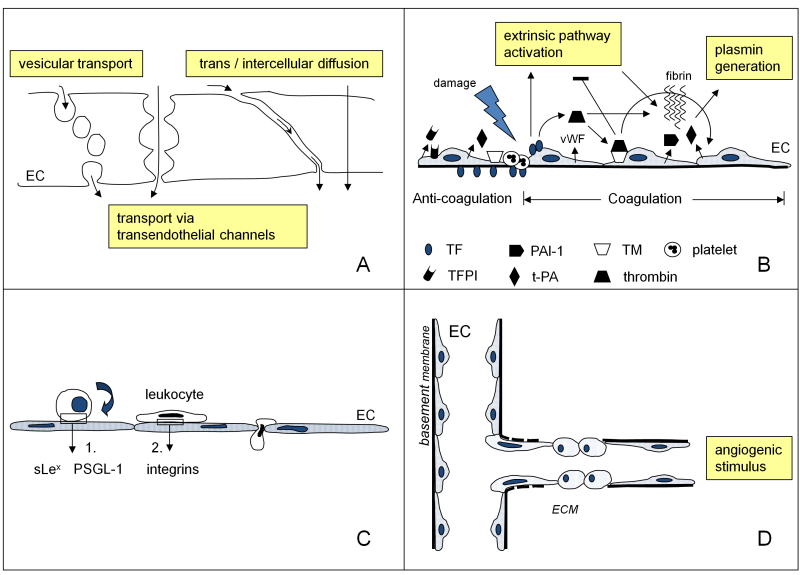


Figure 1.1. Schematic presentation of the major functions of microvascular endothelial cells.
(A) The endothelium forms a semi-permeable barrier for the transport of substances in the blood to the underlying tissue. (B) The endothelium regulates the expression of pro- and anticoagulative substances. (C) The endothelium expresses a variety of cellular adhesion molecules to tether and activate leukocytes and facilitate leukocyte adhesion and transmigration from the blood into underlying tissue. (D) The endothelium actively engages in angiogenesis in wound healing,tumour growth, as well as in a number of physiological processes. EC, endothelial cells; PAI-1, plasminogen activator inhibitor; PSGL-1: P-selectin glycoprotein ligand; sLex, sialyl Lewis x; TFPI, Tissue Factor pathway inhibitor; TM, thrombomodulin. Modified from Griffioen and Molema, with permission[28].

The endothelium is involved in most, if not all, disease states, either as a primary determinant of pathophysiology or as a direct and/or indirect responder to an inciting stimulus[27; 28]. Clinicians rarely examine the status of this cell layer, as it is not amenable to traditional physical diagnostic manoeuvres of inspection, palpation, percussion and auscultation. From a laboratory standpoint, the endothelium sheds several factors and even cells can release from the monolayer, but local organ specific reactions cannot (at present) be unmasked in this way. The differences between endothelial cells in different places in the body, the so called endothelial heterogeneity, and the fact that endothelial cells lose their vascular bed specific phenotype when cultured *ex vivo* and *in vitro*, furthermore makes that this cell layer is difficult to investigate in humans.

There is increasing evidence that the endothelium plays a central role in the pathophysiology of shock[30-32]. Endothelial cells are diverse in function and highly responsive to their extracellular environment[33]. When exposed to certain mediators that circulate in shock states, such as lipopolysaccharide, cytokines, chemokines and growth factors, endothelial cells become activated[34]. The activation state is manifested by enhanced vascular permeability, increased leukocyte adhesion, a shift in the haemostatic balance towards pro-coagulation, and altered regulation of vasomotor tone. This activated state is considered dysfunctional when an overactive endothelium is not capable of restoring the homeostatic state, thereby causing a detrimental effect to the host. In sepsis, endothelial activation and dysfunction are critical determinants of the host response[35]. Sepsis has a vascular phenotype, and in mice organ specific pro-inflammatory endothelial activation can be observed[31]. The role of the endothelium as a mediator of damage to the host can be studied in sepsis models in endothelial specific KO mice. The vascular phenotype in sepsis is mediated by pro-inflammatory endothelial signalling pathways, including the pro-inflammatory NF-κB signal transduction system. Experimental studies using an endothelial specific knock out of NF-κB support the paradigm of the essential role of the endothelium as mediator of septic multiple organ failure[36].

Because of its strategic localisation at the interphase of blood and organs, the endothelium represents a direct possibility of communication to the various organs in the body[37]. Furthermore, the endothelium is an attractive therapeutic target as it has a highly variable phenotype which is amenable to therapeutic modulation with drugs[38].

The endothelial heterogeneity, in which endothelial reactions are different in different parts of the body, could even be used to deliver drugs at specific inflamed sites of the circulation[38-40]. Noteworthy is the fact that the microvascular endothelial compartment represents a pharmacologically neglected target for therapeutic intervention in inflammatory diseases such as sepsis, as the exact effects of drugs on these cells in the organs are almost completely unknown.

1.1.6 Pro-inflammatory endothelial activation, leukocyte recruitment and vascular permeability in shock

Leukocyte recruitment is a process that should be executed in any organ upon demand in response to invading organisms or damage (figure 1.2). During MODS there is an increased leukocyte influx into organs. In almost all organs, the preferred site for leukocyte transmigration into the underlying tissue upon an inflammatory challenge is the post-capillary venule, in which the endothelial cells form tight junctions between each other[27]. The dimensions of leukocytes fitting narrowly in the post-capillary venules make that both cell types physically interact, allowing efficient intercellular communication[41].

Leukocytes must engage in several sequential steps to leave the circulation. Upon an inflammatory insult, one of the first reactions of the endothelial cells is to exocytose the stored, ready-to-release contents of Weibel-Palade bodies. These include the blood coagulation factor von Willebrand factor (VWF), the adhesion molecule P-selectin, and the Tie2 antagonist angiopoietin-2[42]. By this means, a rapid interaction among the activated endothelium, platelets, and neutrophils is created that facilitates leukocyte rolling. Directly afterwards, via gene transcription, the endothelium produces E-selectin, which will be located on its outer membrane to exert its function. The E-selectin interacts with sialyl-Lewis X ligands expressed on the immune cells, leading to rolling of leukocytes on the endothelium. To come to a firm arrest, blood cells must engage in additional binding to endothelial cells. This takes place via the integrin family, specifically leukocyte function–associated antigen type 1 (LFA-1) and the α4β1 integrin (also referred to as very late antigen 4, VLA-4). These integrins bind firmly to adhesion molecules of the immunoglobulin superfamily including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on the endothelial cells, due to which the leukocytes come to an arrest. Thereafter the leukocyte migrates via the transendothelial

route into the subendothelial space and into the tissue[43]. Leukocyte binding to endothelial cells leads to intracellular reactions in both leukocytes (reviewed in [44]) and endothelial cells (reviewed in [45]). The complexity of leukocyte- endothelial binding and transmigration is beyond the scope of this introduction and will not be discussed further.

Angiopoietins (Ang1, Ang2) and Tie2 are molecules involved in the control of vascular integrity and sensitization of vascular endothelial cells to inflammatory and angiogenic stimuli. There has been increasing interest in this system which is reflected by the increasing number of research papers and reviews dealing with this system[46-49]. The Ang/Tie2 system is a signalling system involved in inflammation, coagulation, immunity, cell survival, cell metabolism, cytoskeleton alterations, and cell motion[20]. The current paradigm describes that Ang1 binding to Tie2 increases the phosphorylation status of the intracellular part of Tie2. The competitive binding of Ang2 to the Tie2 receptor decreases its phosphorylation status. From recent studies it can be speculated that Ang/Tie2 signalling system plays a crucial role in the symptoms of MODS, and that intervening in this system can be considered a promising approach towards treatment of MODS.

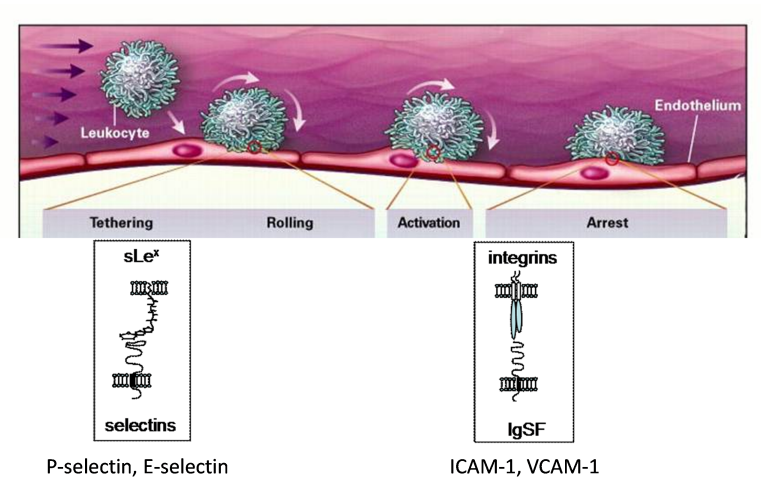


Figure 1.2. Model of endothelial leukocyte interaction in inflammation (enlargement of figure I C) adapted from von Andrian and Mackay[42] and Griffioen and Molema[28].
Endothelial cells present adhesion molecules P-selectin and E-selectin, P-selectin binds to P-selectin glycoprotein ligand (PSLG)-1 expressed on leucocytes and E-selectin binds to sialyl-Lewis X expressed on leukocytes, leading to tethering of leukocytes by the endothelium. Thereafter, integrins on leukocytes bind firmly to adhesion molecules of the immunoglobulin superfamily (IgSF) including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on endothelial cells. Thereafter, leukocytes transmigrate towards the subendothelial tissue (not shown).

In summary, endothelial cells play a vital role in tissue homeostasis. The endothelium is highly heterogeneous in time and place in the body. Moreover, the cells exert location dictated functions, and play a central role in the pathophysiology of many (inflammatory) diseases including shock and MODS.

1.2 AIM OF THE THESIS

Based on the knowledge summarized above, we hypothesised that shock induced stress (being it sepsis or hemorrhage induced) activates microvascular endothelial cells to a pro-inflammatory state, and that microvascular beds in different organs would respond differently to the same stress (figure 1.3). Understanding the nature and molecular control of these processes may allow us to identify targets for therapeutic intervention

As described in part 1.1 of the introduction, multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic shock (HS) and is related to high morbidity and mortality. Interaction of activated neutrophils and endothelial cells is considered to play a prominent role in the pathophysiology of MODS. Until now there is no insight in the kinetics and the molecular basis of endothelial cell activation during the induction of HS. This insight can assist in identifying new rational targets for (early) therapeutic intervention. In **CHAPTER 2**, we therefore examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS followed by resuscitation.

In the **CHAPTER 3** of this thesis we aimed to further study the molecular effects of mechanical ventilation on endothelial cell activation. Mechanical ventilation (MV) is frequently employed in patients with HS. Intubation and MV does protect organs from hypoxia and hypercapnia, on the other hand MV may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. We also examined the role of acute systemic hypoxia which was not induced by shock, to separate the effects of shock, hypoxia and systemic inflammation on endothelial cell activation.

In clinical critical care, the number of adipose patients is increasing and obesity is an independent, 'dose-dependent' risk factor for sepsis morbidity and mortality. Nowadays, adipose tissue is no longer considered as a storage compartment of triglycerides only, but as a highly active metabolic organ. It produces hormones and cytokines, the so called adipokines. Adiponectin is an adipocyte-derived circulating cytokine and the

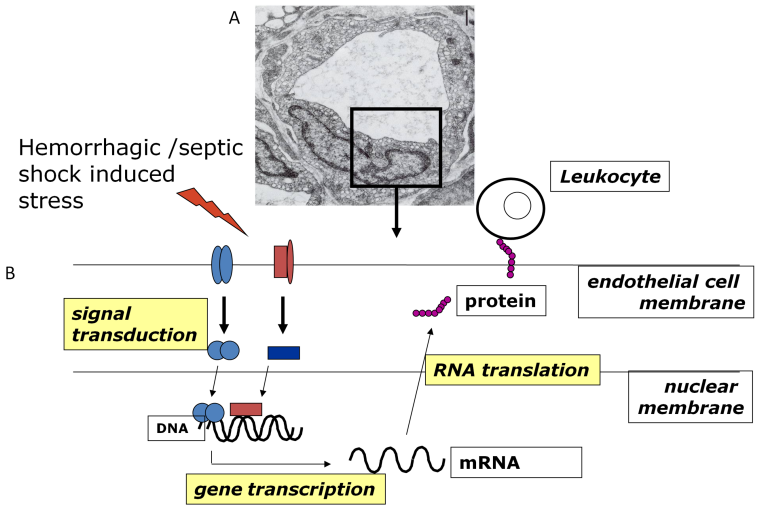


Figure 1.3. Simplified molecular view of pro-inflammatory endothelial activation in shock states.
(A) Electron microscopy image of an endothelial cell surrounding a capillary. (B) Schematic molecular drawing of the area in the black square denoted in A. Shock induced stress on endothelial cells leads to activation of gene transcription in the endothelial nucleus via (mostly unknown) signal transduction pathways. Proinflammatory genes are transcribed into mRNA, which is transported to the endothelial cytoplasm. In the cytoplasm the mRNA is translated into endothelial adhesion molecule proteins (E-selectin, P-selectin, ICAM-1 and VCAM-1, and others) and cytokines and chemokines which are expressed on the luminal side of the endothelial cell membrane respectively exocytosed. These molecules facilitate leukocyte activation and leukocyte influx into organs.

most abundant product of adipose tissue. In **CHAPTER 4**, we employed the mouse caecal ligation and puncture (CLP) model as a sepsis model, and in this study we addressed the role of adiponectine on endothelial activation/dysfunction and vascular leakage.

The Angiopoietin/Tie2 system influences inflammation, vascular leakage and leukocyte recruitment. **CHAPTER 5** describes in detail the pathophysiological mechanisms of its action in shock states. As shown in chapters 2, 3, and 4, hemorrhagic shock as well as CLP induces pronounced vascular activation in the kidney. We hypothesized that the Ang/Tie2 system might control several endothelial responses during shock states. The study presented in **CHAPTER 6** hence dealt with the role of Angiopoietin/Tie2 in MODS. We studied the effects of HS and LPS administration in mice on the Ang/Tie2 system with specific emphasis on acute kidney injury.

To bring our pre-clinical observations to the patient, we lastly studied in **CHAPTER 7** the time course of changes in circulating soluble factors of endothelial activation during human experimental endotoxemia, and human sepsis, and related these to the behaviour of the soluble factors of the Ang/Tie2 system.

In **CHAPTER 8**, we summarize the outcomes of all studies presented in this thesis and put the obtained data and new insights in perspective. The focus of the future perspectives will be on the pitfalls in the way interventions in shock and MODS research were tested in patients in the past. Furthermore we will discuss the caveat that shock and MODS are heterogenic diseases which until now are tested in homogeneous animal models. To finalize chapter 8, we draw a hypothetic roadmap for implementing endothelial cell based therapies in shock and MODS with a specific emphasis on Ang/Tie2 based therapies.

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Chapter 2

Early organ-specific endothelial activation during hemorrhagic shock and resuscitation

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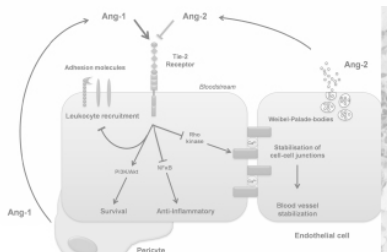

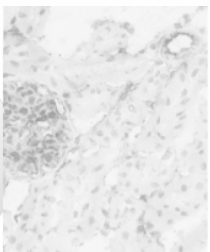

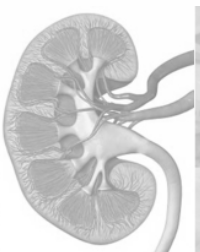

ABSTRACT

Introduction: Multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic shock (HS) and related to high morbidity and mortality. Interaction of activated neutrophils and endothelial cells is considered to play a prominent role in the pathophysiology of MODS. Insight in the nature and molecular basis of endothelial cell activation during HS could assist in identifying new rational targets for early therapeutic intervention. In this study we examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS.

Methods: Anesthetized male mice were subjected to controlled hemorrhage to a mean arterial pressure of 30 mmHg. Mice were sacrificed after 15, 30, 60 or 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a group of mice was resuscitated with 6% hydroxyethyl starch 130/0.4 (Voluven®). Untreated mice, and sham shock mice that underwent instrumentation and 90 minutes of anesthesia without shock served as controls. Gene expression levels of inflammatory endothelial cell activation (P-selectin, E-selectin, VCAM-1 and ICAM-1) and hypoxia responsive genes (VEGF-A and HIF-1α) were quantified in kidney, liver, lung, brain, and heart tissue by quantitative RT-PCR. Furthermore, we examined a selection of these genes with regard to protein expression and localization using immunohistochemical analysis.

Results: Induction of inflammatory genes occurred early during HS and already prior to resuscitation. Expression of adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial genes CD31 and VE-cadherin which function in endothelial cell homeostasis and integrity were not affected during the shock phase, except for VE-cadherin in the liver which showed increased mRNA levels. The rapid inflammatory activation was not paralleled by induction of hypoxia responsive genes.

Conclusion: This study demonstrated the occurrence of early, and organ specific endothelial cell activation during hemorrhagic shock, as presented by induced expression of inflammatory genes. This implies that early therapeutic intervention at the microvascular level may be a rational strategy to attenuate MODS.

Introduction

The development of multiple organ failure (MODS) following hemorrhagic shock is a problem in the care of patients who suffer major bleeding. MODS contributes significantly to morbidity and mortality[1]. Advances in medical care of hemorrhagic shock patients, including the introduction of resuscitation fluids, trauma centers and intensive care units, have resulted in a significant decrease in early deaths caused by hemorrhagic shock. Concomitant with recovering from a previously fatal condition, the systemic inflammatory response initiated by hemorrhagic shock became manifest[1]. The inflammatory response is nowadays considered the leading cause for the development of MODS.

Although the precise mechanisms and pathways leading to organ injury after hemorrhagic shock are still unknown, the neutrophil is thought to be a principal cellular mediator of tissue damage. Migration of neutrophils into tissue during hemorrhagic shock leads to significant organ damage through release of proteases and oxygen derived radicals[2]. The interaction between neutrophils and endothelium is instrumental in the migration of neutrophils into different tissues[3]. This migration is regulated by adhesion molecules on both leukocytes and endothelium, the latter including P-selectin, E-selectin, VCAM-1, and ICAM-1.

Most studies concerning MODS following hemorrhagic shock have focused on the post resuscitation period, in which the shock is compensated by volume infusion. A few studies showed the occurrence of early cytokine production and activation of intracellular signaling pathways which are well established triggers of endothelial cell activation[4-7]. Increased expression of P-selectin in liver and lung after 90 minutes of HS induction implicates early endothelial responses[8], which might be induced by hypoxia[6; 9]. Based on these studies we hypothesized that hemorrhagic shock rapidly activates endothelial cells towards a proinflammatory status. Early endothelial activation could imply options for an early therapeutic window. Considering the organ specific involvement in HS associated MODS, this activation likely presents differently in different organs. It is imperative to unravel the kinetics and pathophysiological mechanisms of endothelial activation in order to develop specific therapeutic strategies that can either prevent or attenuate the effects of hemorrhagic shock on organ function, and related morbidity and mortality. The aim of

this study was to investigate the kinetics, profile and organ specificity of endothelial cell activation during the earliest phase of hemorrhagic shock. We employed a mouse model of controlled arterial pressure of 30 mmHg during a time period of 90 minutes after which designated groups of mice were resuscitated. We examined the expression levels of a series of adhesion molecules (P-selectin, E-selectin, VCAM-1 and ICAM-1) known to contribute to leukocyte recruitment, hypoxia related genes HIF-1α and VEGF-A, and the proinflammatory cytokine TNF-α, by quantitative RT-PCR. Immunohistochemistry was furthermore performed to detect endothelial adhesion molecule and HIF-1α proteins. Besides information on the extent of activation, this approach provided details regarding the microvascular location of endothelial activation within the organs.

Materials and methods

Animals

Eight to twelve-week-old C57Bl/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-hr light/dark cycle. All procedures performed were approved by the local committee for care and use of laboratory animals, and were performed according to strict governmental and international guidelines on animal experimentation.

Hemorrhagic shock model

Mice were anesthetized with isoflurane (inspiratory 1.4 %), N_20 (66%) and 0_2 (33%). Throughout the experiment mice were breathing spontaneously. The animals were kept on a temperature controlled surgical pad (37-38 ˚C). The left femoral artery was canulated with polyethylene tubing with an internal diameter of 0.28 mm and an external diameter of 0.61 mm for monitoring mean arterial pressure (MAP), blood withdrawal and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg within 15-30 minutes. Blood was collected in a heparinized 1 ml syringe to prevent clotting. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30 mmHg during this period. Mice were sacrificed 15, 30, 60 or 90 minutes after MAP of 30 mmHg was achieved. In Europe,

colloidal infusion fluids are frequently used in the resuscitation of shock victims. We therefore resuscitated, following 90 minutes of hemorrhagic shock, additional groups of mice with 6% hydroxyethyl starch 130/0.4 (Voluven®), two times the volume of the blood withdrawn. After 1, 4 or 24 hours following volume resuscitation these mice where sacrificed. Control mice were left untreated and received anesthesia only prior to sacrifice. Sham shock mice underwent instrumentation and were kept under anesthesia for 90 minutes, however no blood was withdrawn. The HS 90 minutes shock and HS sham shock group consisted of five animals, the other groups consisted of three animals per group. After the experimental procedure, brain, heart, lungs, liver and kidney were harvested and snap frozen in liquid nitrogen, and stored at -80 °C until analysis.

RNA isolation and quantitative reverse-transcription polymerase chain reaction

RNA was extracted from twenty (liver, brain and kidney) or thirty (heart and lung) 5 µm thick cryosections and isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoreses. RNA yield (OD260) and purity (OD260/OD280) were measured by ND-1,000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

One microgram of total RNA was subsequently used for the synthesis of first-strand cDNA with SuperScript III RNase minus reverse transcriptase (Invitrogen, Breda, The Netherlands) in a 20 µl final volume containing 250 ng of random hexamers (Promega Benelux) and 40 units of RNase OUT inhibitor (Invitrogen). After the RT reaction, 1 µl cDNA was used for each PCR reaction. Intron-overspanning primers and minor groove binder (MGB) probes used for quantitative RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Foster City, CA, USA) primers included housekeeping gene beta 2 Microglobulin (B2M) (assay ID Mm00437762_m1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915_g1), endothelial cell marker CD31 (platelet endothelial cell adhesion molecule 1, Mm00476702_m1), VE-cadherin (Mm00486938_m1), VEGF-A (Mm00437304_m1), HIF-1α (Mm00468869_m1), and P-selectin (Mm00441295_m1). TNF-α (Mm00443258_m1) primers and probes were purchased as pre-developed assay reagent for gene expression (Applied Biosystems). Primers and probes for E-selectin (forward primer:

5'-CAACGTCTAGGTTCAAAACAATCAG-3', probe: 5'-CACAAATGCAATCGTGGGA-3', reverse primer: 5'-TTAAGCAGGCAAGAGGAACCA-3'), ICAM-1 (forward primer:5'-ATGGGAATGTCACCAGGAATG-3', probe: 5'-CAGTACTGTACCACTCTC-3', reverse primer: 5'-GCACCAGAATGATTATAAGTCCAGTTATT-3') and VCAM-1 (forward primer: 5'-TGAAGTTGGCTCACAATTAAGAAGTT-3', probe: 5'- AACACTTGATGTAAAAGGA-3', reverse primer: 5'-TGCGCAGTAGTAGAGTGCAAGGA-3') were purchased as Assay-by-Design from Applied Biosystems, using the same quality criteria as for Assay-on-Demand.

The final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems, Foster City, CA, USA) for each gene was 900 nM and 250 nM, respectively. Water was used as a negative control, to exclude unspecific signals arising from impurities, and consistently showed no amplification signals.

Taqman real-time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two step cycles of 15 s at 95°C and 60 s at 60°C. Triplicate real-time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (C_t) were averaged. According to the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com), gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the ΔCt value. The average ΔCt value obtained from control, a non-treated, mouse was then subtracted from the average ΔCt value of each sample subjected to the experimental conditions described, yielding the $\Delta\Delta C_t$ value. The gene expression level, normalized to the housekeeping gene and relative to the control, was calculated as $2^{-\Delta\Delta Ct}$.

Localization of adhesion molecule expression, leukocyte infiltration, and HIF-1α using immunohistochemistry

Localization of P-selectin, E-selectin, VCAM-1, and ICAM-1 expression was determined in kidney, liver, heart, lung, and brain by immmunohistochemistry. Leukocyte recruitment was analyzed by immunohistochemical staining with an anti-CD45 pan leukocyte marker antibody. Frozen organs were cryostat cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 minutes. After drying, sections were incubated for 45 minutes at room temperature with primary rat anti-mouse antibodies: anti-E-selectin (MES-1, kindly provided by Dr. D. Brown, Celletech Group, Slough, UK), anti-CD31 (clone

MEC13.3; Pharmingen BD Biosciences, Alphen aan den Rijn, The Netherlands), anti-CD45 leukocyte common antigen (Pharmingen BD Biosciences), anti-VCAM-1 (clone M/K-1.9; ATCC, Manassas VA, USA), and anti-ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After washing, endogenous peroxidase was blocked by incubation with 0.1% H_20_2 in PBS for 20 minutes. This was followed by incubation of 30 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (Rabbit anti Rat-Ig, DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with 2% normal mouse serum. Sections with isotype matched controls, E-selectin, and VCAM-1 specific antibodies were further incubated for 30 minutes at room temperature with HRP-conjugated goat anti-rabbit antibody (Southern Biotech Association, Birmingham, Alabama, USA) diluted 1:100 in PBS. Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie, St.Louis, Missouri, USA) and sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype matched controls, demonstrating specificity of staining with the antigen specific antibodies.

For HIF-1α staining, 5 µm acetone fixed cryosections were incubated for 45 minutes at room temperature with primary HIF-1α goat polyclonal antibody (HIF-1α (Y-15): sc-12542, Santa Cruz Biotechnology, California, USA) in the presence of 5% fetal calf serum in PBS. Endogenous biotin was blocked by Biotin Blocking System (DAKO) according to manufacturer's protocol and peroxidase activity was blocked by incubation with 0.1% H_2O_2 in PBS for 10 minutes. Subsequently, sections were incubated at room temperature for 45 minutes with rabbit anti-goat antibody (DAKO; dilution 1:100 in PBS) in the presence of 2% normal mouse serum. Further staining procedure was performed as described above. Negative control samples were incubated with PBS instead of the primary antibody.

Statistical analysis

Statistical significance of differences was studied by means of the analysis of variance (ANOVA) with post hoc Least Significant difference. Differences were considered to be significant when $p < 0.05$.

RESULTS

Hemodynamic changes induced by hemorrhagic shock

Figure 2.1 illustrates the time course of MAP during the experiment. All groups of mice exhibited initial MAP values in the range of 75-100 mmHg. In sham shock mice the MAP remained stable during 90 minutes between 80-100 mmHg. In the hemorrhagic shock group the MAP was maintained between 20-40 mmHg. At the end of resuscitation, all groups experienced a comparable increase in MAP, which nevertheless did not reach the baseline values observed prior to HS induction. All mice survived the procedure until the end of the experiments.

Baseline gene expression levels in different organs

Quantitative RT-PCR was used to quantitatively measure the basal and HS affected mRNA levels of adhesion molecules and the hypoxia related genes HIF-1α and VEGF-A in the different organs. In control mice (table 2.1) the levels of mRNA of the genes under study differed between genes within one organ, and between organs. CD31 and VE-cadherin expression levels within one organ were within the same magnitude. The low

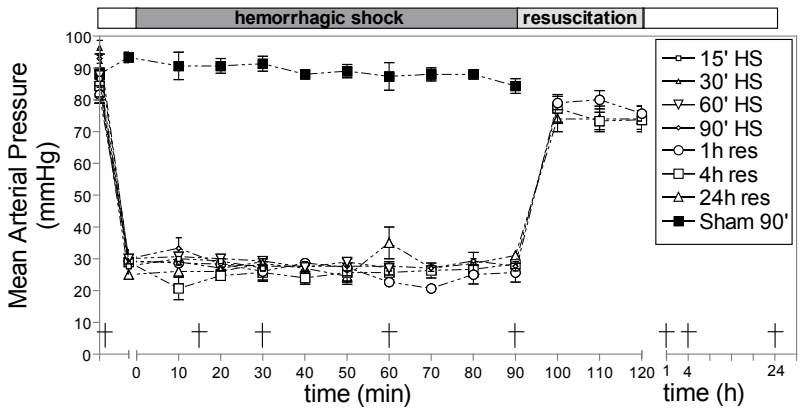

Figure 2.1. Study design and measured mean arterial pressure during experiment.
Hemorrhage was induced by blood withdrawal as described in "Materials and Methods". Mice were sacrificed (†) at start of experiment, or after 15, 30, 60 or 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a group of mice was resuscitated. Sham shock mice (■) underwent instrumentation and 90 minutes of anesthesia. Blood pressure is expressed as mean ± SEM, (n=5 in the 90' HS group and 90' sham shock, other groups n= 3).

ΔCT values of endothelial marker genes CD31 and VE-cadherin in the lung (representing high mRNA levels) most likely reflects the high degree of vascularisation in this organ compared to the other organs. Although the ΔCt values do not directly represent protein levels, these data do imply that organ specific vascular heterogeneity in microvascular endothelial cell status exists.

Kinetics of expression of endothelial adhesion molecules and hypoxia related genes during the shock phase

The kinetics of endothelial cell activation during onset and maintenance of HS were studied by quantitative analysis of P-selectin, E-selectin, VCAM-1 and ICAM-1 mRNA levels (table 2.2A). P-selectin, E-selectin, and ICAM-1 were strongly upregulated at 90 minutes after initiation of hemorrhagic shock compared to sham shock. Furthermore, especially E-selectin expression in the kidney and the lungs showed a trend towards early induction. While the early increase was statistically not significant compared to the activation induced by 90 minutes of sham shock, it did show statistical significance when compared to control mice. Neither CD31 nor VE-cadherin expression changed under the influence of HS, except for VE-cadherin expression in the liver which showed increased mRNA levels. Resuscitation facilitated an additional up regulation of the inflammatory

Table 2.1. Expression levels of genes investigated in this study in healthy mouse organs.

	Kidney	Liver	Heart	Lung	Brain
CD31	5.9 (5.5-6.2)	7.1 (6.8-7.4)	5.5 (5.0-5.6)	0.9 (0.5-1.6)	7.2 (7.2-7.4)
VE-cadherin	6.0 (5,7-6,1)	6.4 (6.3-7.7)	4.4 (4.2-4.6)	-0.3 (-0.6-0.5)	7.1 (7.1-7.2)
P-selectin	14.9 (14,6-15,7)	12.4 (11.6-12.4)	14.4 (12.9-14.8)	8.3 (8.2-8.8)	18.0 (17.0-18.0)
E-selectin	14.6 (14.4-14.7)	17.0 (16.8-17.3)	14.2 (13.6-14.4)	12.3 (11.5-12.6)	18.6 (18.1-18.9)
VCAM-1	9.4 (9.3-9.5)	8.9 (8.6-9.6)	10.2 (9.3-10.2)	6.4 (5.3-6.9)	8.2 (8.1-8.5)
ICAM-1	7.6 (7.4-8.1)	7.7 (7.6-8.3)	10.4 (9.9-10.7)	2.4 (2.1-3.1)	10.7 (10.1-11.1)
HIF-1α	6.1 (5.5-6.4)	7.0 (6.6-7.3)	7.5 (6.0-8.0)	4.4 (4.4-5.0)	6.3 (6.3-6.5)
VEGF-A	4.3 (4.1-4.3)	4.5 (4.1-4.8)	5.7 (4.8-5.7)	0.8 (0.8-1.6)	6.0 (5.9-6.2)

Expression of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in control mice not subjected to any experimental procedure was determined in lung, liver, kidney, heart, and brain using quantitative RT-PCR with GAPDH as housekeeping gene. Data represent values from three animals per group expressed as median ΔCT (minimum-maximum).

Table 2.2A. Kinetics of EC activation PRIOR to resuscitation.

		0	30'	60'	90'	sham 90'
CD31	Lung	1.0 (0.6-1.3)	1.4 (0.2-1.9)	0.6 (0.4-2.0)	0.6 (0.1-1.7)	0.4 (0.1-1.5)
	Liver	0.8 (0.7-1.0)	0.5 (0.4-0.8)	0.6 (0.6-0.6)	0.7 (0.4-1.1)	0.8 (0.6-1.0)
	Kidney	1.0 (0.8-1.3)	0.7 (0.4-0.8)	0.8 (0.5-1.5)	0.5 (0.5-0.7)	0.7 (0.6-0.8)
	Heart	1.1 (1.0-1.5)	1.0 (1.0-1.0)	1.3 (1.1-1.3)	1.2 (0.9-1.3)	1.5 (0.2-2.7)
	Brain	1.0 (0.9-1.0)	0.6 (0.4-0.7)	0.7 (0.6-0.7)	0.7 (0.4-1.1)	0,6 (0.6-0.9)
VE-cadherin	Lung	1.0 (0.6-1.2)	1.2 (1.2-1.6)	0.5 (0.4-1.8)	0.7 (0.1-1.7)	0.3 (0.1-1.7)
	Liver	2.4 (1.0-2.7)	3.3 (2.5-3.4)	3.1 (3.1-3.2)	3.3 (3.0-7.8)‡	4.3 (2.7-5.0)
	Kidney	1.0 (1.0-1.3)	0.9 (0.2-1.0)	1.2 (1.2-1.5)	1.2 (0.9-1.3)	0.8 (0.7-1.0)
	Heart	1.0 (0.9-1.2)	1.1 (1.0-1.1)	1.2 (1.2-1.4)	0.9 (0.9-1.2)	1.3 (0.2-2.4)
	Brain	1.0 (0.9-1.0)	0.7 (0.5-1.0)	1.0 (0.8-1.0)	0.8 (0.5-1.4)	0.9 (0.7-1.2)
P-selectin	Lung	1.0 (0.8-1.1)	2.3 (0.2-20)	1.4 (1.4-7.2)	8.4 (1.1-60)*	1.5 (0.5-2.1)
	Liver	0.6 (0.6-1.0)	0.7 (0.6-1.7)	1.4 (1.0-1.5)	10 (1.8-32)	3.1 (0.4-7.4)
	Kidney	1.0 (0.6-1.2)	2.1 (0.9-4.2)	5.2 (4.7-12)	25 (3.2-112)	4.7 (0.6-7.4)
	Heart	0.4 (0.3-1.0)	0.6 (0.6-3.0)	1.3 (0.5-1.6)	15 (6.4-70)	3.7 (0.5-11)
	Brain	0.5 (0.5-1.0)	1.7 (1.0-2.5)	7.4 (6.5-8.7)	66 (28-397)	26 (1.3-63)
E-selectin	Lung	1.0 (0.8-1.6)	1.5 (0.4-127)	7.6 (5.6-26)	54.5 (9.0-301)*‡	2.5 (2.2-11)
	Liver	1.2 (1.0-1.4)	9.3 (3.4-778)	135 (106-176)	666 (472-2289)‡	637 (1.2-1261)
	Kidney	1.0 (1.0-1.2)	7.4 (2.6-63)	39 (18-90)	133 (18-313)*‡	30 (1.6-64)
	Heart	1.1 (1.0-1.7)	2.9 (2.3-25)	4.7 (3.5-8.8)	52 (16-92)	3.5 (1.8-52)
	Brain	1.0 (0.8-1.4)	9.8 (1.4-25)	34 (21-36)	124 (47-477)*‡	66 (3.9-156)
VCAM-1	Lung	1.0 (0.7-2.1)	2.8 (0.8-15)	3.6 (2.0-6.2)	6.4 (1.6-29)*‡	2.0 (0.9-3.0)
	Liver	0.8 (0.5-1.0)	2.5 (1.2-3.4)	3.4 (2.7-3.4)	4.9 (4.3-9.4)‡	10.8 (1.9-13)
	Kidney	1.0 (0.9-1.1)	1.3 (1.2-3.5)	3.1 (2.5-3.8)	6.0 (1.4-15)*‡	3.8 (0.7-5.3)
	Heart	1.0 (1.0-1.9)	1.5 (1.3-8.7)	3.4 (2.0-4.9)	12.5 (5.4-17)	3.6 (0.6-15)
	Brain	1.0 (0.8-1.1)	0.6 (0.6-0.8)	0.8 (0.6-1.1)	1.8 (1.4-2.1)*‡	1.0 (0.8-1.1)
ICAM-1	Lung	1.0 (0.6-1.2)	1.9 (0.5-5.1)	1.0 (0.7-3.0)	3.1 (0.5-19)*	0.6 (0.3-1.9)
	Liver	1.0 (0.6-1.0)	0.7 (0.7-6.9)	3.9 (3.8-5.9)	24 (17-41)‡	37 (0.6-43)
	Kidney	1.0 (0.7-1.1)	1.4 (1.2-4.4)	5.5 (3.1-6.7)	12 (3.7-24)*‡	3.3 (0.8-4.4)
	Heart	1.2 (1.0-1.8)	3.9 (2.7-30)	12 (6.2-14)	82 (25-195)	6.2 (3.5-125)
	Brain	1.0 (0.7-1.5)	1.8 (0.7-2.8)	2.6 (1.7-4.1)	12 (6.6-33)*‡	5.1 (1.6-7.9)

*Expression levels of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in lung, liver, kidney, heart and brain, analysed by quantitative RT-PCR using GAPDH as housekeeping gene. RNA levels were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n=5 in the 90' HS group and 90' sham shock, other groups n = 3). p < 0.05 values marked with * represent 90 minutes of HS, compared with 90 minutes of sham shock (sham 90'). p < 0.05 values marked with ‡ represent 90 minutes of HS, compared with control, as described in Materials and Methods.*

Table 2.2B. Kinetics of EC activation AFTER resuscitation.

		90'	1 h	4 h	24 h
CD31	Lung	0.6 (0.1-1.7)	0.1 (0.1-0.4)	0.5 (0.1-0.9)	0.8 (0.4-1.7)
	Liver	0.7 (0.4-1.1)	0.5 (0.5-0.8)	0.5 (0.4-0.5)	0.9 (0.6-1.0)
	Kidney	0.5 (0.5-0.7)	0.4 (0.4-0.4)	0.6 (0.3-1.0)	1.3 (0.8-2.7)
	Heart	1.2 (0.9-1.3)	1.0 (0.1-1.3)	0.7 (0.5-0.8)	2.0 (1.5-2.3)
	Brain	0.7 (0.4-1.1)	0.7 (0.6-0.7)	1.1 (0.5-1.2)	2.1 (1.1-2.9)
VE-cadherin	Lung	0.7 (0.1-1.7)	0.2 (0.2-1.7)	0.7 (0.2-1.1)	0.7 (0.2-1.0)
	Liver	3.3 (3.0-7.8)	7.6 (4.9 -8.1)*	6.6 (4.8-9.3)	3.0 (2.4-3.8)
	Kidney	1.2 (0.9-1.3)	1.6 (1.6-1.9)*	2.6 (2.4-2.9)	1.7 (0.8-2.1)
	Heart	0.9 (0.9-1.2)	0.8 (0.1-1.1)	0.9 (0.5-1.3)	1.5 (1.2-1.8)
	Brain	0.8 (0.5-1.4)	1.7 (1.1-1.7)	1.7 (0.9-3.2)	2.1 (1.1-2.4)
P-selectin	Lung	8.4 (1.1-60)	25 (18-65)	6.8 (5.3-28)	2.0 (1.9-6.3)
	Liver	10 (1.8-32)	118 (74-174)*	62 (6.0-75)	0.8 (0.5-1.2)
	Kidney	25 (3.2-113)	264 (255-311)*	94 (33-214)	9.3 (2.2-11)
	Heart	15 (6.4-70)	222 (33-287)*	119 (4.3-184)	2.1 (1.2-2.7)
	Brain	66 (28-398)	482 (253-901)*	26 (7.5-138)	13 (5.8-173)
E-selectin	Lung	55 (9.0-301)	103 (82-234)	3.9 (3.0-9.2)	6.2 (2.7-17)
	Liver	666 (473-2290)	3042 (2196-3059)*	230 (24-379)	6.3 (1.7-7.4)
	Kidney	133 (18-313)	252 (236-465)*	17 (9.5-46)	1.5 (0.6-1.7)
	Heart	52 (15.8-92)	492 (111-795)*	98 (1.5-301)	5.1 (1.6-7.5)
	Brain	124 (47-474)	287 (245-419)	30 (2.3-36)	40 (8.5-151)
VCAM-1	Lung	6.4 (1.6-29)	2.7 (2.6-7.3)	2.1 (2.0-6.4)	1.8 (1.6-2.7)
	Liver	4.9 (4.3-9.4)	12 (12-16)*	4.6 (2.5-7.8)	0.9 (0.9-1.0)
	Kidney	6.0 (1.4-15)	23 (21-27)*	6.4 (3.3-15)	1.5 (0.6-1.8)
	Heart	13 (5.4-17)	37 (2.5-46)*	11 (0.8-2)	1.9 (1.5-2.2)
	Brain	1.8 (1.4-2.1)	2.8 (2.2-3.3)*	0.8 (0.5-1.1)	1.4 (0.9-1.4)
ICAM-1	Lung	3.1 (0.5-19)	3.3 (2.9-7.8)	1.2 (0.9-5.8)	1.2 (0.9-2.8)
	Liver	24 (17-41)	33 (26-41)	9.7 (3.7-14)	1.3 (1.0-1.4)
	Kidney	12 (3.7-24)	25 (22-26)*	16 (4.2-20)	1.6 (0.7-2.6)
	Heart	82 (25-195)	356 (45-365)*	129 (4.4-236)	7.2 (2.2-9.1)
	Brain	12 (6.6-33)	27 (24-29)*	2.1 (1.4-11)	4.2 (1.4-6.6)

*Expression levels of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in lung, liver, kidney, heart, and brain, analysed by quantitative RT-PCR, using GAPDH as housekeeping gene. RNA levels were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n=5 in the 90' HS group, other groups n = 3). 90 minutes of HS was compared with one hour post resuscitation group (1 h). p< 0.05 values are marked with *, as described in Materials and Methods.*

endothelial genes compared with the activation status at 90 minutes of hemorrhagic shock (table 2.2B). It is of note that especially in the liver and the kidney VE-cadherin mRNA levels were strongly upregulated at one hour after resuscitation.

To corroborate the gene expression levels, cell adhesion molecules were immunohistochemicaly analyzed. In kidney, liver, and heart E-selectin protein was strongly expressed at 90 minutes of shock, while in the sham shock group expression was minor or absent (figure 2.2). Immunohistochemical staining did not only show differences in expression between different organs, thereby supporting the gene expression data, but also demonstrated differences in activation between the diverse organ microvascular

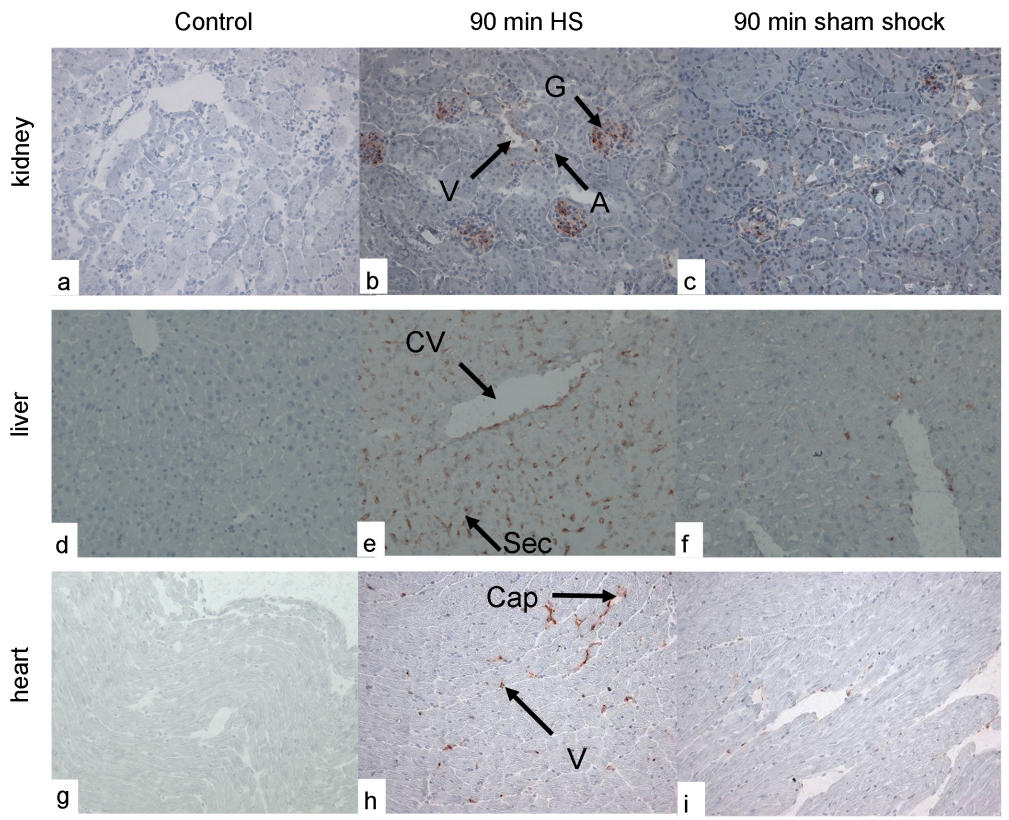

Figure 2.2. Localization of E-selectin expression in kidney, liver and heart during the early phase of HS. *Immunohistochemical detection of E-selectin in healthy mouse tissue (a, d, g), after 90 minutes of HS (b, e, h), and after 90 minutes of sham shock (c, f, i). Staining was performed respectively on kidney (a-c), liver (d-f), and heart (g-i). Original magnification 200x. E-selectin is stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.*

endothelial cells. In the kidney, for example, protein expression of E-selectin was mainly found in glomeruli (figure 2.2), while VCAM-1 expression was seen primarily in the peritubular endothelial cells, arteries and venules, but not in glomeruli (figure 2.3). From this, it was concluded that HS under anesthesia strongly affected the activation status of microvascular endothelial cells in all organs. Unexpectedly, however, anesthesia

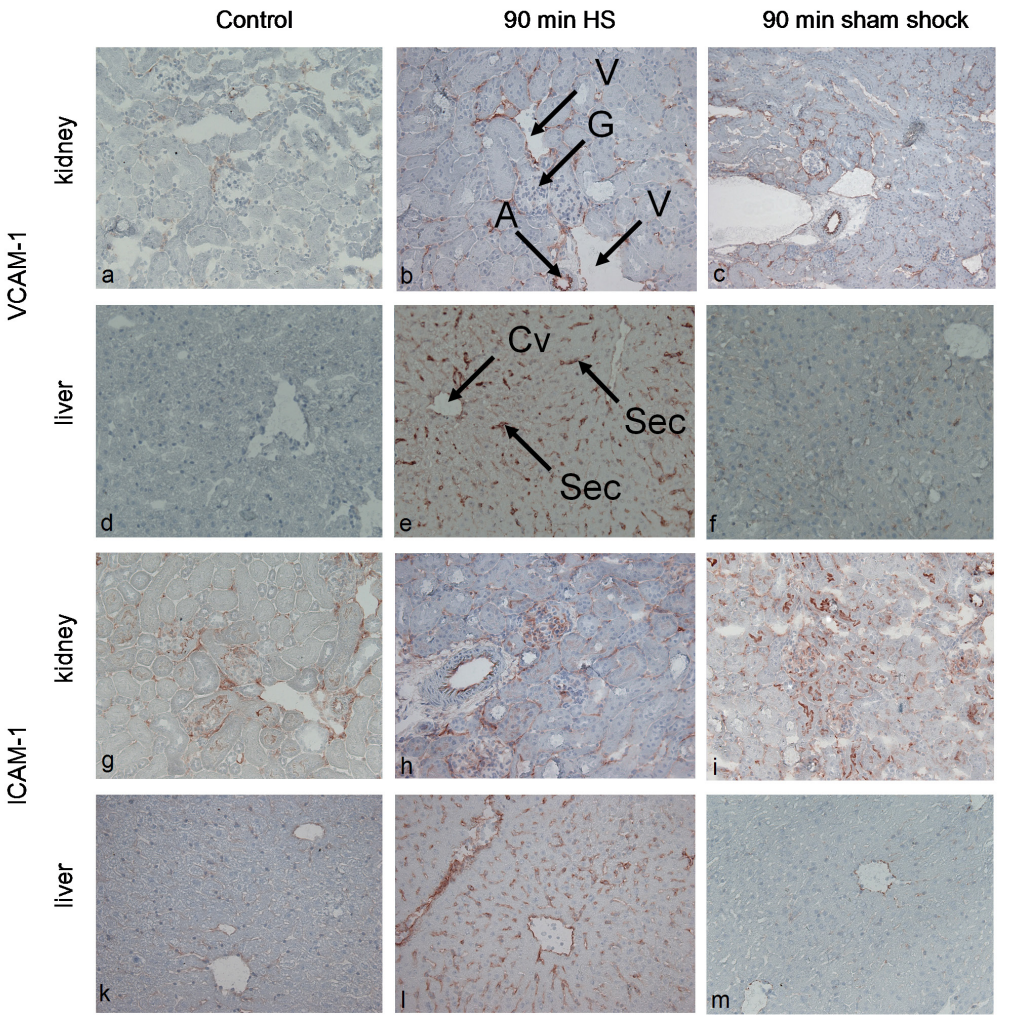

Figure 2.3. Localization of VCAM-1 and ICAM-1 expression in kidney and liver during the early phase of HS. *Immunohistochemical detection of VCAM-1 and ICAM-1 in healthy mouse tissue (a, d, g, j), after 90 minutes of HS (b, e, h, k), and after 90 minutes of sham shock (c, f, i, l). Staining was performed respectively on kidney (a-c, g-i) and liver (d-f, j-k). Original magnification 200x. VCAM-1 and ICAM-1 and CD31 are stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.*

and instrumentation without concurrent induction of HS also affected endothelial cell activation, as observed by upregulation of adhesion molecule mRNA and protein levels (figure 2.2, figure 2.3, table 2.2A).

Hypoxia related genes VEGF-A and HIF-1α were not transcriptionally affected during the 90 minutes of hemorrhagic shock (figure 2.4). Moreover, immunohistochemical staining did not reveal nuclear localization of HIF-1α in any of the organs (data not shown), implying absence of activation of the HIF-1α system during the HS phase.

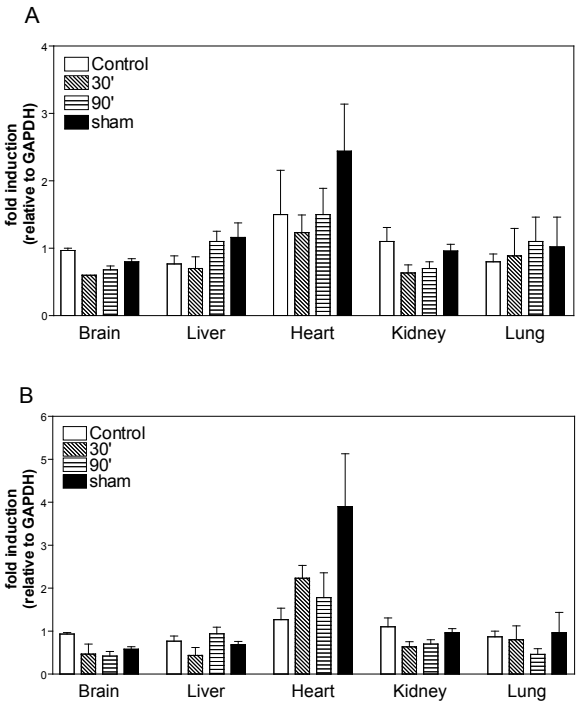

Figure 2.4. Effect of hemorrhagic shock on hypoxia related markers.
Gene expression of HIF-1α (A) and VEGF-A (B) was determined in lung, liver, kidney, heart, and brain by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of hypoxia related genes in comparison to the level of activity that was present in RNA isolates of normal healthy tissue. Data presented are the mean ± SEM of each group (n=5 in the 90' HS group and 90' sham shock, other groups n= 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90'), no significant differences were found.

Early organ activation is paralleled by leukocyte influx

Adhesion molecule expression is instrumental in facilitation of leukocyte migration into the tissues. Influx of leukocytes into tissue, as determined with pan leukocyte marker CD45, was observed in kidney, lung, and liver at 90 minutes of HS (figure 2.5). In contrast, in brain and heart no leukocyte influx was seen after 90 minutes of HS. 24 hours after volume resuscitation, no difference in leukocyte influx could be observed compared to control mice (data not shown).

Upon HS TNF-α is produced in kidney, brain, and heart tissue

Leukocyte-endothelial interactions are often initiated by pro-inflammatory cytokines. These cytokines are either produced locally in the organ where the inflammatory

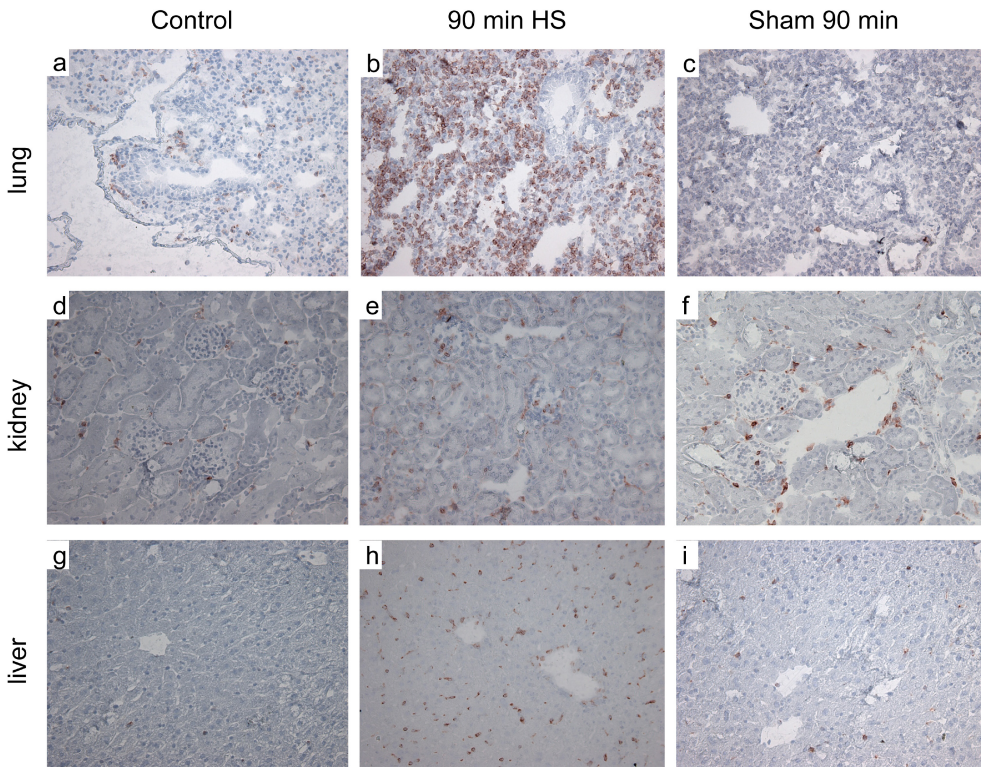

Figure 2.5. Influx of leukocytes in the different organs during HS.
Immunohistochemical staining with an anti-CD45 pan leukocyte antibody showed influx of leukocytes in lungs (a-c), kidney (d-f), and heart (g-i). Staining was performed respectively on healthy mouse tissue (control) (a, d, g) and 90 minutes HS (b, f, h), and 90 minutes sham shock (c, e, i). Original magnification 200x. Leukocytes are stained red, with increased influx of CD45 positive cells is seen at 90 minutes of shock.

response is taking place, or systemically released e.g., by the liver upon exposure to bacterial products. TNF-α gene expression analysis showed a significant TNF production in kidney and heart at 90 minutes after initiation of HS compared to sham shock mice (figure 2.6). Interestingly, in the heart this induction of TNF-α was not paralleled by leukocyte influx (data not shown).

Discussion

The cellular and organ response to hemorrhagic shock is complex and ultimately results in profound changes in gene expression and organ function[10]. The earliest effects of HS on microvascular endothelial cells and its causes remain poorly understood. In models of HS, sample data exist regarding the detrimental influx of leukocytes into organ parenchyma. Since endothelial cell activation is instrumental in leukocyte recruitment, we aimed to investigate the kinetics and organ specificity of microvascular endothelial cell activation during the early phase of hemorrhagic shock. We showed that induction of inflammatory gene expression is an early event which occurs before resuscitation is instituted. Expression of all adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial integrity genes CD31

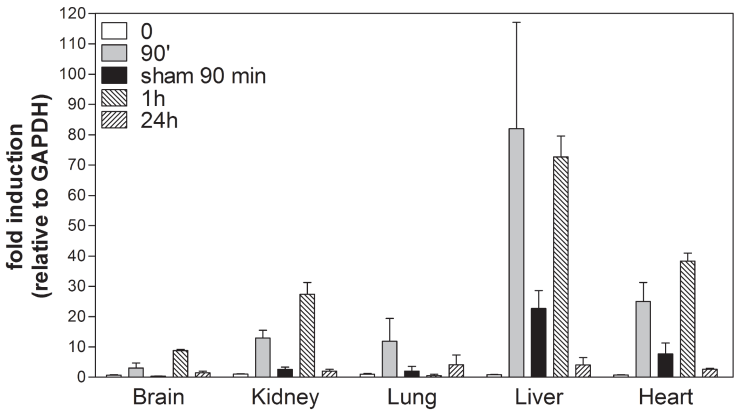

Figure 2.6. Effect of hemorrhagic shock on TNF-α in tissue.
*Gene expression of TNF-α was determined in lung, liver, kidney, heart, and brain tissue by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of TNF-α in comparison to the level of activity that was present in extracts of normal healthy organs. Data presented are the mean ± SEM of each group (n=5 in the 90' HS group and 90' sham shock, other groups n= 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90'). p< 0.05 values are marked with *.*

and VE-cadherin were only affected in the liver, while activation of the hypoxia HIF-1α pathway was absent during the shock period.

To our knowledge we are the first to report the earliest effects of HS on the more complex pattern of microvascular endothelial cell activation and its organ specificity. Although no data on these early endothelial responses have been reported before, Xu et al showed that in liver and lung, P-selectin, and ICAM-1 were upregulated 3 hours after resuscitation in a 90 minutes hemorrhagic shock model[8]. The early endothelial activation during HS found in our study is in accordance with reports of early activation of inflammatory signal transduction routes in HS. In a volume controlled hemorrhagic shock model in which 25% of blood volume was withdrawn the pro-inflammatory p38 mitogen-activated protein kinase (MAPK) activity increased in the kidney 60 minutes after initiation of HS[5]. McCloskey et al observed that Jun N-terminal Kinase (JNK) activation is an early event in the liver. After 30 minutes of HS with a MAP of 25 mmHg they observed an increase of JNK which persisted throughout the duration of the 120-minutes experiment[6]. Although in these studies these activated kinases were not assigned to specific cell types in the organ, these signaling pathways are known to control endothelial adhesion molecules expression[11]. During the progression of hemorrhagic shock condition, the cytokine driven mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NF-kB) intracellular signalling pathways are likely to become activated in the microvascular endothelium[12; 13]. Subsequent signalling via leukocyte integrin – endothelial immunoglobulin superfamily members is implied to take place in microvascular segments in which cell-cell interactions are most prominent. This signalling relays via small GTPase and p38 MAPK[14; 15]. In an early stage of hemorrhage, changes in shear stress may furthermore affect endothelial activation in those microvascular beds where autoregulatory arteriolar control of blood flow cannot be sufficiently controlled[16]. Since most the studies on endothelial responses to inflammatory cytokine and shear stress changes have been performed in in vitro culture systems, and endothelial cells throughout the vascular tree are phenotypically heterogenic, the exact nature of signalling pathways leading to organ specific shock related endothelial activation needs to be identified.

The expression of endothelial adhesion molecules is essential for endothelial-leukocyte interaction[3]. After activation of the endothelium, E-selectin is synthesized by de novo protein synthesis. Its expression on the endothelial cell membrane is induced

a few hours after TNF-α stimulation of endothelial cells in culture[17]. We showed that E-selectin is upregulated at mRNA and protein level within the first 90 minutes after initiation of HS in the kidney, lung and the brain. Of note is the organ specific patterns of endothelial activation, which could theoretically form the basis for an organ specific leukocyte recruitment process. Indeed, the early endothelial upregulation of adhesion molecules was paralleled by early leukocyte recruitment in the liver, kidney and lung. In contrast, the microvascular endothelium in the brain and the heart responded to HS with an increase in different adhesion molecule expression, but no leukocyte recruitment was seen in these organs. A similar organ heterogeneity in leukocyte recruitment was reported by Song et al, who showed an increase in lung neutrophil count after 1 hour, being maximal at 4 hours after hemorrhagic shock, without any change in myocardial neutrophil counts[18]. A more detailed study on chemokine and cytokine expression within the different organs combined with infiltrating leukocyte subset typing may shed light on this organ specificity of leukocyte recruitment.

Cellular hypoxia is considered to be an important mediator of MODS following hemorrhagic shock[19]. During hypoxic conditions HIF-1α accumulates in the cell and forms a stable heterodimer with HIF-1β whereafter it translocates to the nucleus. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumor models[20; 21]. In a rat model of permanent focal ischemia of the brain, Bergeron and colleagues showed that mRNA of HIF-1α was significantly increased after 7.5 hours of vascular occlusion[22]. VEGF-A is a downstream target gene of HIF-1α which is primarily regulated at transcriptional level. and a major controller of vascular permeability and angiogenesis. Under hypoxic conditions upregulation of VEGF-A by HIF-1α occurs within minutes[23]. However, in our model, we did not see any significant induction of mRNA of HIF-1α and VEGF-A during the hemorrhagic shock phase, nor did we see an increased nuclear localization of HIF-1α after 90 minutes of HS. This may imply that either the duration or the severity of hypoxia in the early hemorrhagic shock phase is too short respectively too minor to activate the HIF-1α system. On the other hand, Koury et al showed that in a pressure controlled hemorrhagic shock model in the rat the HIF-1α level as measured by western blotting was increased in the ileac mucosa after 90 minutes of HS accompanied by a MAP of 30 mmHg[24]. While Hierholzer et al observed an increase in HIF-1α DNA binding activity of 3.2 fold in the lung after a 40 mmHg MAP shock period of 2.5 hours[25], no increased

HIF-1α activation in livers of animals subjected to 40 mmHg MAP HS for 60 minutes was found[26]. Our data and those reported by others therefore suggest that although cellular hypoxia may play a role, it is not necessarily a key factor in the upregulation of inflammatory genes in the early phase of HS.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine, with the primary target including vascular endothelial cells. When TNF-α is administered to humans, it produces fever, inflammation, tissue destruction, and, in some cases, shock and death[27]. Effects on endothelial cells include protein independent changes in cell shape and motility as well as induction of proteins that regulate other parameters of the inflammatory response such as vasoregulation, leukocyte adhesion, leukocyte activation, and coagulation[13; 28]. As such TNF-α may be implicated in organ dysfunction in HS. Liu et al showed in rats with a HS to a MAP of 50 mmHg during 60 minutes that the mRNA encoding for TNF-α was upregulated in ileum, kidney, liver and skeletal muscle[13; 29]. Serum TNF-α was increased in a mouse model already after 30 minutes after initiation of shock[12]. Combined with our here reported increase in mRNA TNF-α levels in the various organs, these studies indicate an early TNF-α response in HS. However, a direct relation between local TNF-α production and early activation of endothelial cells respectively leukocyte adhesion cannot be easily inferred. Additional experiments, with induction of HS in TNFR1/R2 knock out mice, need to be performed to get a more detailed view on the role of TNF on early endothelial cell activation in our HS model.

We showed that the combination of surgical instrumentation and anesthesia by itself strongly affected endothelial cell activation status. The administration of anesthetics may contribute to endothelial activation by exerting depressive effects on respiratory and cardiovascular functions, or by direct cellular actions. There also may be an effect associated with the use of heparin which is administered to maintain blood flow through the catheter[30]. Previous studies showing early increased pro-inflammatory IL-6 cytokine expression in mice that underwent a sham shock procedure[31; 32], corroborate our findings of sham shock group pro-inflammatory responses[33].

In summary, our study revealed an early and organ specific endothelial cell activation pattern during hemorrhagic shock which occurred prior to resuscitation, and was not per se hypoxia driven. The early endothelial activation found in this study suggests that a temporal therapeutic window exists which can be employed to attenuate endothelial

cell activation at an early stage during resuscitation, to prevent neutrophil sequestration in organs and subsequent multiple organ dysfunction syndrome. To identify potential targets for early therapeutic interference, we will in future studies, asses complex kinase activity profiles[34] at different timepoints after hemorrhagic shock induction and resuscitation.

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Chapter 3

Hemorrhagic shock induced pro-inflammatory endothelial cell activation in lung and kidney in mice is not influenced by mechanical ventilation and is not hypoxia mediated

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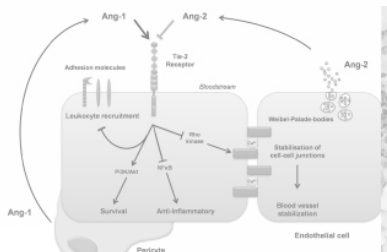

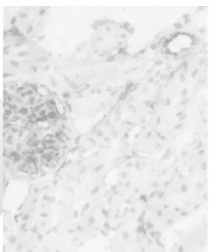

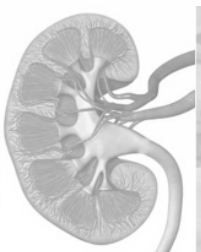

ABSTRACT

Introduction: The interaction between neutrophils and activated endothelium is essential for the migration of neutrophils into tissues, and for the development of multiple organ dysfunction in patients suffering from hemorrhagic shock (HS). HS leads to early and organ specific pro-inflammatory microvascular endothelial activation. Mechanical Ventilation (MV) is frequently employed in patients with HS. Intubation and MV does protect organs from hypoxia and hypercapnia, on the other hand MV may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. Our aim was to investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lung and kidney.

Methods: Anesthetized wild type C57Bl/6 male mice were subjected to controlled hemorrhage. Mice were killed after 90 minutes of HS. After 90 minutes of HS, a group of mice was resuscitated and sacrificed 24 hours after shock induction. To examine the effects of mechanical ventilation, subgroups of mice were mechanical ventilated during the HS insult. To study the effect of acute hypoxia mice were housed in cages with 6, 10 and 21 % oxygen during 2 hours and harvested. Untreated mice served as controls. Gene expression levels of endothelial cell activation, (E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1), inflammation (TNF-α, IL-6 and MCP-1) and hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α) were quantified in kidney and lung, by quantitative reverse-transcription polymerase chain reaction. A selection of these genes was examined with regard to protein expression levels and localization using immunohistochemical analysis. Soluble pro-inflammatory cytokine (TNF-α, IL-6, and CXCL-1) levels in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA).

Results: 90 minutes after shock induction a vascular bed specific, heterogeneous pro-inflammatory endothelial activation represented by E-selectin, VCAM-1 and ICAM-1 expression was seen in kidney and lung. No differences in adhesion molecules between the spontaneous breathing and mechanically ventilated mice were found. Also no differences in endothelial pro-inflammatory activation cytokines and hypoxia influenced genes were found for TNF-α, IL-6, MCP-1 and HIF-1α at 90 minutes after shock induction between the spontaneous breathing and mechanically ventilated mice. During HS,

HIF-1α mRNA was not induced in the kidney, while in the lung HS led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation. To determine the contribution of tissue hypoxia due to decreased oxygen delivery to the pro-inflammatory endothelial activation observed in kidney and lung, we subsequently studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. 2 hours of 6% hypoxia induced upregulation of proinflammatory cytokines IL-6, and MCP-1 mRNA in the lung but not in the kidney, while TNF-α mRNA was unchanged. Two hours of 6% oxygen did not however induce the expression of E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice.

Conclusions: Hemorrhagic shock leads to an early and reversible pro-inflammatory endothelial activation in kidney and lung. This proinflammatory cytokine response during HS can induce an endothelial pro-inflammatory activation. HS induced endothelial activation is not augmented nor prevented by mechanical ventilation during the shock phase. Hypoxia alone does not lead to endothelial activation.

INTRODUCTION

The development of multiple organ dysfunction syndrome (MODS) is a complication in patients who suffer from major bleeding[1]. Advances in medical care of HS patients, including the introduction of resuscitation fluids, trauma centers, intensive care units, and mechanical ventilation have resulted in a significant decrease in early deaths caused by HS. But instead of dying, patients can develop MODS. The inflammatory response is considered the leading cause for the development of MODS. Two failing organs are the lung and the kidney. Lung failure, the so called Acute Respiratory Distress Syndrome (ARDS), and acute kidney injury (AKI) are strongly associated with patient morbidity and mortality[2]. To treat symptoms of ARDS and AKI, patients are treated with mechanical ventilation and renal replacement therapy. The precise mechanisms leading to MODS after HS are still largely unknown. One of the proposed mechanisms is infiltration of neutrophils into the tissues, leading to significant organ damage through release of proteases and oxygen-derived radicals. The interaction between neutrophils and endothelium is essential for the migration of neutrophils into tissues[3]. This migration is regulated by adhesion molecules on both leukocytes and endothelium, the latter including E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1)[4]. We have recently shown that HS leads to early and organ specific pro-inflammatory microvascular endothelial activation[5]. This organ and microvascular bed specific endothelial activation is also seen in animal models subjected to septic shock, where it coincides with increased vascular leakage[6; 7]. HS occurs frequently in the operating theatre as a result of difficult to control surgical bleeding. Patients with HS due to other causes including trauma or gastrointestinal bleeding require resuscitation and procedures to control the bleeding[8; 9]. Therefore HS patients will be frequently intubated and mechanically ventilated to allow intervention procedures and warrant a patent airway and gas exchange[10]. Mechanical ventilation (MV) during conditions of hemorrhagic shock can act as a double-edged sword. On the one hand intubation and mechanical ventilation may protect organs from hypoxia and hypercapnia, on the other hand it may initiate an inflammatory reaction[11] and induce pro-inflammatory activation in the lung and in distant organs[12; 13]

To follow up on our earlier observation of hemorrhagic shock induced microvascular endothelial priming during the shock phase[5], our aim was to investigate the beneficial, neutral or harmful consequences of mechanical ventilation in mice subjected to HS on microvascular endothelial activation in the lung and kidney. Mechanical ventilation is started to prevent hypoxia and threatened oxygen delivery. HS by definition decreases oxygen delivery[14], yet the role of decreased oxygen availability in endothelial activation is not clear. To study the effect of a decreased oxygen delivery in vivo on endothelial cell (EC) activation we also evaluated endothelial proinflammatory adhesion molecule expression in hypoxic mice.

Materials and methods

Animals

8- to 12-wk-old C57Bl/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12:12-h light-dark cycle. All procedures were approved by the local committee for care and use of laboratory animals and were performed according to national and international guidelines on animal experimentation.

Mouse shock model

The mouse hemorrhagic shock model has been extensively documented elsewhere[15; 16]. In short, mice were anesthetized with isoflurane (inspiratory, 1.4%), N_2O (66%), and O_2 (33%). The left femoral artery was canulated for monitoring mean arterial pressure (MAP), blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain a pressure constant hemorrhagic shock model with a MAP at 30 mmHg during this period. A subset of mice was resuscitated after 90 minutes of hemorrhagic shock with 6% hydroxyethyl starch 130/0.4 (Voluven®; Fresenius-Kabi, Bad Homburg, Germany) at two times the volume of blood withdrawn and sacrificed at 24 hours post HS induction. During sacrifice, blood was withdrawn via aortic puncture under isoflurane anaesthesia, and the kidneys and

lung were excised, snap-frozen in metal cups on liquid nitrogen, and stored at –80°C until analysis.

Mouse mechanical ventilation model

The mechanical ventilation model employed has been described previously[11; 17]. In short mice were anesthetized with isoflurane inspiratory 3.0 % induction in N_2O (2 liter/min), and O_2 (1 liter/min). Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). After intubation anaesthesia was continued as described above. Endotracheal tube position was confirmed by bilateral chest excursions. Subsequently animals were connected to the ventilator (MiniVent®; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Tidal volume was set at 180 µl, frequency was set at 150/min. All animals received 4 cm H_2O PEEP.

Mouse hypoxia model

To examine the role of acute hypoxia on endothelial pro-inflammatory and hypoxia driven genes, a subset of mice was housed for 2 hours in respiratory cages to manipulate oxygen concentration. Oxygen concentration was set at 21%, 10% and 6% respectively. Hypoxia exposed mice and unexposed control mice were sacrificed under isoflurane anaesthesia, after which blood was withdrawn and kidneys and lung were harvested and handled as described above.

Gene expression analysis by quantitative RT-PCR

RNA was extracted from 20 x 5-µm cryosections from kidney and mouse lung, and isolated using the RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield and purity were measured by an ND-1,000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The Assay-on-Demand primers (ABI Systems, Foster City, CA) used in the PCR included the housekeeping gene GAPDH (assay ID Mm99999915_g1), E-selectin (assay ID Mm00441278_m1), ICAM-1 (assay ID Mm00516023_m1), VCAM-1 (assay ID Mm00449197_m1), TNF-α (assay ID

Mm00443258_m1), IL-6 (assay ID Mm00446190_m1), MCP-1 (assay ID Mm00441242_m1), HIF-1α (assay ID Mm00468869_m1), VEGF-A (assay ID Mm00437304_m). Duplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. According to the comparative CT method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene, yielding the ΔCT value. The relative mRNA level was calculated by $2^{-\Delta CT}$ and per group averaged.

Cytokine analysis

TNF-α, interleukin (IL)-6, and CXCL-1 levels in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA) (TNF-α, IL-6, CytoSet, BioSource, CA; CXCL-1: ELISA kit, R&D Systems, Minneapolis, MN). Lower detection limits: 32 pg/ml for tumour necrosis factor-α; 160 pg/ml for IL-6; 160 pg/ml for for CXCL-1.

Localization of adhesion molecule expression using immunohistochemistry

Localization of CD31, E-selectin, VCAM-1, and ICAM-1 expression was determined in kidney by immunohistochemistry. Snap frozen organs were cryostat cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 minutes. After drying, sections were incubated for 45 minutes at room temperature with primary rat anti-mouse antibodies recognizing CD31 (clone MEC13.3; Pharmingen BD Biosciences, Alphen aan de Rijn, The Netherlands), E-selectin (MES-1, kindly provided by Dr. D. Brown, UCB Celltech, Brussels Belgium), and ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After washing, endogenous peroxidase was blocked by incubation with 0.1% H_20_2 in PBS for 20 minutes. This was followed by incubation for 30 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (rabbit anti rat-Ig, DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with 2% normal mouse serum. Sections with isotype matched controls and E-selectin antibodies were further incubated for 30 min at room temperature with HRP-conjugated goat anti-rabbit antibody (Southern Biotech Association, Birmingham, Alabama, USA) diluted 1:100 in PBS. Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie, St.Louis, Missouri, USA) and sections were counterstained with Mayer's

hematoxylin (Klinipath, Duiven, The Netherlands).

Statistical analysis

Statistical significance of differences was studied by means of Student's t-test or ANOVA with post hoc comparison using Dunnet correction. First 90 minutes time points and 24 hours time points HS were compared with HS combined with mechanical ventilation. When there were no differences, HS and MV at the same time points were pooled, for increased statistical power. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered to be significant when $p < 0.05$.

RESULTS

Mice under anesthesia were intubated and mechanically ventilated or allowed to breathe spontaneously while blood was withdrawn to reach a MAP of 30 mmHg. No differences in blood pressure were observed between the groups, neither during the

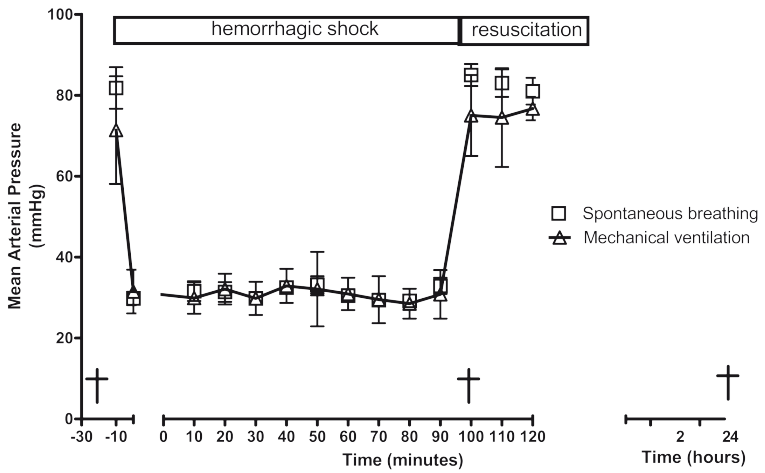

Figure 3.1. Study design and measured mean arterial pressure in mice during hemorrhagic shock in the presence or absence of mechanical ventilation.

Hemorrhage was induced by blood withdrawal as described in 'Materials and Methods'. Groups of mice were sacrificed (†) at the start of the experiment (control), or after 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a subgroup of mice was resuscitated and sacrificed 24 hours after shock induction. Spontaneous breathing mice (□) were compared with mechanically ventilated mice (▲) . Data are expressed as mean ± SD, n>5 per group.

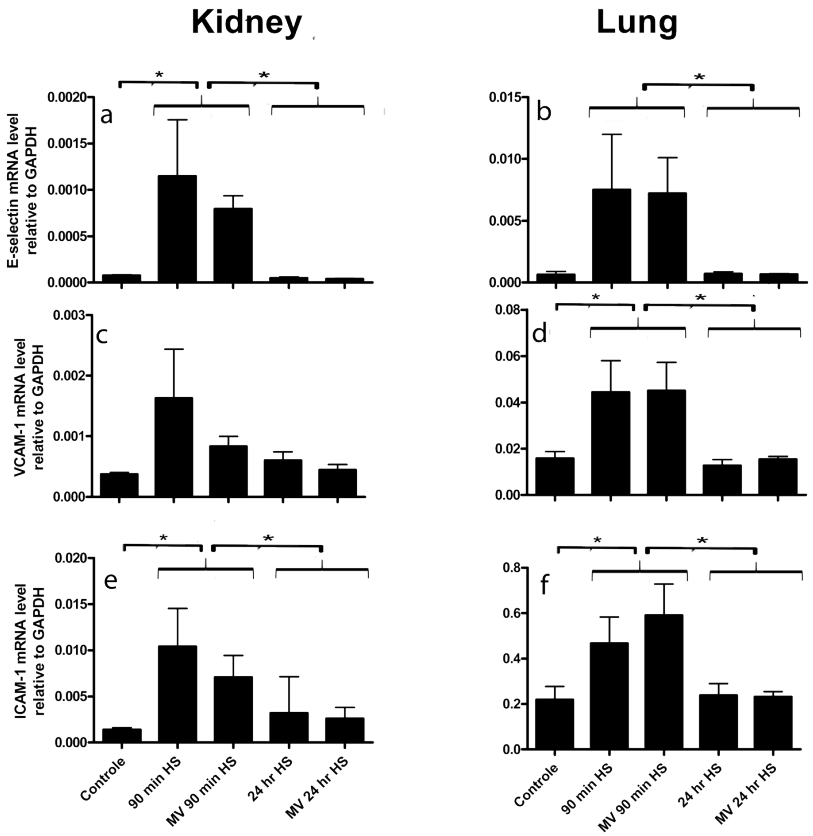

Figure 3.2. Kinetics of mRNA changes of endothelial cell adhesion molecules during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.
*Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f) in kidney (a, c, e) and lung (b, d, f) were analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5, * p< 0.05.*

shock phase nor during the post shock phase (figure 3.1). The initial blood pressure after induction in the spontaneous breathing group (mean 82 mmHg, SD 16.2) was statistically not significantly different than the blood pressure in the mechanical ventilation group (mean 71 mmHg, SD 13.8). Mice in all the groups remained normoxic and did not became hypercapnic (data not shown).

Ninety minutes after shock induction, an endothelial pro-inflammatory activation was observed reflected by increased E-selectin, VCAM-1, ICAM-1 mRNA expression levels (figure 3.2). After 24 hours the pro-inflammatory endothelial activation genes in both the mechanical ventilation and the spontaneously breathing group were back to baseline

level (figure 3.2). No differences between the spontaneous breathing and mechanically ventilated mice were found for E-selectin, VCAM-1, and ICAM-1 at 90 minutes or 24 hours after shock induction. The lung and the kidney have different endothelial response patterns during HS. VCAM-1 mRNA levels were upregulated in the lung after shock (figure 3.2d) but unchanged in the kidney (figure 3.2c). In the kidney and the lung, both mRNA of E-selectin and ICAM-1 were upregulated after 90 minutes of HS (figure 3.2a, 3.2b, 3.2e and 3.2f).

To further extend our knowledge on microvascular bed specific differences of endothelial activation in HS, we examined these endothelial pro-inflammatory activation

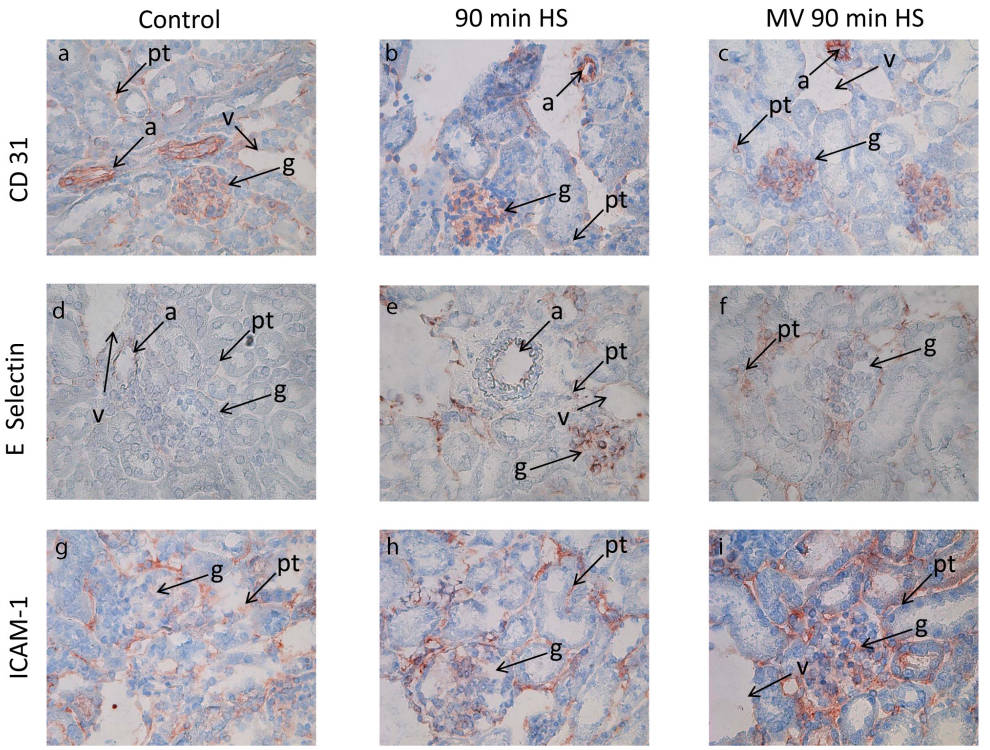

Figure 3.3. Endothelial marker gene CD31 and endothelial cell adhesion molecules E-selectin and ICAM-1 expression during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.

Immunohistochemical detection of CD31, E-selectin, and ICAM-1 in mouse kidneys. Staining was performed in healthy mouse tissue (a, d, g), after 90 minutes of HS (b, e, h), and after 90 minutes of mechanical ventilation in HS (c, f, i). Original magnification 200x. CD31 (a, b, c), E- selectin (d, e, f), and ICAM-1 (g, h, i) are stained red, while cells are stained blue. Specific renal microvascular beds are indicated by arrows: a = arteriole, g = glomerulus, pt = peritubular vasculature, and v = venule.

differences within the kidney by immunohistochemistry of different renal microvascular beds. The pan endothelial marker protein CD31 was expressed in all microvascular beds including arterioles, glomeruli, the peritubular vascular capillaries and venules in control mice (figure 3.3a) and did not change after shock induction with or without (figure 3.3b and 3.3c) mechanical ventilation. In the kidney, E-selectin was absent in the control kidney, but was strongly upregulated 90 minutes after shock induction in glomerular capillaries both with and without mechanical ventilation (figure 3.3d, 3.3e, 3.3f). In contrast in peritubular and arteriolar vascular beds E-selectin could not be detected after shock induction (3.3e and 3.3f). In control kidneys, constitutive ICAM-1 expression was observed in all vascular beds. In HS kidney increased ICAM-1 expression was observed most prominently in the peritubular capillaries (figure 3.3h and 3.3i). Overall no visible differences were found in the expression of adhesion molecules in the kidney related to MV.

Endothelial pro-inflammatory activation can be caused by pro-inflammatory cytokines[18] and hypoxia[19]. First, we investigated the mRNA expression levels in lungs and kidneys of the pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 in time. No differences between the spontaneous breathing and mechanically ventilated HS mice were found for TNF-α, IL-6, and MCP-1 90 minutes after shock induction (figure 3.4a-f). In the kidney and lung, 90 minutes HS induced a small increase in mRNA for TNF-α (figure 3.4a, 3.4b) and IL-6 (figure 3.4c and 3.4d), while MCP-1 mRNA levels were unchanged. (figure 3.4e, 3.4f). To investigate whether the pro-inflammatory cytokines TNF-α, CXCL-1 and IL-6 were produced in remote organs we measured soluble cytokine proteins in plasma. While TNF-α protein in plasma was not changed at 90 minutes (figure 3.5a), levels of the pro-inflammatory cytokines CXCL-1 and IL-6 were significantly increased after 90 minutes (figure 3.5b, 3.5c). Mechanical ventilation during 90 minutes of hemorrhagic shock did not affect these HS induced changes in IL-6 and CXCL-1 in the systemic circulation (figure 3.5). At 24 hours after the shock period, all pro-inflammatory cytokines in the plasma were back to baseline (figure 3.5).

Hypoxia can induce HIF-1α both via transcriptional control and via posttranslational processes affecting the protein level, with an increase in VEGF-A as one of the downstream consequences. We therefore investigated whether shock induced changes in cellular oxygen levels influenced HIF-1α and VEGF-A, in our model. During the shock

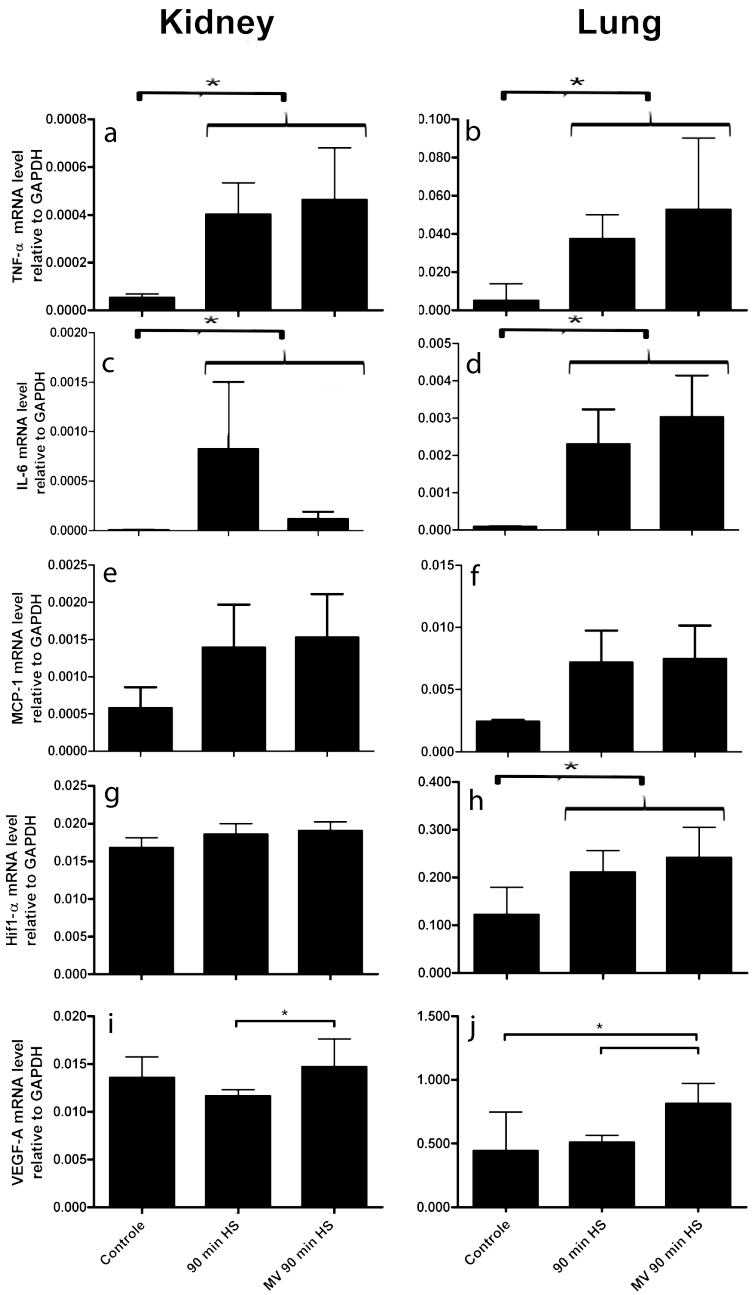

Figure 3.4. Kinetics of expression of pro-inflammatory and hypoxia related genes in kidney and lung during hemorrhagic shock in the presence or absence of mechanical ventilation.

*Gene expression levels of the pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 and the hypoxia related molecules HIF-1α and VEGF-A in kidney and lung analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5. p < 0.05 values are marked with * .*

period, HIF-1α mRNA was not induced in the kidney, while in the lung it led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation (figure 3.4g and 3.4h). VEGF-A mRNA showed a small but significant upregulation in the 90 minutes with MV compared to HS alone, both in lung and kidney compared to the spontaneous breathing mice (figure 3.4i and 3.4j).

The upregulation of HIF-1α in the lung and the upregulation of VEGF-A in the mechanical ventilation group implies that a hypoxic condition may have occurred during the shock

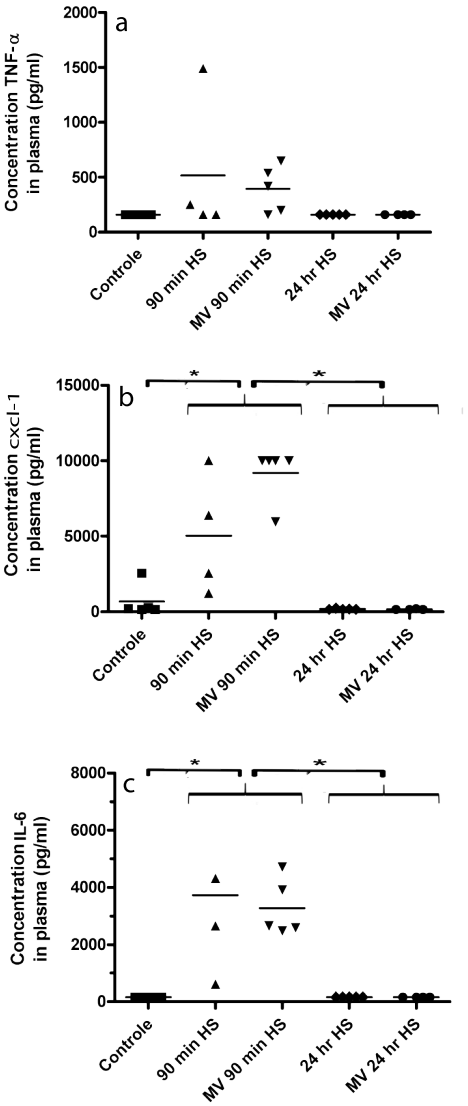

Figure 3.5. Pro-inflammatory cytokines in plasma during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.

*Levels of TNF-α, CXCL-1, and IL-6 in control (C), HS during 90 minutes, with and without mechanical ventilation and 24 after the HS insult with or without mechanical ventilation. Data are expressed as mean ± SD, n>4 for all groups. * p < 0.05 compared with control mice.*

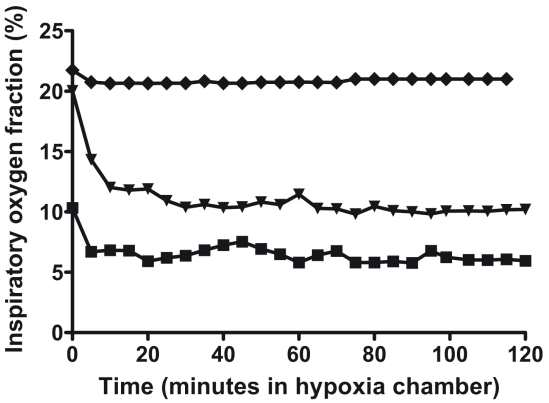

Figure 3.6. Inspiratory oxygen concentration of mice housed in hypoxic cages for 120 minutes.
Levels of inspiratory oxygen were controlled during a 120 minutes stay in hypoxic cages. Inspiratory oxygen levels were maintained at 21% (diamonds), 10% (triangles) and 6% (squares). Mice were sacrificed after 120 minutes.

period in the absence and presence of mechanical ventilation. To determine the potential contribution of tissue hypoxia to the pro-inflammatory endothelial activation observed, we next studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. To expose the mice to hypoxia during a similar period of time as the HS mice, including the time needed for instrumentation and anesthesia, mice were housed for 120 minutes in hypoxic cages with three different oxygen levels (figure 3.6). Neither 120 minutes of 6% oxygen, nor 10% oxygen (data not shown), induced the expression of the pro-inflammatory genes E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice (figure 3.7). Surprisingly, this acute and severe hypoxia also did not increase the mRNA expression levels of HIF-1α and VEGF-A in lung and kidney (figure 3.7m-p). The severe hypoxia, did, however induce a pro-inflammatory response in the lung; 2 hours of 6% hypoxia led to the upregulation of pro-inflammatory cytokines IL-6 and MCP-1 mRNA, while TNF-α mRNA was unchanged (figure 3.7). In the kidney no differences in mRNA for IL-6, MCP-1 and TNF-α were observed between normal oxygen levels and severe hypoxia (figure 3.7g-k).

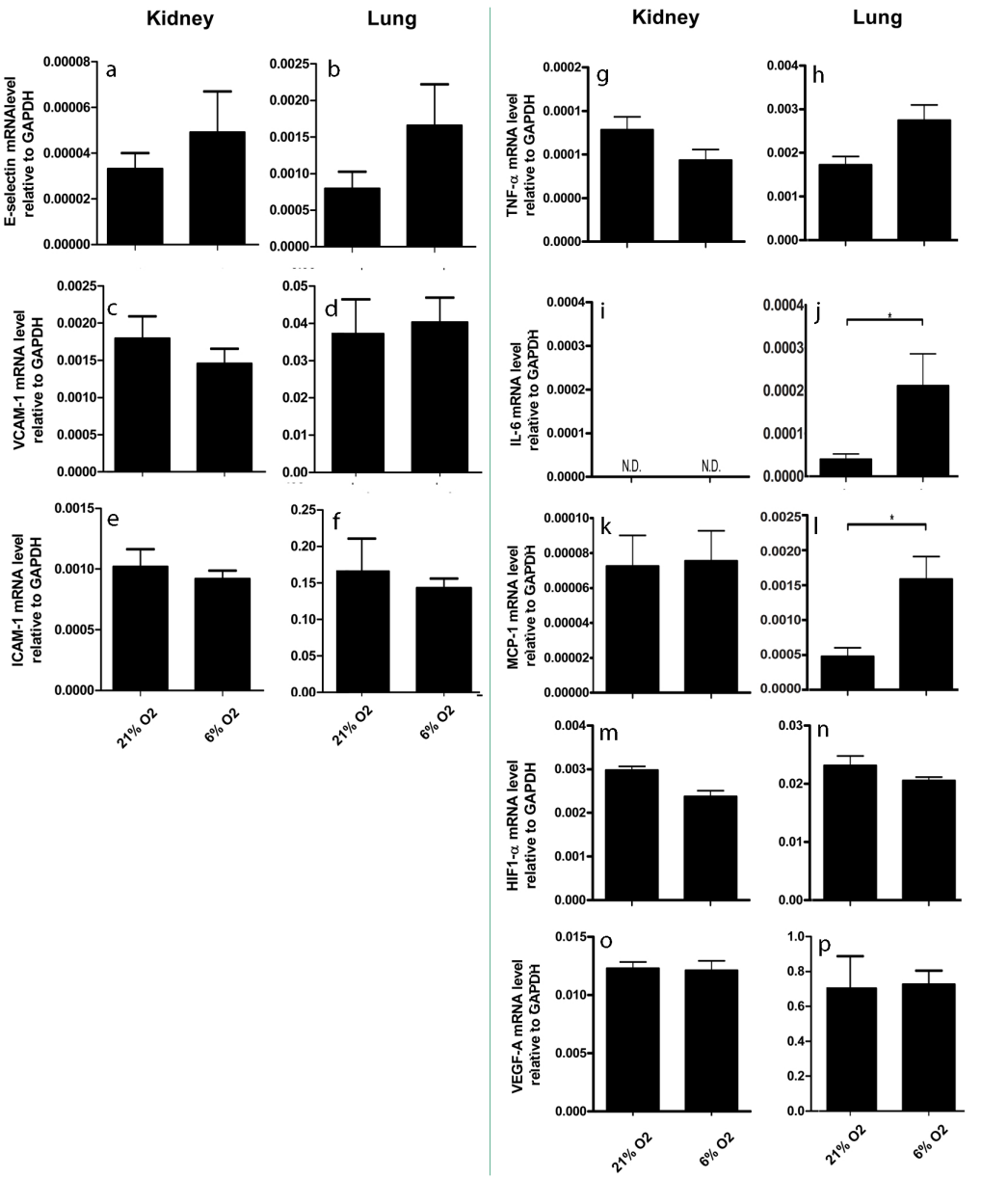

Figure 3.7. Kinetics of mRNA of endothelial cell adhesion molecules, pro-inflammatory cytokines and hypoxia related genes during acute hypoxia.

Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f), and the pro-inflammatory cytokines TNF-α (g, h), IL-6 (i, j), and MCP-1 (k, l) and the hypoxia related molecules HIF-1α (k, l) and VEGF-A (m, n) in kidney (a, c, e, g, i, k, m, o) and lung (b, d, f, h, j, l, n, p). Gene expression was analysed by quantitative RT-PCR using GAPDH as housekeeping gene after 120 minutes of acute hypoxia, during normoxia (21%) and hypoxia (6%). Data are expressed as mean ± SEM , n>5. N.D. = not detectable.

Discussion

The interaction between neutrophils and activated endothelium is crucial for the development of multiple organ dysfunction in patients suffering from hemorrhagic shock, but the effects of MV on this endothelial activation is not known. In this study of mechanical ventilation during hemorrhagic shock in mice, we demonstrated that the pro-inflammatory endothelial cell activation in the lung and the kidney was not prevented nor augmented by mechanical ventilation. Furthermore, we found that the organ specific endothelial activation in HS is not induced by an impaired oxygen delivery but merely by a systemic pro-inflammatory response induced by the hemorrhagic shock.

To our knowledge, we are the first to report the effect of mechanical ventilation during HS and its effects on EC activation in mice. Mechanical ventilation alone is able to induce an inflammatory response in mice and man[11; 20]. In mice, mechanical ventilation induced TNF-α, IL-6 and IL-1β in mice lung already 60 minutes after MV initiation, whereas plasma protein levels followed some time thereafter[11]. Moreover mechanical ventilation in mice led to endothelial activation in lung and distant organs within 120 minutes after MV initiation[12]. The data in our studies show that, compared to the HS insult, no additional detrimental effects on endothelial inflammation are induced when mechanical ventilation is performed in haemorrhagic shock animals. This implies that the insult given is too mild or too short to add additional detrimental effects.

Our results on the early pro-inflammatory cytokine release during HS corroborate several other animal studies. For example, Liu and Dubick showed in rats with an HS to a MAP of 50 mmHg during 60 minutes that the mRNA encoding for TNF-α was up-regulated in ileum, kidney, liver, and skeletal muscle[21]. Serum TNF-α was increased in a mouse model already 30 minutes after initiation of shock[22].

Cellular hypoxia is considered to be an important mediator of MODS after HS[23]. During hypoxic conditions, HIF-1α accumulates in the cell, translocates to the nucleus and forms a stable heterodimer, after which it induces gene transcription. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumour models[24; 25]. Vascular endothelial growth factor A is a downstream target gene of HIF-1α that is primarily regulated at the transcriptional level and a major controller of vascular permeability in shock states[6]. Under hypoxic conditions, up-regulation of VEGF-A by HIF-1α occurs

within minutes[26]. In cellular experiments using the human umbilical vein cell line, EA.hy926, incubation for 16 hours in 1% oxygen, induced HIF-1 nuclear translocation. This was accompanied by ICAM-1 and E-selectin mRNA upregulation compared to cells that were incubated in normoxia[19]. In Human Aortic Endothelial Cells (HAEC) however incubated for 8 hours in 4% oxygen hypoxia no upregulation of E-selectin, VCAM-1 or ICAM-1 could be observed by Illumina gene microarray analysis[27]. In our model however, we did not see any significant induction of mRNA of HIF-1α nor VEGF-A during HS alone, while mechanical ventilation combined with HS led to a small increase in HIF-1α and VEGF-A mRNA. Moreover HIF-1α and VEGF-A were not affected during our acute hypoxia experiments in mice without shock. Our data are contrary to Koury et al.[28], who showed that in a pressure-controlled HS model in the rat, HIF-1α levels as measured by Western blotting were increased in the ileac mucosa after 90 minutes of HS of a MAP of 30 mmHg. Hierholzer et al.[29] observed an increase in HIF-1α DNA binding activity of 3.2-fold in the lung after a 40 mmHg MAP shock period of 2.5 h. No increased HIF-1α activation in livers of animals subjected to 40 mmHg MAP HS for 60 minutes was found[30]. From these studies it becomes clear that there are large organ, insult and time-frame dependent differences that regulate HIF-1α responsiveness in HS models. Our data and those reported by others therefore suggest that severe whole body hypoxia is not the driving factor for EC activation in HS in kidney and lung, but that large inter-organ differences might exist.

Our animal model of HS with or without MV has several limitations. We used a short shock period to mimic the short and severe non-resuscitated HS seen in the clinic, while the described pro-inflammatory effects of MV might be more pronounced after longer periods of MV[11; 12]. Possibly, after the shock insult has resolved, ongoing MV may lead to additional organ damage. This clinical important question can however not be tested in this small rodent model because of instabilities of this model after prolonged MV. A larger animal model with more physiological monitoring might in the future provide more insight in the consequences of longer mechanical ventilation exposure after the shock insult on EC activation. Furthermore in our hypoxic cage experiments we did not measure blood gases nor oxygen delivery capacity. In man however, it has been shown that 12% normobaric hypoxia is sufficient to cause hyperlactatemia as an indicator of insufficient oxygen transport[31]. Therefore we hypothesised that 6% oxygen is sufficient

to induce an impaired oxygen delivery. This oxygen concentration leads to an estimated pO_2 of 4-5 kPa and an oxygen saturation of 50-60%. In our model the severe hypoxia induced a proinflammatory response in the lung; 2 hours of 6% hypoxia lead to the upregulation of IL-6 and MCP-1 mRNA. It is of note that in the hypoxic animals blood gases were uncontrolled. It cannot be excluded that the severe hypoxic animals became hypercapnic secondary to a decrease in ventilation, i.e. hypoxic ventilatory decline and/or muscle fatigue during the 2 hours exposure period. Lastly the translation of our rodent results to HS patients is difficult. In our experiments, we used young male, otherwise healthy mice, which do not model older patients with multiple co morbidities. These effects of aging and co-morbidity on the systemic and microvascular responsiveness to HS and mechanical ventilation are now under investigation.

The early endothelial activation found in this study suggests that the therapeutic window to attenuate endothelial cell activation takes place early during the shock insult. This EC activation is induced at least in part by a pro-inflammatory response induced by pro-inflammatory cytokines, while in the mouse whole body cellular hypoxia does not induce endothelial adhesion molecules in the same time frame. Mechanical ventilation perse does not add to the pro-inflammatory endothelial activation seen in lung and kidneys in our model of HS. Further studies will investigate potential therapeutic strategies to diminish pro-inflammatory endothelial activation in HS on endothelial dysfunction.

Acknowledgements

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Chapter 4

Adiponectin deficiency accentuates sepsis morbidity and mortality associated with endothelial dysfunction

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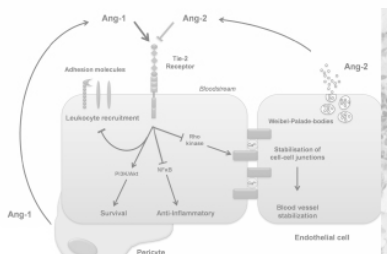

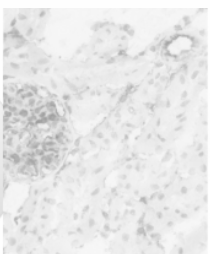

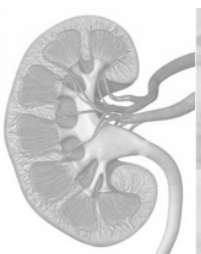

ABSTRACT

Increasing evidence suggests that the endothelium plays a critical role in sepsis pathophysiology. Adiponectin is an adipocyte-derived anti-inflammatory cytokine that has been shown to attenuate endothelial activation. Previous studies have demonstrated that sepsis is associated with reduced circulating levels of adiponectin. Thus, we hypothesized that sepsis-mediated adiponectin deficiency results in accentuated endothelial activation and secondary multi-organ dysfunction. We show that circulating levels of adiponectin are reduced in endotoxemia, but increased in cecal ligation puncture (CLP).

Quantitative RT-PCR for adiponectin and its receptors revealed no changes respectively significant reduction in gene expression in either model of sepsis, the pattern of response being model and organ specific. Adiponectin deficiency resulted in increased expression of endothelial adhesion and coagulation molecules in the lung, liver and kidney during sepsis, increased macrophage and neutrophil infiltration, and vascular leakage in the liver and kidney during experimental sepsis. This was accompanied by impaired survival following CLP and increased blood levels of interleukin (IL)-6, sVEGFR1, and soluble endothelial adhesion molecules sE-selectin and sICAM-1. Finally, adiponectin deficiency promoted end-organ injury in the liver and kidney while the lungs were not affected. These data suggest a protective role of adiponectin in sepsis and a role in diminishing endothelial dysfunction during sepsis.

Introduction

Over 750,000 cases of severe sepsis are diagnosed every year in the US. The mortality rate continues to be unacceptably high[1]. Increasing evidence suggests that the endothelium plays an important role in sepsis pathophysiology (reviewed in[2]). In animal and human models of sepsis, endothelial dysfunction is manifested by increased expression of cell adhesion molecules, enhanced production of procoagulants and cytokines, and altered release of nitric oxide[3; 4]. Collectively, these changes lead to increased leukocyte trafficking, fibrin deposition, vasomotor dysfunction, hemostatic imbalance, vascular permeability and inflammation.

Obesity is thought to be an independent risk factor for sepsis morbidity and mortality[5; 6]. Adipose tissue is a highly active endocrine organ. Adipocytes produce a number of adipokines, of which adiponectin is the most abundant. Circulating levels of adiponectin are reduced in patients with obesity, insulin resistance, atherosclerosis and related inflammatory disorders[7-9]. Adiponectin exerts profound anti-inflammatory and anti-atherosclerotic actions via its receptors, AdipoR1 and AdipoR2, which are expressed in liver and skeletal muscle, endothelial cells, macrophages, and smooth muscle cells *in vivo* and *in vitro*[10-12]. Previous studies have shown that the vasculoprotective effects of adiponectin are mediated by a suppression of endothelial cell activation, including cell adhesion molecule expression and nitric oxide release.

Recent studies have implicated a protective role of adiponectin in sepsis. However, the extent to which adiponectin deficiency is associated with endothelial cell dysfunction in sepsis remains largely unknown. In the present study, we show that mice that are null for adiponectin have increased mortality and morbidity in sepsis, including worsened endothelial cell dysfunction. Our findings suggest that adiponectin may exert a protective role in sepsis at the level of the vasculature.

MATERIALS AND METHODS

Sepsis models and tissue sample preparation

Male C57Bl/6 (Charles River), adiponectin knockout mice (adipoq$^{-/-}$, C57Bl/6j background)[13] and wild-type littermates at eight weeks of age were used for the study. In the endotoxemia model, mice were injected i.p. with 16 mg/kg lipopolysaccharide (LPS). CLP was performed as previously described[14; 15]. Sham operation was performed the same way as CLP procedure except the caecal puncture. Blood samples were collected in different groups at 16 h after LPS or CLP. Subsequently, animals were systemically perfused with PBS and organs were harvested and snap-frozen for RNA isolation and histological analysis. In subgroups of LPS-injected and control mice, epididymal white fat was harvested, and primary mouse adipocytes were separated from the stromal vascular fraction as previously described[16]. The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee approved all animal studies.

Measurement of mouse cytokines, soluble endothelial adhesion molecules, blood urea nitrogen levels and alanine aminotransferase levels

To harvest plasma or serum samples from mice, blood samples were collected by heart puncture into heparinized and non-treated tubes respectively, centrifuged, and the supernatant was stored until use at -80°C. To obtain serum, blood samples were coagulated overnight at 4˚C before centrifugation. Plasma levels of mouse adiponectin, leptin, soluble vascular growth factor receptor-1 (sVEGFR1, also known as Flt-1), interleukin-6 (IL-6), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1) and sE-selectin were measured by commercially available ELISA kits (R&D systems, Minneapolis, USA) according to the manufacturer's instructions. Blood urea nitrogen (BUN) and alanine aminotransferase (ALT) were measured as previously described[17; 18].

Tissue RNA isolation and quantitative RT-PCR analysis

Tissue RNA was isolated and purified cDNA was prepared for real-time PCR was performed as previously described[15; 19; 20].

Permeability assay

Sixteen hours after the CLP procedure, mice were anesthetized and injected i.v. with 200 µl Evans blue dye (1% in saline). Forty minutes later, mice were systemically perfused via heart puncture with PBS containing 2 mM EDTA for 5 minutes. Organs (lung, liver, and kidney) were harvested and minced, and then incubated in formamide for 3 days to extract Evans blue dye. Absorbances were measured at 620 nm.

Survival studies

Survival studies were performed in the CLP model. Both male adiponectin knockout mice and age-matched wild-type littermates were used for the study. Survival was assessed from 0 up to 96 h after CLP operation.

Statistical analysis

A one way analysis of variance followed by a Bonferroni correction was used to compare cytokine levels, vascular leakage, ALT, BUN levels and gene expression. The Wilcoxon log-rank test was used for the survival studies. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered significant when $p < 0.05$.

RESULTS

Plasma adiponectin levels are reduced in mouse models of sepsis

To investigate the effect of sepsis on circulating levels of adiponectin, mice were subjected to CLP or endotoxemia. Compared with unperturbed control mice, CLP resulted in reduced levels of adiponectin (6.9 ± 0.86 µg/ml in vs. 4.8 ± 0.37 µg/ml) (figure 4.1A). Surprisingly, however, sham-operation was associated with a further reduction in plasma levels (3.6 ± 0.26 µg/ml). Compared with non-injected controls, mice with endotoxemia (LPS 16 mg/kg i.p.) revealed significantly lower plasma adiponectin levels (8.2 ± 1.5 µg/ml vs. 5.9 ± 0.56 µg/ml) (figure 4.1B).

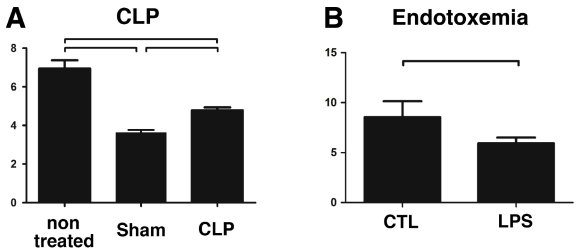

Figure 4.1. Plasma levels of adiponectin in murine sepsis models.
Twenty-four hours after onset of sepsis, adiponectin plasma levels in non-treated (CTL), Sham operated (Sham) and CLP operated mice (CLP) (A). Adiponectin plasma levels in non-treated (CTL) and 16 mg/kg i.p. injected LPS (LPS) mice (B). Data are expressed as mean ± SEM (n=8). - are statistical significant with P <0.05 (six mice per group).

mRNA levels of adiponectin and its receptors are altered in an organ-specific manner in mouse models of sepsis

We next performed real-time PCR analysis of adiponectin and its receptors, adiponectin receptor-1 and adiponectin receptor-2, using total RNA from various organs of CLP- or LPS-treated mice and their respective controls. The highest baseline expression of adiponectin mRNA was detected in epididymal adipose tissue (451.2 ± 34.6 mRNA copies/10^6 18S copies) followed by skeletal muscle (39.3 ± 10.5 mRNA copies/10^6 18S copies) (figure 4.2A). Low mRNA levels were found in other organs. In mice subjected to CLP, adiponectin mRNA expression was significantly decreased in lung (5.9-fold), kidney (4.7-fold) and epididymal fat tissue (1.8-fold), as compared with non-treated mice (figure 4.2A). Similar changes were observed in sham-operated mice. There were no detectable changes in adiponectin receptor1 mRNA levels in any of the organs tested (figure 4.2B). However, adiponectin receptor-2 revealed reduced mRNA expression in small intestine (2.5- fold) and epididymal fat tissue (2.8-fold) in CLP treated mice, compared with non-treated control and sham-operated mice, respectively (figure 4.2C).

In endotoxemia, adiponectin mRNA levels were significantly reduced in skeletal muscle (3.2-fold) and epididymal fat tissue (2.4-fold) (figure 4.2D). Adiponectin receptor1 mRNA expression was significantly decreased in brain (2.4-fold), lung (1.4-fold), skeletal muscle (1.3-fold) and fat tissue (2.0-fold) (figure 4.2F). Adiponectin receptor2 mRNA expression was significantly downregulated in lung (1.3-fold), liver (2.1-fold), kidney (1.4-fold), small intestine (3.8-fold), skeletal muscle (1.9-fold) and adipose tissue (12-fold) (figure 4.2G).

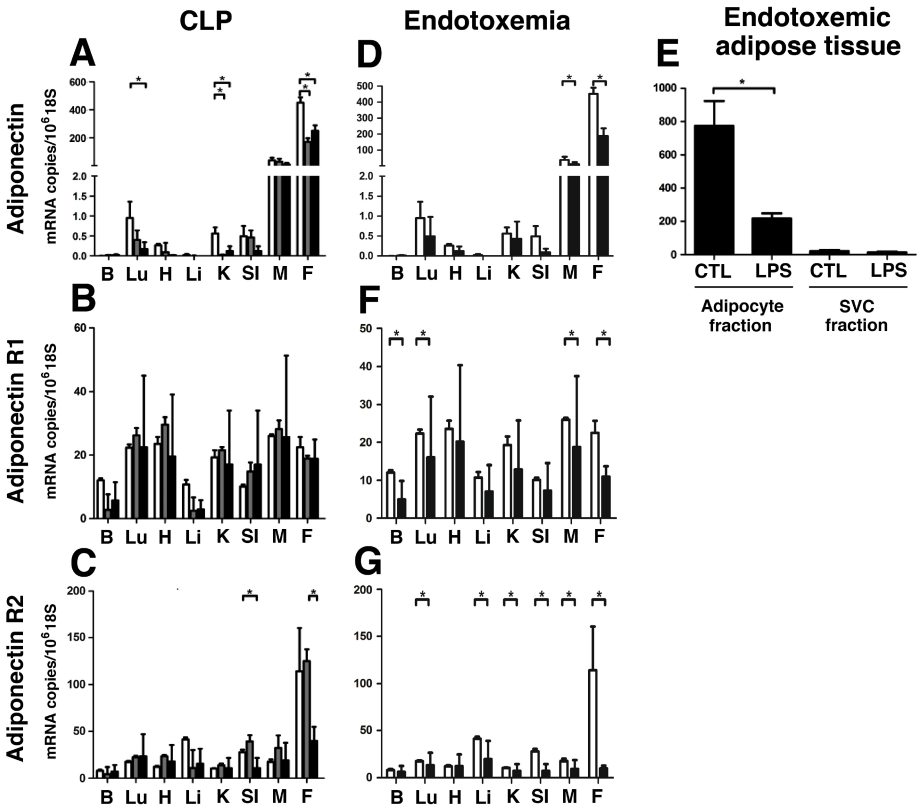

Figure 4.2. mRNA levels of adiponectin, adiponectin receptor 1 and - 2 during endotoxemia and CLP in various organs and in endotoxemic primary adipocytes.

*Quantitative RT-PCR analyses (mRNA copy number per 106 copies of 18S) of adiponectin (A, D), adiponectin receptor 1 (B, F), and Adiponectin receptor 2 (C, G) gene expression in multiple organs in CLP and endotoxemia models, respectively. White, grey and black bars represent non-treated, sham-operated and CLP treated mice, respectively (A-C). White and black bars represent non-treated and LPS-treated mice, respectively (D, F, G). Adiponectin mRNA expression in isolated adipocytes and stromal vascular cell fraction (SVC) isolated from epididymal fat tissue, in the absence or presence of LPS for 24 hrs (E). Data are expressed as mean ± SEM (n=6), * are statistical significant with P <0.05. B: brain, Lu: lung, H: heart, Li: liver, K: kidney, SI: small intestine, M: skeletal muscle, and F: epididymal fat pad.*

Thus, sepsis results in organ-specific changes in mRNA expression of adiponectin and its receptors.

To identify the cell type(s) that express adiponectin, adipocytes and stromal vascular cells (SVC) were separated from LPS-treated or non-treated mouse epididymal fat pads as previously described[16]. Under normal conditions, adiponectin was preferentially expressed in the adipocyte fraction (figure 4.2E). Adiponectin in the adipocyte fraction but not the SVC fraction was significantly reduced in endotoxemia (figure 4.2E).

Adipoq$^{-/-}$ mice have increased mortality in CLP

To determine whether adiponectin plays a protective role in sepsis, we carried out a survival study of adipoq$^{-/-}$ mice subjected to CLP. As shown in figure 4.3 none of the adiponectin deficient mice survived the observation period of 96 h after CLP, compared to 35.7% survival in the wild-type littermates (p=0.0080) (figure 4.3). Thus, adiponectin has a protective effect in sepsis.

Adipoq$^{-/-}$ mice have increased circulating levels of inflammatory markers and endothelial adhesion cell adhesion molecules in CLP

To investigate the role of adiponectin in inflammation and endothelial activation, we measured circulating levels of established sepsis biomarkers (IL-6, sVEGFR1 and leptin) as well as the soluble forms of endothelial adhesion molecules (sICAM-1, sVCAM-1 and sE-selectin). When subjected to CLP, plasma sVEGFR1 (1.9-fold) and IL-6 (7.3-fold) were significantly elevated in adipoq$^{-/-}$ vs. adipoq$^{+/+}$ mice, whereas leptin levels were induced to comparable levels in knockout and wild-type mice (figure 4.4A-C). In response to CLP, adipoq$^{-/-}$ mice also demonstrated higher circulating levels of sICAM-1 (figure 4.4D) and sE-selectin (figure 4.4F), but not sVCAM-1 (figure 4.4E) compared with wild-type control (adipoq$^{+/+}$) animals. In Sham-operated animals (adipoq$^{-/-}$ or adipoq$^{+/+}$) no differences could be observed in any of the above markers.

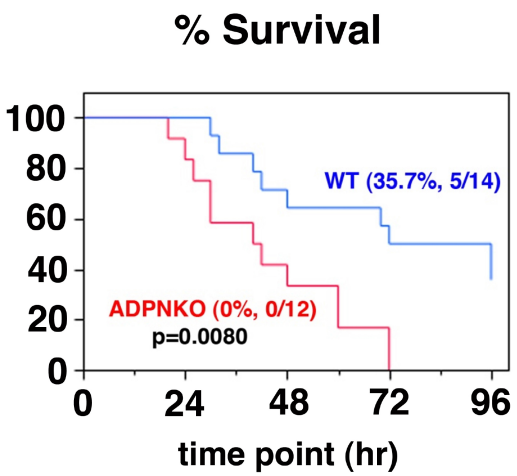

Figure 4.3. Impaired survival in adiponectin deficient mice during polymicrobial sepsis.
Survival studies on adiponectin KO (n=12) and wildtype (n=14) mice after CLP-induced polymicrobial sepsis.

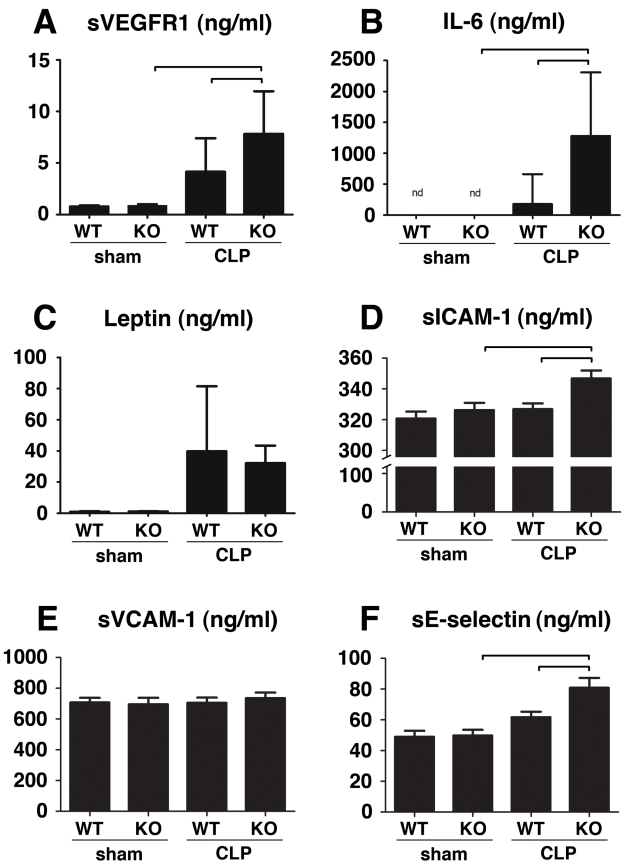

Figure 4.4. Effects of adiponectin deficiency on plasma levels of sVEGR1, IL-6, leptin, sICAM-1, sVCAM-1 and sE-selectin during CLP-induced microbial sepsis.

Twenty-four hours prior to plasma preparation, mice were treated with CLP or sham. Plasma levels of sVEGFR1 (A), IL-6 (B) and leptin (C), sICAM-1 (D), sVCAM-1 (E) and sE-selectin (F) are shown. Data are expressed as mean ± SEM (n=4) of three independent experiments; - are statistical significant with P <0.05. nd: non detectable levels.

Adipoq$^{-/-}$ mice have organ-specific changes in mRNA expression of endothelial activation markers in CLP

To determine the extent to which adiponectin deficiency influences endothelial cell activation, we employed real-time PCR to measure mRNA expression of a panel of vascular-related genes in organs commonly affected by sepsis, namely the liver, kidney and lung. At 16 h following CLP, adipoq$^{-/-}$ mice demonstrated significantly higher mRNA expression (compared with adipoq$^{+/+}$ mice) of ICAM-1 in the liver, kidney and lung (3.9-fold, 3.5-fold and 1.6-fold, respectively) (figure 4.5A, G, M), E-selectin in the liver,

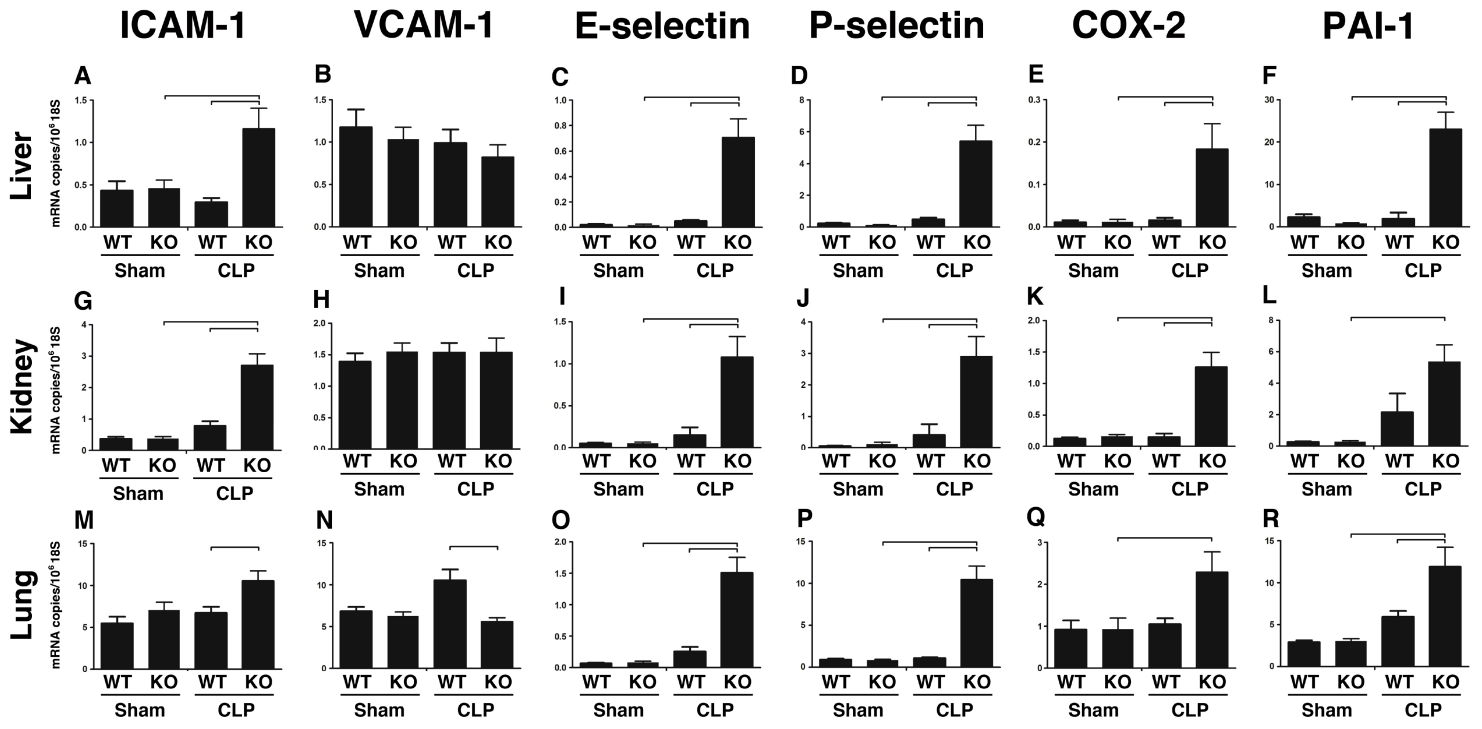

Figure 4.5. Effects of adiponectin deficiency on mRNA expression of endothelial cell adhesion and coagulation related molecules during CLP-induced microbial sepsis.

The results of quantitative RT PCR analyses (mRNA copy number per 106 copies of 18S) of ICAM-1 (A,G,M), VCAM-1 (B,H,N), E-selectin (C,I,O), P-selectin (D,J,P), COX-2 (E,K,Q), and plasminogen activator inhibitor-1 (F,L,R) in the liver (A–F) and kidney (G–L) and lung (M-R) of Sham operated (Sham) respectively CLP (CLP) operated wild type (WT) and adiponectin knock out (KO) mice. All data are mean ± SEM (n≥3). - are statistical significant with P <0.05.

kidney and lung (14-fold, 7.1-fold and 5.9-fold, respectively) (figure 5C, I, O), P-selectin in the liver, kidney and lung (11-fold, 7.1-fold and 9.6-fold, respectively) (figure 4.5D, J, P), cyclooxygenase-2 (COX-2) in the liver, kidney and lung (11-fold, 8.5-fold and 2.2-fold, respectively) (figure 4.5E, K, Q), and plasminogen activator inhibtor-1 (PAI-1) in the liver, kidney and lung (12-fold, 2.5-fold and 2.0-fold, respectively) (figure 4.5F, L, R). In contrast, there were no differences in VCAM-1 mRNA expression between adipoq$^{-/-}$ and adipoq$^{+/+}$ mice subjected to CLP in the liver and kidney (figure 4.5B, H), whereas adiponectin knockout actually attenuated CLP-mediated induction of VCAM-1 in the lung (figure 4.5N). There were no differences in gene expression between control and adiponectin-null mice following sham surgery.

In response to CLP, adipoq$^{-/-}$ mice have increased infiltration of leukocytes and barrier dysfunction in the kidney and liver

We next wished to determine whether the increased expression of endothelial cell adhesion molecules in adiponectin-null mice is associated with tissue influx of macrophages and neutrophils. To that end, we carried out immunohistochemistry using antibodies against Mac-1 (a marker of monocytes/macrophages) and Ly6G (a marker of neutrophils). As shown in figure 4.6A-D, CLP resulted in elevated Mac-1 and Ly6G staining in the kidney and liver of knockout mice compared with wild-type mice. CLP-mediated influx of macrophages in adipoq$^{-/-}$ mice occurred primarily in the glomerular and peritubular regions of the kidney and the sinusoids of the liver.

Vascular leakage is another hallmark of sepsis. To determine the effect of adiponectin deficiency on barrier function in sepsis, we injected Evans Blue in mice subjected to CLP and quanititated extravasation of the dye in liver, kidney and lung. Compared with wild-type controls, adiponectin knockout resulted in increased CLP-mediated vascular permeability in the liver (1.5-fold) (figure 4.6E) and kidney (1.9-fold) (figure 4.6F), but not the lung (figure 4.6G).

Adipoq$^{-/-}$ mice have worsened renal and liver function in CLP

We next investigated the effects of adiponectin deficiency on liver and kidney function during sepsis. Adiponectin deficiency resulted in increased levels of both blood urea nitrogen (BUN) (2.2-fold) (figure 4.7A) and alanine aminotransferase (ALT) (3.1-fold)

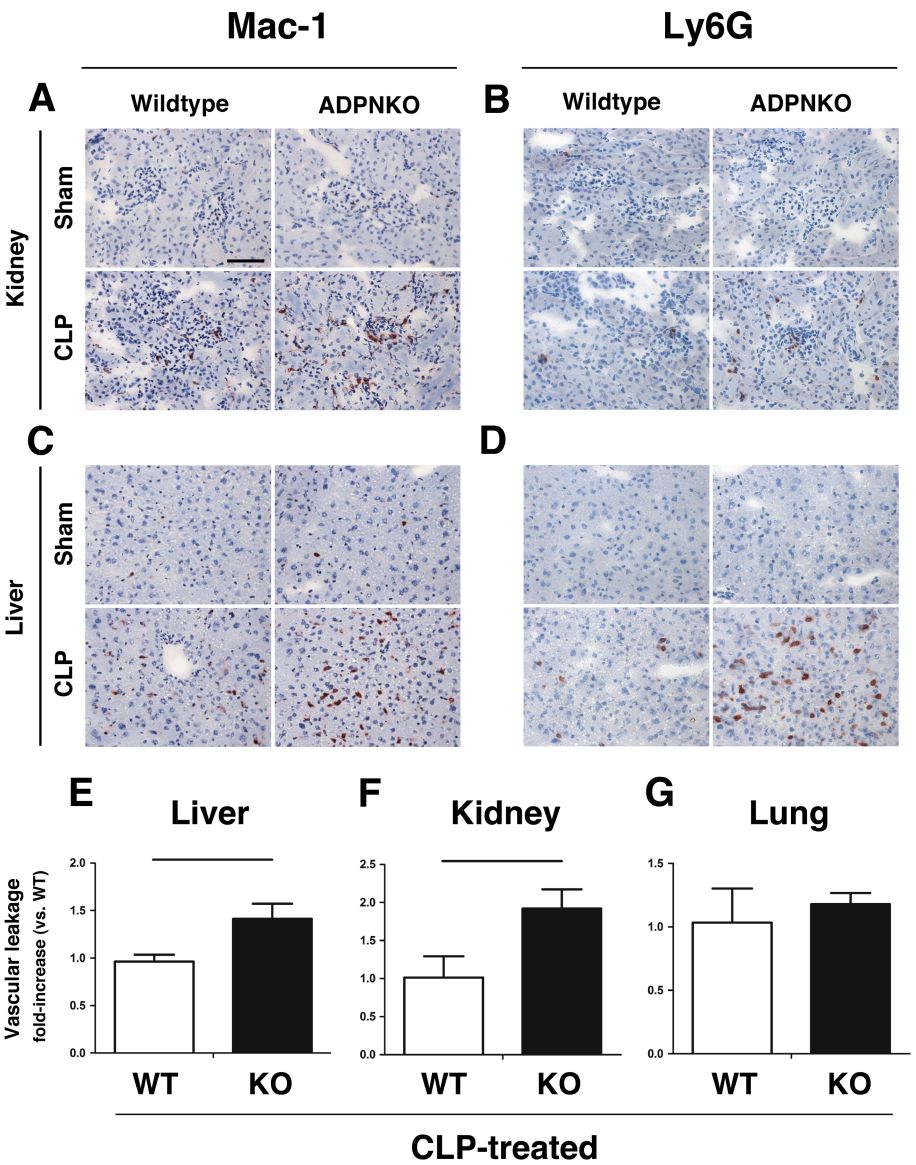

Figure 4.6. Effects of adiponectin deficiency on polymorphonuclear neutrophils and macrophage infiltration, and vascular barrier function in kidney and liver in polymicrobial sepsis.

Immunohistochemical detection of macrophage influx in mouse kidneys (A) and liver (C), of Sham mouse tissue, respectively CLP exposed mice as assessed by Mac-1 in mouse (Mac-1, red; Hematoxylin blue). Immunohistochemical detection of neutrophil influx as assessed by Ly6G in mouse kidneys (B) and liver (D), of sham-operated mouse tissue, respectively CLP exposed mice (Ly6G: red, Hematoxylin: blue). Twenty-four hours prior to i.v. injection of Evans blue dye, mice were treated with CLP. Quantitative data of Evans blue extravasation in the liver (E), kidney (F) and lung (G) is shown. Data is normalised for the OD 620nm in control organs and expressed as mean ± SEM (n=3) of two independent experiments. Scale bar in panel A applies to other panels B, C and D (scale bar= 50 µm).

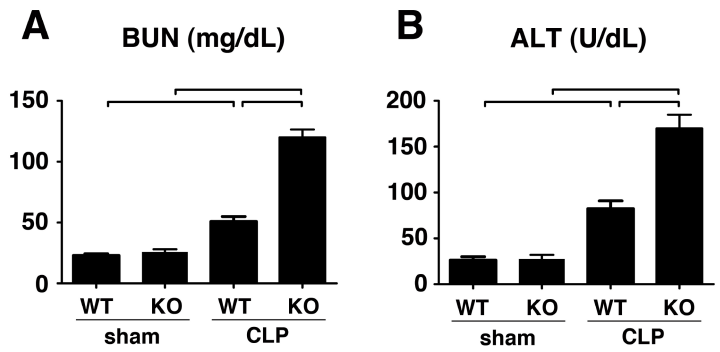

Figure 4.7. Impaired renal and liver function in adiponectin deficient mice during polymicrobial sepsis. *Kidney function as assessed by blood urea nitrogen (BUN) (A), liver function as assesed by alanine aminotransferase (ALT) (B). Data is expressed as mean ± SEM (n≥4). - are statistical significant with P <0.05.*

(figure 4.7B) compared to sham-operated mice. Adiponectin deficiency resulted in further induction of both BUN (2.3-fold) (figure 4.7A) and ALT (2.1-fold) (figure 4.7B) levels during sepsis, as compared to CLP in wild-type mice.

DISCUSSION

Adiponectin, a cytokine that is specifically expressed in adipocytes, has anti-inflammatory and vasculoprotective properties. Previous studies have shown that adiponectin levels are reduced in mice subjected to CLP[21] or endotoxemia[22]. Moreover, a small study in humans demonstrated an association between low levels of adiponectin and septic shock[23]. Collectively, these published findings suggest that adiponectin may have a protective role in sepsis. Consistent with this hypothesis, a recent report showed that adiponectin-null mice have increased mortality following CLP[24].

The goal of the present study was to extend these findings and determine the effect of adiponectin deficiency on endothelial cell activation and dysfunction. Our data confirm that adiponectin levels are indeed reduced in response to endotoxemia or CLP, compared with non-injected and sham-operated controls, respectively. Also consistent with previous reports, we found that adiponectin deficiency is associated with reduced survival in CLP. Finally, we demonstrated that adiponectin deficiency is indeed associated with increased endothelial dysfunction in sepsis.

In contrast to the lower levels of plasma adiponectin in sepsis, mRNA levels adiponectin

were comparable in tissues examined from CLP and sham-operated controls. There are several possible explanations for this discrepancy. First, transcriptional levels may be significantly reduced in a non-sampled tissue, such as subcutaneous or mesenteric fat. Second, post-transcriptional modifications, including hydroxylation and glycosylation of the adiponectin transcript may affect mRNA stability and translation efficiency[25; 26]. Finally, it is possible that sepsis inhibits the release of adiponectin into the circulation.

Recent studies have implicated a role for adiponectin in attenuating endothelial cell activation and dysfunction. For example, under *in vitro* conditions, adiponectin has been shown to stimulate the production of nitric oxide[27]. Adiponectin also inhibits tumor necrosis factor (TNF)-α-induced expression of cell adhesion molecules in endothelial cells[28]. Mice that are null for adiponectin demonstrate impaired endothelium-dependent vasorelaxation, increased endothelial expression of E-selectin and VCAM-1, increased leukocyte rolling and adhesion in the microcirculation, and reduced production of endothelial nitric oxide[29]. In a mouse thioglycollate-induced inflammation model, thioglycollate challenge resulted in increased expression of VCAM-1 and ICAM-1 in the aortas of adipoq$^{-/-}$ compared with adipoq$^{+/+}$ mice[24]. This effect was reversed with systemic administration of recombinant adiponectin. Our data demonstrating that CLP induces circulating levels of endothelial cell adhesion molecules and mRNA expression of endothelial activation markers (and secondary leukocyte infiltration) provides further evidence for a vasculoprotective role of adiponectin, specifically in the setting of sepsis.

In contrast to the increased basal expression of endothelial VCAM-1 in adipoq$^{-/-}$ mice[29] and the super-induction of VCAM-1 with thioglycollate challenge in these animals[24], CLP did not result in increased plasma levels of sVCAM-1 or VCAM-1 mRNA expression in septic or non-septic adipoq$^{-/-}$ mice. The differences in results may relate to differences in the model employed (basal expression vs. thioglycollate challenge vs. CLP). Alternately, it is possible that VCAM-1 mRNA and protein expression is induced in untested vascular beds, and/or that protein expression is induced at a post-transcriptional level.

Adiponectin has been shown to protect cultured endothelial cells from TNF-α-mediated barrier dysfunction associated with increased actin stress fibers, intercellular gap formation and β-tubulin disassembly[30]. Our data show that adiponectin deficiency exacerbates CLP-mediated vascular permeability in the liver and kidney. It is possible that this effect is simply a marker of sepsis morbidity. However the observation that

the lung is spared argues against this possibility, and makes it more likely that the loss of barrier function in the knockout mice is directly related to the loss of adiponectin signaling at the level of the endothelium.

CLP in adipoq$^{-/-}$ resulted in higher BUN compared with adipoq$^{+/+}$ controls. Given that the knockout mice also demonstrated increased adhesion molecule expression and leukocyte infiltration as well as enhanced barrier dysfunction in the kidney, it is possible that impaired adiponectin signaling in renal endothelium is responsible for this effect. Similarly, the increased ALT in septic knockout animals corresponds to changes in cell adhesion molecule expression, leukocyte infiltration and vascular permeability in that organ, and is thus consistent with a loss of impaired adiponectin signaling in the liver.

In summary, our data strongly suggest a mechanistic link between adiponectin and sepsis. Adiponectin deficiency exacerbates a spectrum of sepsis-induced vascular phenotypes, which in turn, may contribute to multi-organ dysfunction and death in this disease. Further studies are required to determine mechanisms underlying acquired adiponectin deficiency, as occurs in obesity, and to connect the adiponectin-deficient state to the organ-specific endothelial dysfunction we have reported here. Finally, the prognostic value of measuring circulating adiponectin and the therapeutic potential of adiponectin in patients with sepsis syndrome remain to be determined.

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Chapter 5

Bench-to-bedside review: Angiopoietin signalling in critical illness - a future target?

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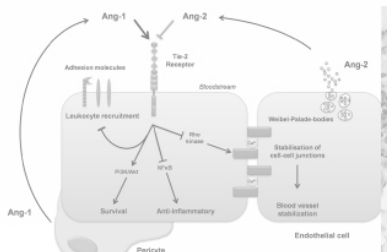

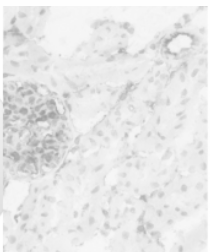

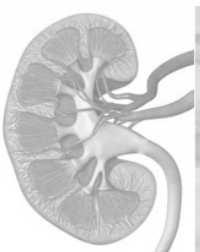

ABSTRACT

Multiple organ dysfunction syndrome (MODS) occurs in response to major insults such as sepsis, severe haemorrhage, trauma, major surgery and pancreatitis. The mortality rate is high despite intensive supportive care. The pathophysiological mechanism underlying MODS are not entirely clear, although several have been proposed. Overwhelming inflammation, immunoparesis, occult oxygen debt and other mechanisms have been investigated, and – despite many unanswered questions - therapies targeting these mechanisms have been developed. Unfortunately, only a few interventions, usually those targeting multiple mechanisms at the same time, have appeared to be beneficial. We clearly need to understand better the mechanisms that underlie MODS. The endothelium certainly plays an active role in MODS. It functions at the intersection of several systems, including inflammation, coagulation, haemodynamics, fluid and electrolyte balance, and cell migration. An important regulator of these systems is the angiopoietin/Tie2 signalling system. In this review we describe this signallingsystem, giving special attention to what is known about it in critically ill patients and its potential as a target for therapy.

INTRODUCTION

Critical illness is a life threatening disease by definition. Patients treated for their critical illness in the intensive care unit have infection, trauma, major surgery, hemorrhagic shock, pancreatitis or other major insults. Despite maximal supportive care, severely ill patients treated in intensive care units are still likely to die, usually after an episode of increasing failure of multiple organs. The outcome is frequently detrimental usually after an episode of increasing failure of multiple organs[1.]

The mechanisms underlying this multiple organ dysfunction syndrome (MODS) are not known[2], although several have been proposed, including overwhelming infection or immune response, immune paralysis, occult oxygen debt, and mitochondrial dysfunction [3-5]. Although these potential mechanisms have features in common, it is not clear whether MODS is a final common pathway or when it is engaged. The innate and adaptive immune systems, coagulation, and hormonal and neuronal signalling are undoubtedly involved and are all connected. For example, the hypoxic response is linked to innate immunity and inflammation by the transcription factor nuclear factor-κB (NF-κB)[6]. It is no coincidence that the few interventions that appear to be of benefit, although this is still under debate, have pleiotropic mechanisms of action[7-9]. Thus, it seems reasonable to study the intersections between and within cellular and molecular systems to elucidate the interactions and to develop therapeutic options.

One of the central cellular players in this system is the endothelial cell (EC). Once thought to serve as an inert vascular lining, ECs are highly heterogeneous and constitute an active disseminated organ throughout the circulatory system. ECs form the border between every organ and the bloodstream and thus with the rest of the body. The EC receives and gives signals, stores active substances of multiple systems, and regulates the passage of fluids, electrolytes, proteins and cells. The EC has a time and place dependent phenotype that is dynamically controlled, and its reactions to stimuli are specific to organ and vascular bed[10-13]. The EC merits robust investigation in critical illness, as in vascular medicine[14].

ECs fulfil three functions. First, they participate in the formation of new blood vessels. This is important in embryogenesis and organogenesis in normal physiology and in wound repair, but it is considered pathologic in tumour growth and diabetes[15]. Second,

in the adult organism, ECs help to maintain homeostasis, including fluid, electrolyte and protein transport, and cell migration into and out of the vessel, and to regulate blood flow. Third, ECs react and respond to disturbances of homeostasis (for example, in inflammation, coagulation and hypoxia/reperfusion).

All three functions are involved in MODS, in which ECs are shed, blood flow regulation is hampered, vessels become leaky, cells migrate out of the vessel and into the surrounding tissue, and coagulation and inflammation pathways are activated[16]. The machinery involved - receptors, signalling pathways and effectors - is largely the same in each function, but the net effect is determined by the balance between the parts of the machinery and the context[15].

The angiopoietin/Tie2 signalling system (Ang/Tie system) appears to be crucial in all three functions[17; 18]. The Ang/Tie system, which was discovered after vascular endothelial growth factor (VEGF) and its receptors, is mainly restricted to EC regulation and is the focus of this review. Accumulating evidence suggests that this system is nonredundant and is involved in multiple MODS-related pathways. All components of potential pathophysiological mechanisms in MODS should be viewed within their own context, because all systems are mutually dependent. Thus, examination of the Ang/Tie system might offer insight into the mechanisms underlying MODS and provide opportunities for therapeutic intervention.

Is the Ang/Tie system involved in critical illness?

The notion that the Ang/Tie system contributes to disease pathogenesis is supported by clinical studies and studies in animal models, and by the relation between symptoms of critical illness and disturbances in this system. In mice, Ang-2 over-expression in glomeruli causes proteinuria and apoptosis of glomerular ECs[19]. In a rat model of glomerulonephritis, Tie2 is over-expressed by ECs, and Ang-1 and Ang-2 are over-expressed by podocytes in a time dependent way during the repair phase [20]. Therefore, Ang/Tie might be involved in renal failure and repair.

Lung dysfunction is common in critical illness, and evidence of Ang/Tie involvement has been found in animal models. In a rat model of acute respiratory distress syndrome, Ang-1 reduces permeability and inflammation, whereas Tie2 deficiency increases

damage[21]. In an experimental model of asthma, Ang-1 mRNA was decreased, and Ang-1 supplementation decreased alveolar leakage and NF-κB dependent inflammation[22]. In hypoxia induced pulmonary hypertension in rats, decreased activity of the Tie2 pathway is contributed to right ventricular load, and his effect was antagonized by Ang-1[23]. On the other hand, a causative role for Ang-1 in pulmonary hypertension has also been suggested[24]. In hyperoxic lung injury, Ang-2 is involved in lung permeability and inflammation[25].

Ang/Tie also may contribute to critical illness in patients with pulmonary conditions. Ang-1 and Ang-2 concentrations in sputum from asthma patients correlated with airway microvascular permeability[26]. In patients with exudative pleural effusion, the Ang-2 level was increased whereas Ang-1 was unchanged[27]. Ang-2 levels are associated with pulmonary vascular leakage and the severity of acute lung injury. Plasma of patients with acute lung injury and high Ang-2 disrupts junctional architecture *in vitro* in human microvascular ECs[28; 29].

Patients with cardiovascular disorders also exhibit changes in the Ang/Tie system. Circulating Ang-1 concentrations are stable in patients with atrial fibrillation, but Ang-2 concentrations are increased, along with markers of platelet activation, angiogenesis and inflammation[30]. Patients with hypertension resulting in end-organ damage have increased levels of circulating Ang-1, Ang-2, Tie2 and VEGF[31]. Congestive heart failure is associated with elevated plasma levels of Ang-2, Tie2 and VEGF, but normal levels of Ang-1[32]. A similar pattern is seen in acute coronary syndrome[33].

Circulating levels of components of the Ang/Tie system have been measured in patients admitted to the critical care unit. In trauma patients plasma Ang-2, but not plasma Ang-1 and VEGF, is increased early after trauma, and the level correlated with disease severity and outcome[34]. In children with sepsis and septic shock, Ang-2 levels in plasma were increased and once again correlated with disease severity, whereas Ang-1 levels were decreased[35]. The same Ang-1/Ang-2 pattern is seen in adults with sepsis[28; 36-39]. The results of studies of the Ang/Tie system in humans are summarized in table 5.1. In sepsis, VEGF and its soluble receptor sFLT-1 (soluble VEGFR-1) are also increased in a disease severity-dependent manner[40-42]. The picture that emerges from these studies is that the Ang/Tie signalling system appears to play a crucial role in the symptoms of MODS. Findings in animal models and in patients suggest that Ang-1 stabilizes ECs and

Table 5.1. Clinical studies performed in critically ill patients on systemic levels of ANG1, ANG2 and soluble Tie2

Article	Publication Year	Patients	Number of patients in the study	Ang-1	Ang-2	Soluble TIE2	Clinical effects
Lee (ref 33)	2004	Acute coronary syndrome	82 acute myocardial infarction; 44 unstable angina; 40 Stable coronary artery disease and 40 HC	Not statistically different	Increased in AMI compared to SA, USA and HC	Increased in AMI compared to HC, SA and USA	Not determined
Kugathasan (ref 23)	2005	Pulmonary arterial hypertension	6 idiopatic pulmonary hypertension; 7 pulmonary hypertension associated with other disease, 8 HC	Not statistically different	Not statistically different	mRNA for Tie2 is upregulated in the lungs of pulmonary hypertension patients compared to controls	Not determined
Parikh (ref 28)	2006	Sepsis	ICU patients: 17 severe sepsis; 5 mild sepsis; 29 HC	Not statistically different	Increased in severe sepsis compared to HC	Not determined	Ang-2 levels correlate with a low PaO2/FiO2 level
Callagher DC (ref 180)	2007	Vascular leakage in interleukin 2 therapy	14 Il2 therapy: 4 IL2 and Bevacizumab	Not determined	Increased during therapy	Not determined	Ang-2 levels rise during the days Il2 therapy is given. High levels on day 3 are prognostic for stopping Il2 therapy beacause of vascular leakage
Gallagher DC (ref 29)	2007	ARDS patients	45 ICU; 18 ARDS	Not determined	Increased in patients with ARDS compared to ICU patients. Within ARDS group increased in non survivors	Not determined	Higher levels of Ang-2 on day patient meet ARDS criteria. Ang-2 levels in ARDS patients are correlated with mortality
Giuliano JS (ref 35)	2007	Septic shock children	ICU patients 20 SIRS; 20 Sepsis; 61 Septic shock; 15 HC	Ang-1 is lower in septic shock vs sepsis and SIRS	Increased in septic shock compared to HC, SIRS and sepsis	Not determined	Not determined
Orfanos (ref 37)	2007	ICU patients	ICU patients: 6 no SIRS; 8 SIRS; 16 sepsis; 18 severe sepsis; 13 septic shock	Not determined	Increased in severe sepsis compared to no SIRS and sepsis	Not determined	Ang-2 levels show a relation with severe sepsis and TNF-α levels
Scholz (ref 181)	2007	Liver Cirhosis	180 liver cirrhosis, 40 HC	Not determined	Increased in cirrhosis compared to control	Not determined	Not determined
Ganter (ref 34)	2008	Trauma patients in ER	208 trauma	Unchanged	Increased within 30 minutes of emergenecy room admission	Not determined	Ang-2 levels correlate with met ISS and mortality. Ang-2 is higher in non survivors compared with survivors
van der Heiden (ref 39)	2008	Septic and non septic critically ill patients	24 sepsis, 88 non septic critically ill, 15 HC	Ang-1 is lower in sepsis and in critically illness compared to controls	Increased in sepsis and non sepsis crically ill patients. Septic patients have higher levels compared to non sepsis critically ill patients	Not determined	Ang-2 levels are associated with pulmonary permeability oedema and the severity of ALI in septic and non septic critically ill patients
Lukasz (ref 36)	2008	Critically ill patients	94 critically ill patient on a medical ICU, 30 HC	Ang-1 is negatively correlated wih SOFA score	Ang-2 is positively correlated with sofa score	Not determined	Ang-1 correlates negatively and Ang-2 correltates positively with SOFA scores
Sinner (ref 38)	2008	Critically ill patients	20 nonseptic (ICU), 10 sepsis, 12 severe sepsis, 24 septic shock	Not determined	Ang-2 increases with the severity of sepsis	Not determined	Increase in Ang-2 associated with severity of illness and hospital mortality

Abreviations used in the table: HC: healthy controls, Il2: Interleukin 2, ARDS: Acute Respiratory Distress Syndrome,AMI: acute myocardial infarction, SA: stable angina, USA: unstable angina, ISS: International Severity Score, SOFA score: Sequential Organ Failure Assessment score.

Ang-2 prepares them for action. The close relation with VEGF is also apparent.

The angiopoietin signalling system

Ligands and receptors

The angiopoietin signalling system consists of four ligands and two receptors (figure 5.1). The ligands are Ang-1 to Ang-4, the best studied being Ang-1 and Ang-2[17;43-45]. The role of Ang-3 (the murine orthologue of Ang-4) and Ang-4 are much less clear[18]. Angiopoietins are 70 kDa glycoproteins that contain an amino-terminal angiopoietinspecific domain, a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen homology domain [17; 44; 46; 47]. Ang-1 and Ang-2 bind to Tie2 after polymerization of at least four (Ang-1) and two (Ang-2) subunits[48; 49]. The dissimilarity between Ang-1 and Ang-2 signalling lies in subtle differences in the receptor binding domain that lead to distinct intracellular actions of the receptor; differential cellular handling of both receptor and ligands after binding and signalling initiation may also play a role[49;50].

The receptors are Tie1 and Tie2[51]. Tie2 is a 140-kDa tyrosine kinase receptor with homology to immunoglobulin and epidermal growth factor[47; 52]. Tie receptors have an amino-terminal ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain[51]. Ligand binding to the extracellular domain of Tie2 results in receptor dimerization, autophosphorylation and docking of adaptors, and coupling to intracellular signalling pathways[47; 53-55]. Tie2 is shed from the EC and can be detected in normal human serum and plasma; soluble Tie2 may be involved in ligand scavenging without signalling[56]. Tie2 shedding is both constitutive and induced; the latter can be controlled by VEGF via a pathway that is dependent on phosphoinositide-3 kinase (PI3K) and Akt[57]. Shed soluble Tie2 can scavenge Ang-1 and Ang-2[56]. Tie1 does not act as a transmembrane kinase; rather, it regulates the binding of ligands to Tie2 and modulates its signalling[58-60].

Origin of ligands and distribution of receptors

Ang-1 is produced by pericytes and smooth muscle cells (figure 5.1). In the glomerulus, which lacks pericytes, Ang-1 is produced by podocytes[61]. Ang-1 has high affinity for the extracellular matrix, and so circulating levels do not reflect tissue levels, which in

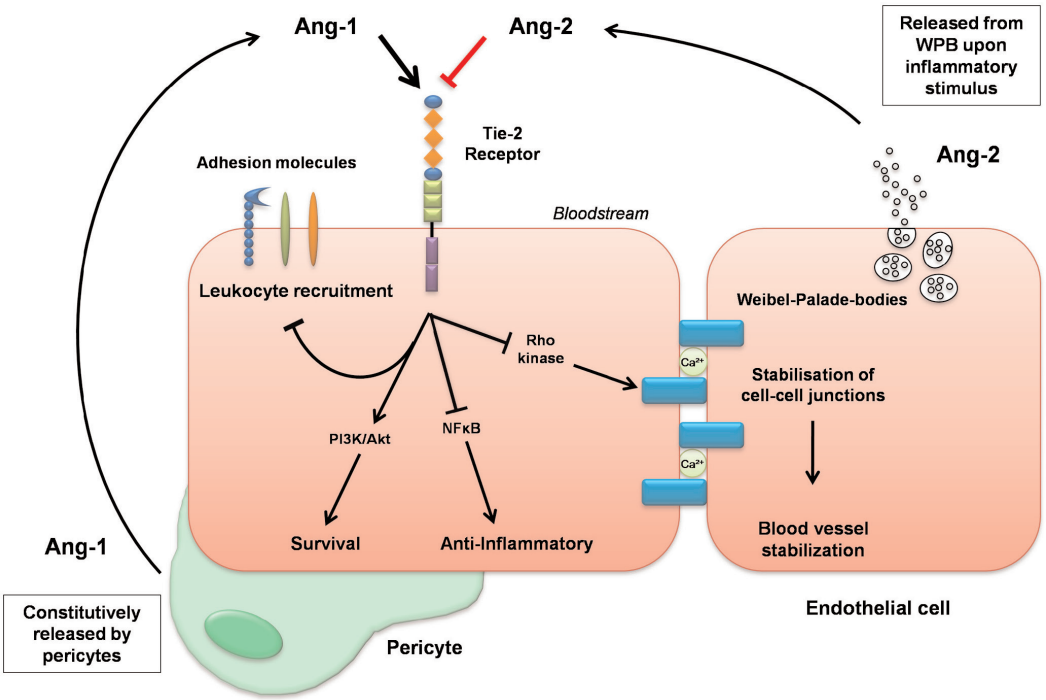

Figure 5.1. A schematic model of the angiopoietin-Tie2 ligand-receptor system.
Quiescent endothelial cells are attached to pericytes that constitutively produce Ang-1. As a vascular maintenance factor, Ang-1 reacts with the endothelial tyrosine kinase receptor Tie2. Ligand binding to the extracellular domain of Tie2 results in receptor dimerization, autophosphorylation, docking of adaptors and coupling to intracellular signalling pathways. Signal transduction by Tie2 activates the PI3K/Akt cell survival signalling pathway, thereby leading to vascular stabilization. Tie2 activation also inhibits the NF-κB-dependent expression of inflammatory genes, such as those encoding luminal adhesion molecules (for example, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin). Ang-2 is stored and rapidly released from WPBs in an autocrine and paracrine fashion upon stimulation by various inflammatory agents. Ang-2 acts as an antagonist of Ang-1, stops Tie2 signalling, and sensitizes endothelium to inflammatory mediators (for example, tumour necrosis factor-α) or facilitates vascular endothelial growth factor-induced angiogenesis. Ang-2-mediated disruption of protective Ang-1/Tie2 signalling causes disassembly of cell-cell junctions via the Rho kinase pathway. In inflammation, this process causes capillary leakage and facilitates transmigration of leucocytes. In angiogenesis, loss of cell-cell contacts is a prerequisite for endothelial cell migration and new vessel formation. Ang, angiopoietin; NF-κB, nuclear factor-κB; PI3K, phosphoinositide-3 kinase; WPB, Weibel-Palade body.

part is probably responsible for the constitutive phosphorylation of Tie2 in quiescenct endothelium[62-65]. Ang-2 is produced in ECs and stored in Weibel-Palade bodies (WPBs)[66;67]. The release of Ang-2 from WPBs by exocytosis can be regulated independently of the release of other stored proteins[68]. Tie2 is expressed predominantly by ECs, although some substes of macrophages and multiple other cell types express Tie2 at low levels[69;70]. In ECs, Tie2 is most abundant in the endothelial caveolae[71].

Genetics and transcriptional regulation of components of the Ang/Tie system

Ang-1 and Ang-2 genes are localized on chromosome 8. Functional polymorphism have not been identified in the Ang-1 gene, but three have been identified in the coding region of Ang-2[72]. In ECs under stress, Ang-2 mRNA expression is induced by VEGF, fibroblast growth factor 2 and hypoxia[44; 73]. Upregulation of Ang-2 induced by VEGF and hypoxia can be abolished by inhibiting tyrosine kinase or mitogen-activated protein kinase[73]. Ang-2 mRNA expression can be downregulated by Ang-1, Ang-2, or transforming growth factor[74]. After inhibition of PI3K by ortmannin, Ang-2 mRNA poduction is induced by the transcription factor FOXO1 (forkhead box O1)[75]. EC-specific Ang-2 promoter activity is regulated by Ets-1 and the Ets family member Elf-1[76; 77]. Because Tie2 signalling is required under circumstances that usually hamper cell metabolism, its promoter contains repeats that ensure transcription under difficult circumstances, including hypoxia[78].

The Tie2 downstream signalling pathway

Tie2 is present in phosphorylated form in quiescent and activated ECs throughout the body[62]. Signalling is initiated by autophosphorylation of Tie2 after Ang-1 binding and is conducted by several distinct pathways[54; 71; 79; 80]. Tie2 can also be activated at cell-cell contacts when Ang-1 induces Tie2/Tie2 homotypic intercellular bridges[63]. In human umbilical vein endothelial cells (HUVECs), Ang/Tie signalling resulted in 86 upregulated genes and 49 downregulated genes[81; 82]. Akt phosphorylation by PI3K with interaction of nitric oxide is the most important intracellular pathway[51; 83-86] however, ERK1/2, p38MAPK, and SAPK/JNK can also participate in Ang/Tie downstream signalling [71; 81; 84; 87-90]. Endothelial barrier control by Ang-1 requires p190RhoGAP, a GTPase regulator that can modify the cytoskeleton[80]. The transcription factors FOXO1, activator protein-1 and NF-κB are involved in Ang/Tie-regulated gene transcription[75; 91-93]. Ang-1 induced signalling is has also been implicated in cell migration induced by reactive oxygen species[94]. ABIN-2 (A20-binding inhibitor of NF-κB 2), an inhibitor of NF-κB, is involved in Ang-1-regulated inhibition of endothelial apoptosis and inflammation in HUVECs[93]. However, the downstream signalling of Tie2 varies depending on cell type and localization and whether a cell-cell or cell-matrix interaction in involved, which results in spatiotemporally different patterns of gene expression. For example, Ang-1/Tie2 signalling leads to Akt activation within the context of cell-cell interaction, but it

leads to ERK activation in the context of cell-matrix interaction. The microenvironment of the receptor in the cell membrane plays a central role in this signal differentiation. Adaptor molecules such as DOK and SHP2 and the availability of substrate determine which protein is phosphorylated[95].

Signal regulation

After binding of Ang-1, and to a lesser extent Ang-2, Tie2 is internalized and degraded, and Ang-1 is shed in a reusable form[50]. VEGF is an important co-factor that can exert different effects on Ang-1 and Ang-2 signalling[88]. Ang-2 is antiapoptotic in the presence of VEGF but induces EC apoptosis in its absence[96]. Autophosphorylation and subsequent signalling are inhibited by heteropolymerization of Tie1 and Tie2[59]. Although the Ang/Tie system appears to play its role mainly in paracrine and autocrine processes, its circulating components have been found in plasma. The significance of this finding in health and disease has yet to be determined.

Summary

The Ang/Tie system is an integrated, highly complex system of checks and balances (figure 5.1)[45; 54]. The response of ECs to Ang-1 and Ang-2 depends on the location of the cells and the biological and biomechanical context[97; 98]. It is believed that PI3K/Akt is among the most important downstream signalling pathways and that VEGF is one of the most important modulators of effects. Below we describe in more detail how this system responds to changes in homeostatic balances under various conditions of damage and repair.

Ang/Tie signalling system in health and disease

Angiogenesis, inflammation and homeostasis are highly related, and the Ang/Tie system lies at the intersection of all three processes[99; 100]. The Ang/Tie system is critically important for angiogenesis during embryogenesis, but in healthy adults its function shifts toward maintenance of homeostasis and reaction to insults. Except for follicle formation, menstruation and pregnancy, angiogenesis in adults is disease related. Neoplasia-associated neoangiogenesis and neovascularization in diabetes and rheumatoid arthritis

are unfavourable events, and improper angiogenesis is the subject of research in ischaemic disorders and atherosclerosis. Finally, failure to maintain homeostasis and an inappropriate reaction to injury are detrimental features in critical illness.

Angiogenesis

Angiogenesis is dependent on multiple growth factors and receptors and their signalling systems and transcriptional regulators[101]. This process is complex and encompasses the recruitment of mobile ECs and endothelial progenitor cells, the proliferation and apoptosis of these cells, and reorganization of the surroundings[102]. To form stable new blood vessels, the response must be coordinated in time and space, and the Ang/Tie system is involved from beginning to end. To prepare for angiogenesis, Ang-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism[44; 103]. Before vascular sprouting starts, focal adhesion kinase and proteinases such as plasmin and metalloproteinases are excreted[85]. Often, this stage is preceded by activation of innate immunity and inflammation[104]. Apparently, the machinery to clean up after the work has been finished is installed before the work is commenced, again illustrating the close relations among the different processes[104].

Ang-1 maintains and, when required, restores the higher order architecture of growing blood vessels[43; 44; 105; 106]. This is achieved by inhibiting apoptosis of ECs by Tie2-mediated activation of PI3K/Akt signalling[107-109]. Ang-1/Tie2 signalling is involved in angiogenesis induced by cyclic strain and hypoxia[110; 111]. Although the role is less clear, Tie1 might be involved in EC reactions to shear stress[112]. Ang-1 is a chemoattractant for ECs[83-85], and both Ang-1 and Ang-2 have proliferative effects on those cells[98; 113]. At the end of a vascular remodelling phase, Ang-2 induces apoptosis of ECs for vessel regression in competition with the survival signal of Ang-1[106]. This apoptotic process requires macrophages, which are recruited by Ang-2[70; 116].

ECs require support by surrounding cells such as pericytes, podocytes, and smooth muscle cells[64]. These cells actively control vascular behaviour by producing signalling compounds (for instance, Ang-1 and VEGF) that govern the activity and response of ECs[61]. To attract ECs, Ang-1 secreted by support cells binds to the extracellular matrix. In quiescent ECs, this binding results in Tie2 movement to the site of cell-cell interaction. In mobile ECs, Ang-1 polarizes the cell with Tie2 movement abluminal site[63]. In tumour

angiogenesis and in inflammation, Ang-2 recruits Tie2 positive monocytes and causes them to release cytokines and adopt a pro-angiogenic phenotype[111].

Homeostasis

The Ang/Tie system provides vascular wall stability by inducing EC survival and vascular integrity. However, this stability can be disrupted by Ang-2 injection, which in healthy mice causes oedema[28; 79; 116] that can be blocked by systemic administration of soluble Tie2[116]. Ang-2 can impair homeostatic capacity by disrupting cell-cell adhesion through E-cadherin discharge and EC contraction[28; 117]. In contrast, through effects on intracellular signalling, the cytoskeleton and junction-related molecules, Ang-1 reduces leakage from inflamed venules by restricting the number and size of gaps that form at endothelial cell junctions[80; 118; 119]. Ang-1 also suppresses expression of tissue factor induced by VEGF and tumour necrosis factor (TNF)-a, as well as expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin. As a result, endothelial inflammation is suppressed[120-124].

In primary human glomerular ECs *in vitro,* Ang-1 stabilizes the endothelium by inhibiting angiogenesis, and VEGF increases water permeability[124]. Similar observations were made in bovine lung ECs and immortalized HUVECs, in which Ang-1 decreased permeability, adherence of polymorphonuclear leucocytes and interleukin-8 production [123].

Injury

Reaction to injury can be seen as an attempt to maintain homeostasis under exceptional conditions. ECs can be affected by several noxious mechanisms. The Ang/Tie system is considered crucial in fine-tuning their reaction to injury and in containing that reaction. Ang-2-deficient mice cannot mount an inflammatory response to peritonitis induced chemically or with Staphylococcus aureus[125], but they can mount a response to pneumonia, suggesting the existence of inflammatory reactions for which Ang-2 is not mandatory. Ang-2 sensitizes ECs to activation by inflammatory cytokines. In Ang-2-deficient mice, leucocytes do roll on activated endothelium but they are not firmly attached, owing to the lack of Ang-2-dependent upregulation of adhesion molecules and the dominance of Ang-1-regulated suppression of adhesion molecules[120; 121; 123; 125].

In bovine retinal pericytes, hypoxia and VEGF induce Ang-1 and Tie2 gene expression acutely without altering Ang-2 mRNA levels. The opposite occurs in bovine aortic ECs and microvascular ECs, underscoring the heterogeneity of ECs from different microvascular beds[73; 126; 127].

Lipopolysaccharide (LPS) and pro-inflammatory cytokines can shift the Ang/Tie balance, rouse ECs from quiescence and provoke an inflammatory response. In rodents LPS injection induces expression of Ang-2 mRNA and protein and reduces the levels of Ang-1, Tie2 and Tie2 phosphorylation in lung, liver and diaphragm within 24 hours, which may promote or maintain vascular leakage. The initial increase in permeability is probably due to release of Ang-2 stored in WPBs[128]. In a mouse model of LPS-induced lung injury, pulmonary oedema was found to be related to the balance between VEGF, Ang-1 and Ang-4[129]. In a comparable model, Ang-1-producing transfected cells reduced alveolar inflammation and leakage[130].

In choroidal ECs, TNF induces Ang-2 mRNA and protein before affecting Ang-1 and VEGF levels[131]. In HUVECs, TNF-induced upregulation of Ang-2 is mediated by the NF-κB pathway[132], and TNF-induced Tie2 expression can be attenuated by both Ang-1 and Ang-2. Without TNF stimulation, only Ang-1 can reduce Tie2 expression[133]. Ang-2 sensitizes ECs to TNF, resulting in enhanced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin[74; 125; 134]. By inhibiting those endothelial adhesion molecules, Ang-1 decreases leucocyte adhesion[122].

Angiopoietins can mediate the synthesis of platelet-activating factor by ECs to stimulate inflammation[90]. Moreover, both Ang-1 and Ang-2 can translocate P-selectin from WPBs to the surface of the EC[135], and both can also increase neutrophil adhesion and chemotaxis and enhance those processes when they are induced by interleukin-8[86; 136; 137].

In a rat model of haemorrhagic shock, Ang-1 reduced vascular leakage, and it inhibited microvascular endothelial cell apoptosis in vitro and in vivo[107; 138]. In this model, Ang-1 promoted cell survival was partly controlled through integrin adhesion[139]. It has been suggested that EC apoptosis in haemorrhagic shock contributes to endothelial hyperpermeability[140-142]. Apoptosis is one of the reactions to MODS related injury as demonstrated in hypoxia/reperfusion[143].

Cell adhesion

Ang-1 and Ang-2 are involved in cell-cell and cell-matrix binding[139; 144-146]. Endothelial permeability is greatly dependent on cell-cell adhesion. The major adherens junction is largely composed of vascular-endothelial cadherin. This complex can be disrupted by VEGF, leading to increased vascular permeability[147; 148], which can be antagonized by Ang-1[149; 150]. ECs can also bind to the matrix through the binding of Ang-1 to integrins, which can mediate some of the effects of Ang-1 without Tie2 phosphorylation[146; 151]. At low Ang-1 concentrations, integrin and Tie2 can cooperate to stabilize ECs[151]. Ang-2 might play a role in inflammatory diseases such as vasculitis by disrupting the cell-cell junction and inducing denudation of the basal membrane[152]. Ang-1 can mediate the translocation of Tie2 to endothelial cell-cell contacts and induce Tie2-Tie2 bridges with signal pathway activation, leading to diminished paracellular permeability[63].

Summary

In the mature vessel, Ang-1 acts as a paracrine signal to maintain a quiescent *status quo*, whereas Ang-2 induces or facilitates an autocrine EC response[74; 153] . In general, Ang-1 can be viewed as a stabilizing messenger, causing continuous Tie2 phosphorylation, and Ang-2 as a destabilizing messenger preparing for action[17]. Attempts to unravel the exact molecular mechanisms that control the system are complicated by microenvironment-dependent endothelial phenotypes and reactivity and by flow typedependent reactions to dynamic changes[154; 155; 13]. Hence, the EC must be viewed in the context of its surroundings - the pericyte at the abluminal site, and the blood and its constituents on the luminal site[65]. The Ang/Tie system certainly functions as one of the junctions in signal transduction and plays a key role in multiple cellular processes, many of which have been linked to MODS.

Targeting the Ang/Tie system in critical illness

A therapy should intervene in the right place and at the right time, with the proper duration of action and without collateral damage[156; 157]. The Ang/Tie system is involved in many processes and lies at the intersection of molecular mechanisms of disease. Thus, interventions targeting this system might have benefits. As in other pleiotropic systems, however, unexpected and unwanted side effects are a serious risk. The absence of

redundant systems to take over the function of Ang/Tie2 has the advantage that the effect of therapeutic intervention cannot easily be bypassed by the cell. On the other hand, because the cell has no escape, the effect may become uncontrolled and irreversible. Moreover, the exact function of the Ang/Tie system in the pathological cascade is not fully established. What we see in animal models and in patients is most probably the systemic reflection of a local process. We do not know whether this systemic reflection is just a marker of organ injury or even a mediator of distant organ involvement.

Of the three main functions of the Ang/Tie system, it is mainly angiogenesis that has been evaluated as a therapeutic target. So far, the focus of Ang/Tie modulation has been on inhibiting angiogenesis related to malignant and ophthalmological diseases and to complications of diabetes[158; 159]. In peripheral arterial occlusive disease, stimulation of angiogenesis seems a logical strategy to ttenuate the consequences of ongoing tissue ischaemia. In a rat model of hind limb ischaemia, combined livery of Ang-1 and VEGF genes stimulated collateral vessel development to the greatest extent[160; 161]. Thus far, therapy directed at VEGF has reached the clinic, but not therapy directed at Ang/Tie[162].

Targeting homeostasis and repair/inflammation in critically ill patients is an attractive option and has already led to the development of new drugs[45; 158; 163]. From current knowledge, one can speculate about the best options for therapy aimed at the Ang/Tie system. In critical illness, Ang-1 is considered to be the 'good guy' because it can create vascular stability and thus its activity should be supported. In contrast, Ang-2 appears to be a 'bad guy' that induces vascular leakage, so its activity should be inhibited[164].

Production of recombinant Ang-1 is technically challenging as Ang-1 is 'sticky' because of its high affinity for the extracellular matrix[165]. However, stable Ang-1 variants with improved receptor affinity have been engineered. A stable soluble Ang-1 variant has anti-permeability activity[165]. When injected intraperitoneally in mice, human recombinant Ang-1 can prevent LPS-induced lung hyperpermeability[80]. In diabetic mice, a stable Ang-1 derivative attenuated proteinuria and delayed renal failure[166], and manipulating the Ang-1/Ang-2 ratio changed infarct size[167]. A more profound Ang-1 effect can be achieved by locally stimulating Ang-1 production. In experimental acute respiratory distress syndrome, transfected cells expressing Ang-1 reduced alveolar nflammation and leakage[130]. An adenovirus construct encoding Ang-1 protected mice from death in an LPS model, and Ang-1 gene therapy reduced acute lung injury in a rat model[21; 168; 169]. In

hypertensive rats, a plasmid expressing a stable Ang-1 protein reduced blood pressure and end-organ damage[170]. If used in a disease with a limited duration, as critical illness should be, virus/plasmid-driven production of Ang-1 could easily be shut down when it is no longer needed.

Manipulating Ang-2 activity is also difficult. Ang-2 stored in WPBs is rapidly released and must be captured immediately to prevent autocrine/paracrine disruption of protective Ang-1/Tie signalling. Soluble Tie2 or Ang-2 inhibitors should be effective[26; 171]. Neutralizing antibodies against Ang-2 might also be an option. Replenishment of Ang-2 stores could be abolished by small interfering RNA techniques or spiegelmer/aptamer approaches[25; 172; 173].

However, no bad guy is all bad, and no good guy is all good. For example, Ang-1 has been linked to the development of pulmonary hypertension[174]. Also, under certain circumstances Ang-2 can act as a Tie2 agonist and exert effects similar to those of Ang-1 - an unexplained finding that illustrates our limited understanding of the Ang/Tie system[75]. Complete blockade of Ang-2 might also amper innate immunity and revascularization.

Finding the right balance and timing will be the major challenge when developing therapies to target the Ang/Tie system. In the meantime, we might have already used Ang/Tie-directed therapy with the most pleiotropic of all drugs - corticosteroids. In the airways, steroids suppressed Ang-2 and increased Ang-1 expression[26; 171; 175] . Interventions further downstream targeting specific adaptor molecules, signalling pathways, or transcription factors have yet to be explored.

Diagnostic and prognostic opportunities

In patients with malignant disease, the Ang/Tie system might serve as a tumour or response marker. In patients with multiple myeloma, normalization of the Ang-1/Ang-2 ratio reflects a response to treatment with anti-angiogenesis medication[176]. In patients with non-small-cell lung cancer, Ang-2 is increased in serum and indicates tumour progression[177]. After allogeneic stem cell transplantation in patients with high-risk myeloid malignancies, the serum Ang-2 concentration predicts disease-free survival[178], possibly reflecting a relation between cancer-driven angiogenesis and Ang-2 serum level.

In nonmalignant disease, the levels of Ang/Tie system components correlate with disease severity[28; 34-36; 37; 39]. However, current data are insufficient to justify the use of

serum soluble Tie2/Ang levels for diagnostic and prognostic purposes. In critical illness, assessment of the Ang/Tie system in patients with different severities of disease and with involvement of different organ systems might help to define our patient population and allow us to rethink our concepts of MODS. In this way, such work may lead to enhanced diagnosis and prognostication in the future[2].

CONCLUSIONS

Accumulating evidence from animal and human studies points to the involvement of the Ang/Tie system in vascular barrier dysfunction during critical illness. Many processes in injury and in repair act through this nonredundant system. Thus far, only preliminary studies in critically ill patients have been reported. Methods to manipulate this system are available but have not been tested in such patients. The response to treatment is difficult to predict because of the pleiotropic functions of the Ang/Tie system, because the balance among its components appears to be more important than the absolute levels, and because the sensitivity of the endothelium to disease-related stimuli varies, depending on the environment and the organ involved. To avoid disappointment, further experimental and translational research must be carried out, and Ang/Tie modulation must not be introduced into the clinic prematurely. Implementing the results of this research in critical care represents an opportunity to show what we have learned[2]. Ang/Tie signalling is a very promising target and must not be allowed to become lost in translation[179].

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Chapter 6

Shock induced stress induces loss of microvascular endothelial Tie2 in the kidney which is not associated with reduced glomerular barrier function

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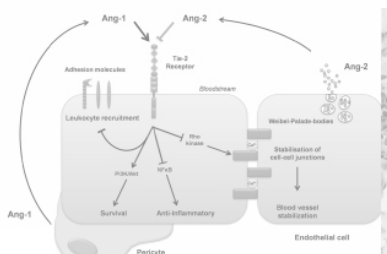
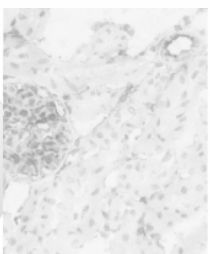
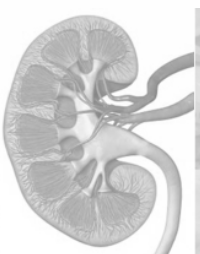



ABSTRACT

Both hemorrhagic shock and endotoxaemia induce a pronounced vascular activation in the kidney which coincides with albuminuria and glomerular barrier dysfunction. We hypothesized that changes in Tie2, a vascular restricted receptor tyrosine kinase shown to control microvascular integrity and endothelial inflammation, underlie this loss of glomerular barrier function. In healthy murine and human kidney, Tie2 is heterogeneously expressed in all microvascular beds, although to different extent. In mice subjected to hemorrhagic and septic shock, Tie2 mRNA and protein were rapidly, and temporarily, lost from the renal microvasculature, and normalized within 24 hours after initiation of the shock insult. The loss of Tie2 protein could not be attributed to shedding as both in mice and healthy volunteers subjected to endotoxaemia, sTie2 levels in the systemic circulation did not change. In an attempt to identify the molecular control of Tie2, we activated glomerular endothelial cell cultures and human kidney slices in vitro with LPS or TNF-α, but did not observe a change in Tie2 mRNA levels. In parallel to the loss of Tie2 in vivo, an overt influx of neutrophils in the glomerular compartment which coincided with proteinuria, was seen. As neutrophil – endothelial cell interactions may play a role in endothelial adaptation to shock, and these effects can not be mimicked in vitro, we depleted neutrophils prior to shock induction. While this neutrophil depletion abolished proteinuria, Tie2 was not rescued, implying that Tie2 may not be a major factor controlling maintenance of the glomerular filtration barrier in this model.

Introduction

Acute kidney injury (AKI) after shock states is an often lethal complication of hemorrhagic and septic shock. Aggressive management of shock with supportive therapy has not substantially lowered the more than 50% 60-day mortality of AKI patients treated in Intensive Care units[1]. AKI is characterized by a sudden loss of the ability of the kidneys to excrete wastes, maintain fluid balance, and conserve electrolytes[2] and by the occurrence of proteinuria[3].

A number of potential mechanisms have been described to underlie the occurrence of proteinuria in AKI[4; 5], including loss of microvascular integrity. One of the molecular systems controlling microvascular integrity is the Angiopoietin/Tie2 system[6]. Tie2 is a 140 kD tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology[7], that has specificity for Angiopoietin (Ang)-1 and Ang-2 binding[8; 9]. Ang-1 induced Tie2 signaling is considered essential for endothelial integrity and provides quiescent endothelial status with anti-inflammatory properties[10]. In contrast, competition of Ang-1/Tie2 binding by Ang-2 induces inhibition of Tie2 signal transduction and is associated with inflammatory and vascular leakage disorders, similar to a diminished Ang-1/Tie2 signaling due to other causes[11-14]. Both hemorrhagic shock and endotoxaemia induce a pronounced vascular activation in the kidney which coincides with vascular leakage and glomerular barrier dysfunction[15-17]. An increase in Ang-2 has until now been assigned as being the dynamic factor of the system, which upon endothelial release from Weibel Palade bodies competes with Ang-1 for binding to Tie2, and thereby creates a condition of endothelial destabilization[18]. Ang-2 overexpression in podocytes led to increased proteinuria in adult mice[14], while in a diabetic mouse model the administration of Ang-1 exerted protective effects with diminished proteinuria[19]. Also in human proteinuric diseases like systemic lupus erythematosus, Ang-2 serum levels correlated positively with proteinuria[20]. Although not considered actively regulated, preliminary observations in our critical illness models showed differences in Tie2 mRNA expression during shock onset. We therefore hypothesized, that a change in Tie2 expression may be one of the molecular responses of the Angiopoietin/Tie2 system that underlies maladaptive behavior in shock, including loss of microvascular integrity in the kidney.

To test this hypothesis, we studied the spatiotemporal changes in Tie2 mRNA

and protein expression in the renal microvasculature of mice during endotoxic and hemorrhagic shock as models of AKI, and investigated the relation between Tie2 changes and proteinuria as a measure glomerular barrier dysfunction. The initial observations justified a further study into the role of neutrophils in the changes in Tie2 expression. For this, we depleted the neutrophils by antibody treatment prior to shock induction, and investigated its consequences for Tie2 expression and proteinuria. The observations were extended to humans, by studying a human volunteer endotoxaemia model and human kidney slices exposed to sepsis mediators.

Materials and Methods

Animals

Eight- to 12-week-old C57Bl/6 male mice (20-30g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All procedures were approved by the local committee for care and use of laboratory animals and were performed according to governmental and international guidelines on animal experimentation.

Mouse shock models

The mouse hemorrhagic shock (HS) model has been extensively documented elsewhere[15]. In short, mice were anesthetized with isoflurane (inspiratory, 1.4%), N_2O (66%), and O_2 (33%). The left femoral artery was canulated for monitoring mean arterial pressure (MAP), blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30 mmHg during this period. The mice were resuscitated after 90 minutes of HS with 6% hydroxyethyl starch 130/0.4 (Voluven®; Fresenius-Kabi, Bad Homburg, Germany) at two times the volume of blood withdrawn. After 4, 8, or 24 hours post volume resuscitation, blood was withdrawn via aortic puncture under isoflurane anesthesia, and the kidneys were excised, snap-frozen in metal cups on liquid nitrogen, and stored at -80˚C until analysis.

For the induction of endotoxaemia, mice were intraperitoneally (i.p.) injected with LPS (Escherichia coli, serotype 026:B6l; Sigma, St. Louis, MO) at 5 mg/kg (15,000 EU/g) body weight. 4, 8 and 24 hours later, blood was drawn and organs harvested as described above. Control mice were left untreated and were sacrificed under isoflurane anesthesia, after which blood was withdrawn and kidneys were harvested and handled as described above.

In indicated experiments, mice were housed in a metabolic cage for 24 hours at 7 days before the experimental procedure, to obtain a control urine sample. Metabolic cages were used to obtain urine samples from mice in healthy and diseased conditions. Control albumin/creatinin ratios were assessed by housing mice in metabolic cages for 24 hours 7 days prior to the insult and from 0 until 4 hours, 0 until 8 hours, and 8 until 24 hours after LPS induced shock. A subgroup of LPS treated mice was i.p. injected with 0.5 mg anti-NIMP antibody to selectively deplete the neutrophils prior to shock induction[21]. One day after this procedure mice were i.p. injected with LPS at a similar dose as described above. These mice were housed in metabolic cages for urine collection immediately after LPS administration and sacrificed 8 hours later under isoflurane anesthesia, blood was withdrawn via an aortic puncture, and the kidneys were harvested, snap-frozen in metal cups on liquid nitrogen, and stored at -80˚C until analysis.

Human endotoxaemia

For the human endotoxaemia model, human volunteers who participated in a drug intervention study were injected with a dose of 4 ng/kg body weight (10,000 endotoxin units/µg) LPS (Escherichia coli, batch EC-6, US Pharmacopeia, Twinbrook Parkway, Rockville, MD, USA). The local Investigations Review Board approved the study. Written informed consent was obtained from all subjects before enrolment in the study. Data from this study have been reported extensively elsewhere[22]. From this cohort, plasma stored at -80˚C was analyzed for soluble Tie2.

In vitro cell culture and organ slice incubation

Conditionally immortalized Human Glomerular Endothelial cells (ciGEnC)[23] were cultured in EBM medium in 12-well culture dishes at a density of 100,000 cells/well for 24h at 33˚C, followed by 5 days at 37˚C under 5% CO_2/95% O_2 before introducing them in

an experiment. The ciGEnC culture medium consisted of EBM-2 medium supplemented with 5% fetal calf serum (FCS) and EGM-2 MV singleQuots (Lonza Group Ltd, Basel, Switzerland). In the experiments described here ciGEnC were used up to passage 40.

Confluent ciGEnC were activated for 4 hours with 0.1, 1 and 10 ng/ml TNF-α (Boehringer, Ingelheim, Germany) and 1, 50 and 1,000 ng/ml LPS. After incubation cells were microscopically analyzed with regard to their morphology and consistently were found to be adherent and viable.

For kidney slice incubations, human kidney tissue was obtained as tumor free surgical waste from patients subjected to kidney carcinoma surgery. The three patients were all male, age between 60 and 66 years, with normal kidney function. Tissue was prepared for precision cut tissue slices within 15 minutes. Tissue cylinders were prepared with an 8 mm diameter motor-driven coring tool, and further processed into 250 µm thick slices with a mechanical slicer as described earlier[24]. Slices were incubated individually in 12-well culture plates (Costar 3512; Corning Glassworks, Corning, NY) in 1.3 ml of Williams Medium E with Glutamax-I, supplemented with D-glucose (25 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). For activation, 10 ng/ml LPS was added to the medium at the start of the incubation period. The culture plates were placed at 37°C and slices were incubated under humidified carbogen on an orbital shaker (45 rpm). The condition of precision-cut slices was evaluated at different incubation time points by microscopic examination of hematoxylin and eosin stained cryosections. Intracellular ATP levels were measured in slice homogenates with ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics Nederland, Almere, The Netherlands) to judge the overall metabolic condition of the tissue. Immunohistochemical staining of Tie2 was performed on 5 µm cryosections and gene expression analysis was performed with RNA isolated from frozen slices as described below.

Laser microdissection of renal microvasculature

From mice kidneys, 5 µm cryosections mounted on 1.35 µm polyethylene-naphtalene membranes attached to normal 1 mm slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany) were fixed in acetone and stained with Mayer's hematoxylin, washed with diethyl pyrocarbonate treated water, and air-dried. ECs from small arterioles (6 x 10^5 μm^2) and postcapillary venules (1.3 x 10^6 μm^2), as well as glomeruli (3 x 10^6 μm^2), were

dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology).

Gene expression analysis by quantitative RT-PCR

RNA was extracted from 20 x 5 µm cryosections from mouse kidney, 250 µm human kidney slices and cells, and isolated using the RNeasy Mini Plus Kit (Qiagen, Leusden, The Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield (OD260) and purity (OD260/OD280) were measured by an ND-1,000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The Assay-on-Demand primers (ABI systems, Foster City, USA) used in the PCR reaction included housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915_g1 for mouse and assay Hs99999905_m1 for human), Tie2 (assay ID Mm00443242_m1 for mouse and assay Hs00176096_m1 for human), E-selectin (assay ID Hs00174057_m1 for human), VEGF-A (assay ID Mm00437304_m1 for mouse), and VEGFR-2 (assay ID Mm00440099_m1 for mouse). Duplicate real time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. According to the comparative CT method described in the ABI manual gene expression was normalized to the expression of the housekeeping gene, yielding the ΔCT value. The average, relative mRNA level was calculated by $2^{-\Delta CT}$.

Localization of proteins by immunohistochemistry

Localization of Tie2, CD31, E-selectin and neutrophils was determined using immunohistochemistry. Frozen kidneys were cryostat-cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 minutes. After drying, sections were incubated for 45 minutes at room temperature with primary rat anti-mouse antibodies in the presence of 5% fetal calf serum (table 6.1). After washing, endogenous peroxidase was blocked by incubation with 0.1% H_2O_2 in phosphate-buffered saline (PBS) for 20 minutes. This was followed by incubation for 30 minutes at room temperature with horseradish peroxidase-conjugated secondary antibodies (table 6.1). Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected

Table 6.1. Antibodies and their controls used for immunohistochemistry and leukocyte depletion.

	Ab name / epitope	Provider	Isotype	Dilution used	Application
Primary Abs					
Mouse Tie2	Tek4	eBioscience	IgG1	1:50	IHC
Hu Tie2	sc324	Santa Cruz		1:50	IHC
Mouse CD31	PECAM-1	BD Pharmingen	IgG2a	1:100	IHC
Mouse E-selectin	MES-1	Dr. D. Brown, United Kingdom	IgG2a	1:10	IHC
Neutrophil	Ly6G	BD Pharmingen	IgG2a	1:50	IHC
Control IgG for rat Ab		Antigenix America	IgG1, IgG2a, IgG2b	1:50	IHC
Neutrophil	Anti-NIMP	HBt	IgG2b	n.a.	PMN depl.
Control IgG		Sigma	IgG	n.a.	Control depl.
Secondary Abs					
Rabbit anti rat-HRP	P0459	Dako		1:40	IHC
Envision kit, rabbit polymer	K4009	Dako			IHC
Goat anti rabbit-HRP	4050-05	Southern Biotech		1:50	IHC
Rabbit anti rat (preadsorbed)		Vector		1:300	IHC

with 3-amino-9-ethylcarbazole (Sigma-Aldrich Chemie, St. Louis, MO, USA), and sections were counterstained with Mayer hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype-matched controls (table 6.1), demonstrating specificity of staining with the antigen specific antibodies.

Quantification of Tie2 protein levels by ELISA

To quantify the amount of Tie2 protein in the renal tissues of mice, 15 x 10 µm kidney slices were homogenized in 50mM Tris-HCl buffer (pH 7.5), containing 150mM NaCl and protein inhibitor cocktail (Sigma-Aldrich Chemie) and centrifuged at 13.000g for 15 minutes. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, USA), before quantification of Tie2 by ELISA (mouse Tie2 MTE200, R&D Systems Inc. Minneapolis, USA) according to the manufacturer's instructions. Tie2 levels were normalised to total protein concentrations in the tissue homogenate and expressed as pg Tie2 per µg total protein.

The level of soluble Tie2 in the plasma was analysed using a commercially available Tie2 ELISA (human DTE200 and mouse MTE200; R&D Systems) according to the manufacturer's instructions. The DTE200 ELISA kit was previously used to measure changes in soluble Tie2 in different patients groups[25; 26]. During this investigation, we validated the MTE200 ELISA for suitability to measure soluble Tie2 using commercially available soluble mouse Tie2 (R&D systems; 762-T2).

Kidney function measured by albumin:creatinin ratio

To assess glomerular barrier function, the micro-albumin and creatinine levels were measured in mouse urine using a commercial available kit (Exocell Inc., Philadelphia, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical significance of differences was studied by means of the a Student's t-test or ANOVA with post hoc comparison using Bonferroni correction. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism software (GraphPad Prism Software Inc., San Diego, California, USA). Differences were considered to be significant when $p < 0.05$.

RESULTS

In healthy mouse kidney, Tie2 is expressed in all vascular beds to different extents

To examine the expression pattern of Tie2 in healthy mouse kidney, we immunohistochemically stained tissue for Tie2 protein (figure 6.1A). Tie2 is located in all microvascular beds, with a clear differential level of expression between the microvascular segments that can be histologically discriminated. Pronounced expression of Tie2 was observed in arterioles, glomeruli, and peritubular endothelium, while the expression was lower in the endothelium of the postcapillary venules. These vascular bed specific differences were corroborated by Tie2 mRNA levels in microvascular segments microdissected from mouse kidneys prior to gene expression analysis. Most Tie2 mRNA was localized in glomeruli while the least was seen in venules (figure 6.1B).

Tie2 expression is diminished in the kidney in different shock states

After initiation of hemorrhagic shock and LPS induced shock, Tie2 was rapidly lost, both at the mRNA and protein level. 24 hours after the shock insult the mRNA in hemorrhagic shock had normalized, while an increase in mRNA was seen in the LPS treated groups (figure 6.2A). Of note is the fact that 24 hours after the shock insult, levels of Tie2 protein in both groups of shock subjected mice had normalized. The decrease in mRNA content was accompanied by a reduction in Tie2 protein levels in kidney homogenates in both

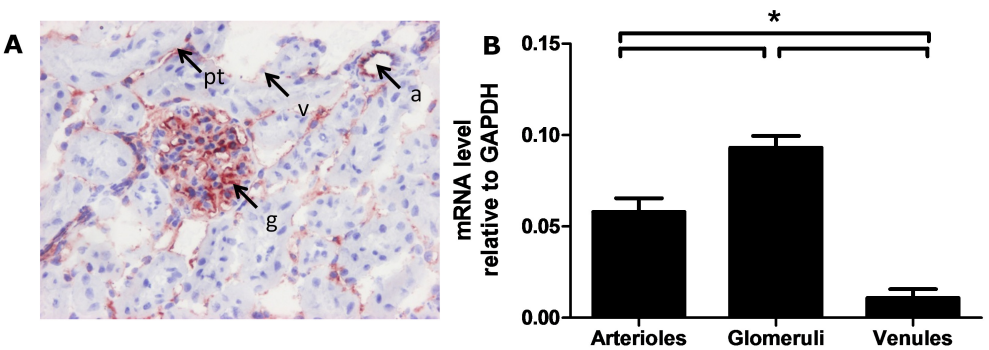


Figure 6.1. Tie2 is expressed in different microvascular beds in healthy mouse kidney.
*(A) Protein expression detected by immunohistochemical staining. Arrows point at different microvascular beds: arteriole (a), glomerulus (g), peritubular vasculature (pt), and venule (v). (B) Expression of Tie2 mRNA levels by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in three microvascular beds laser microdissected from kidney. Mean values ± SD of 3 mice per group, * p <0.05.*

models, with the most prominent reduction visible in the LPS model (figure 6.2B). Immunohistochemical detection of Tie2 revealed that the protein was lost from all vascular beds, i.e., the arteriolar, glomerular, peritubular and venular vasculature (figure 6.2C).

Tie2 is not shed in LPS mediated shock

The cause of Tie2 protein downregulation in LPS shock can be either internalization and degradation or shedding of the membrane associated protein[27; 28]. To determine the potential occurrence of Tie2 shedding during and after the shock period, we measured

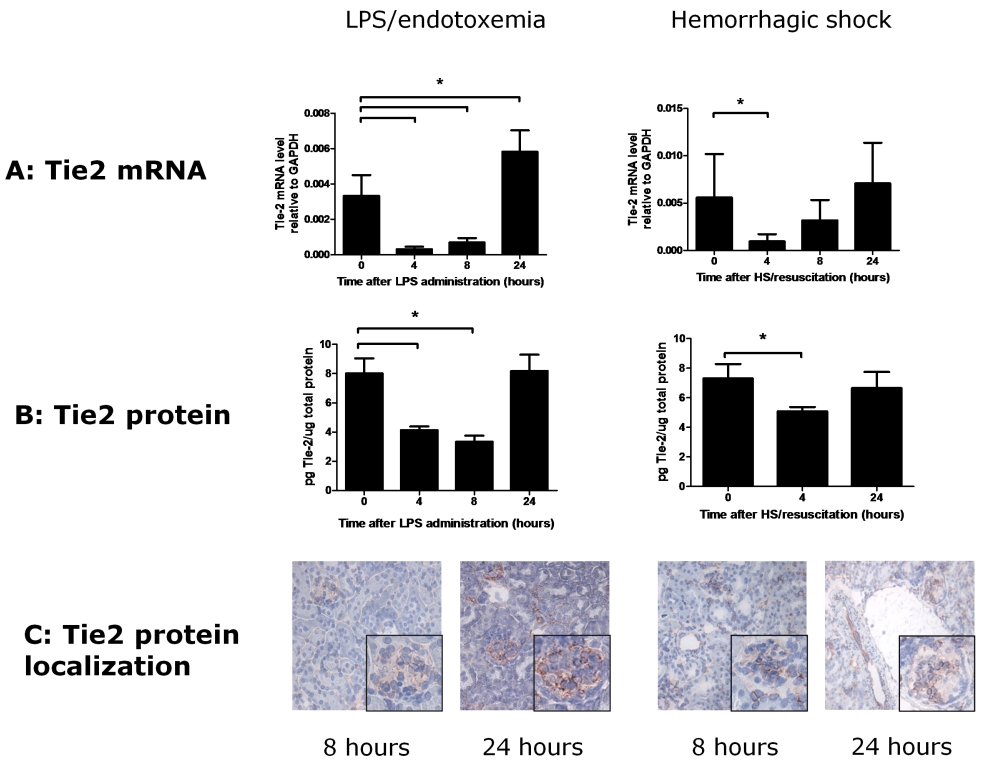


Figure 6.2. Spatiotemporal changes in renal Tie2 mRNA and protein expression in mice subjected to LPS induced shock and hemorrhagic shock followed by resuscitation.

*In the endotoxemia model, LPS was administered at a dose of 0.5 mg/kg mice, while in the hemorrhagic shock model mice were subjected to blood withdrawal to a mean arterial pressure of 30 mmHg for 90 minutes, after which they were resuscitated with Voluven® as described in Materials and Methods. (A) mRNA levels shown are relative to GAPDH as housekeeping gene and determined by quantitative RT-PCR as described in Materials and Methods. Mean values ± SD of at least 5 mice per group , * p<0.05. (B) Protein levels were measured in kidneys homogenates by ELISA as described in Materials and Methods. Mean values ± SD of at least 8 mice per group, * p <0.05. (C) Representative light microscopy pictures of the microvascular localization of Tie2 protein after LPS or hemorrhagic shock respectively and resuscitation 8 hours and 24 hours after induction of shock, assessed by immunohistochemistry. Original magnification 200x, insert 400x * p <0.05.*

soluble Tie2 in the systemic circulation after LPS induced shock. In mice, no increased shedding occurred during the first 24 hours after LPS administration (figure 6.3A). Similar to the mouse model, no shedding of Tie2 into the plasma could be observed in human endotoxaemia (figure 6.3B). These mouse and human data suggest that the diminished Tie2 protein expression observed in the kidney is not due to systemic protein shedding.

Endothelial cell loss of Tie2 cannot be induced in in vitro and ex vivo conditions

In order to determine the molecular mechanism underlying shock induced loss of Tie2 from endothelial cells, we incubated glomerular endothelial cells with LPS and with TNF-α, which is one of the rapid responder cytokines in vivo after LPS administration[29] (figure 6.4A and 6.4B). Neither low, nor relatively high concentrations of LPS or TNF-α, changed the mRNA levels of Tie2 in vitro. The strong induction of E-selectin mRNA expression under these pro-inflammatory conditions ruled out an overall non-responsiveness of the cells toward LPS and TNF-α.

As glomeruli contain mesangial cells and podocytes next to endothelial cells, theoretically these non-endothelial cells could have contributed to the observed Tie2 decrease in vivo. Compared to glomerular endothelium, however, their Tie2 expression level was more than 100 – 1,000 fold lower, and no effect of both short term and long term LPS exposure on Tie2 mRNA levels could be detected (supplemental figure 6A).

To determine whether a possible interplay between cells determined the main

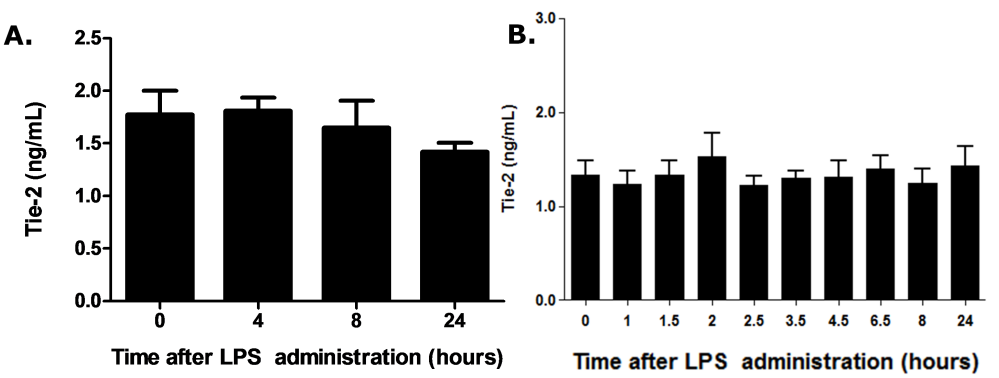


Figure 6.3. Soluble Tie2 levels in plasma of mice and humans administration did not change after LPS administration.

Quantitation of soluble Tie2 protein levels in plasma from LPS challenged mice (A; n=4) and LPS challenged humans (B; n=6) was performed using ELISA. Results are expressed as the mean ± SD, p <0.05.*

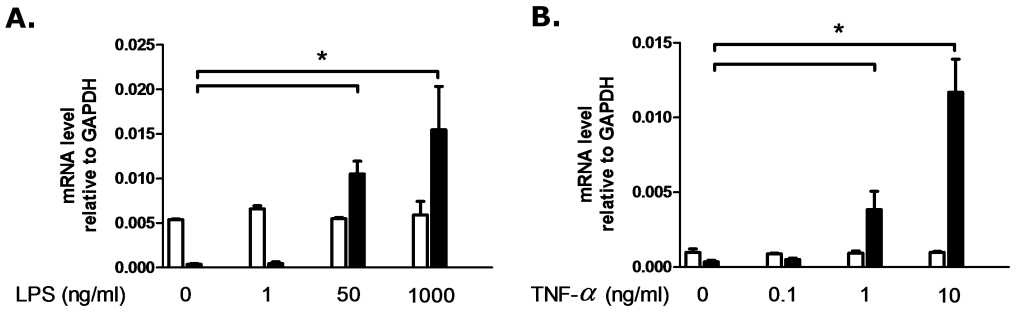
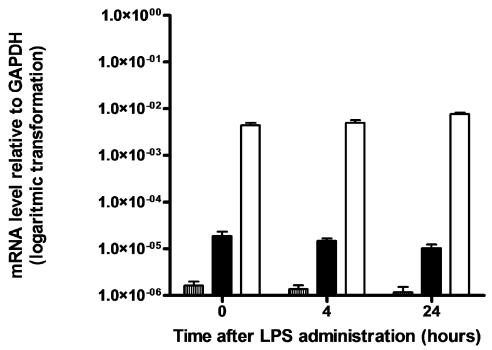


Figure 6.4. In vitro, LPS nor TNF-α stimulation of glomerular endothelial cells did not affect Tie2 gene expression, while E-selectin was strongly induced.

*Conditionally immortalized glomerular endothelial cell (ciGEnC) were exposed for 4 hours to LPS (A) or TNF-α (B), at increasing concentrations. White bars represent Tie2 mRNA levels, black bars E-selectin mRNA levels. Values are mean of 3 ± SD, * p<0.05.*

cause of Tie2 gene and protein expression loss, we incubated 250 µm precision cut human kidney slices in the absence and presence of LPS. In human kidneys, Tie2 was expressed in all microvascular beds in a pattern similar to that in mice (figure 6.5A). Upon ex vivo incubation of the slices in normal medium for 8 hours, Tie2 mRNA levels significantly dropped compared to levels in control kidney snap frozen directly prior to slice production. These lower mRNA levels were still well above the detection limit of



Supplemental figure 6A. In vitro, cells from glomerular origin express Tie2 to a different extent which is not affected by exposure to LPS.

Mesangial cells, podocytes and endothelial cells derived from human glomeruli were analysed for Tie2 mRNA expression levels in the absence and presence of 1 µg/ml LPS. mRNA was harvested and analysed by real-time RT-PCR as described in Materials and Methods and relative to GAPDH as house keeping gene. Striped bars represent mesangial cells, black bars represent podocytes, white bars represent glomerular endothelial cells. Values are mean of 3 ± SD.

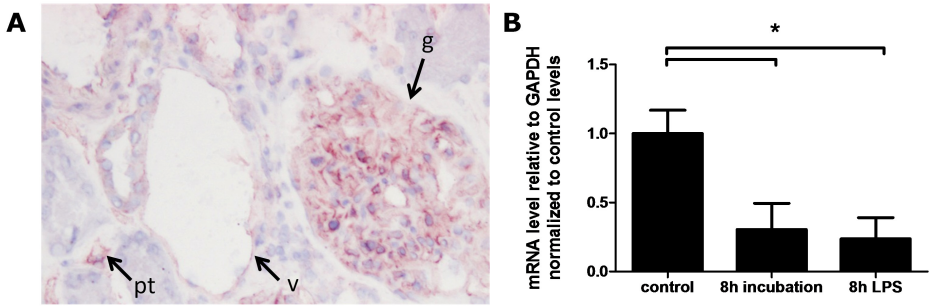


Figure 6.5. Tie2 expression in human kidney slices does not change under the influence of LPS.
*(A) Immunohistochemical detection of Tie2 in human kidney. Tie2 protein expression is visible in glomeruli (g), peritubular (pt), and postcapillary venule (v) endothelial cells. (B) Tie2 expression in human kidney tissue in a controlled, ex vivo precision cut tissue slice incubation system. After incubation for 8 hours in medium with and without 50 µg/ml LPS, slices were harvested and processed for mRNA expression analysis. Tie2 mRNA expression decreased significantly upon incubation in medium for 8 hours, yet no additional effect of exposure to LPS on Tie2 mRNA expression levels were observed, * p <0.05.*

the analytical procedure. Loss of Tie2 was not accompanied by a concurrent drop in ATP content of the slices (ATP data not shown). Incubation of the slices with LPS for 8 hours had, however no extra effect on the Tie2 mRNA levels (figure 6.5B).

Tie2 reduction is paralleled by, but not directly related to, neutrophil influx and loss of glomerular barrier function integrity

In mice, LPS administration resulted in a rapid increase in expression of inflammatory proteins. For example, E-selectin was strongly expressed by glomerular and arteriolar endothelium, while scattered expression occurred in the peritubular microvasculature, and limited expression was observed in the postcapillary venules, which normalized within 24 hours (figure 6.6A). This inflammatory response was accompanied by a loss of glomerular barrier integrity as evidenced by the occurrence of a gradual increase in urinary albumin/creatinine ratio from 0 to 24 hours after the initiation of the insult (figure 6.6B).

By semi-quantitative analysis we showed that neutrophils represent the main responding white cell population in this model. Glomerular neutrophil influx was at a maximum at 4 hours after LPS injection (figure 6.7B). Leukocyte-endothelial cell interactions can contribute to changes in the molecular status of the endothelium, and

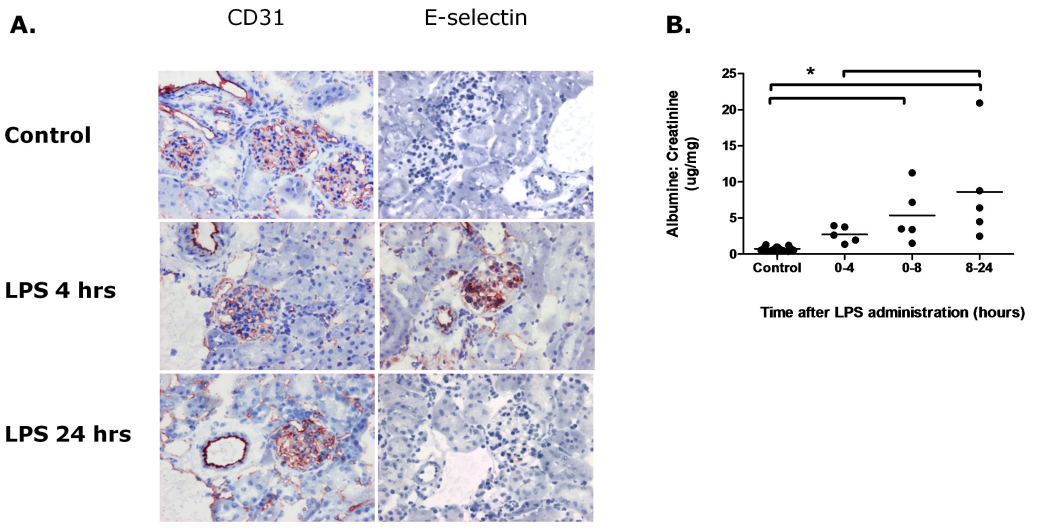


Figure 6.6. LPS administration to mice induced proinflammatory microvascular endothelial cell activation in parallel with proteinuria due to loss of glomerular barrier function.
*(A) Immunohistochemical staining of CD31 and E-selectin at two different time points after i.p. LPS administration show a minor loss of CD31 mainly from peritubular endothelial cells during the initial stage of shock, while at the same time E-selectin expression was mainly induced in arteriolar, glomerular and peritubular endothelium. Original magnification 200x. Representative sections of biopsies from 5 mice per group are shown, *p <0.05. (B) After LPS administration to mice (0.5 mg/kg) loss of glomerular barrier function became visible by an increase in urine albumin/creatinin ratio.*

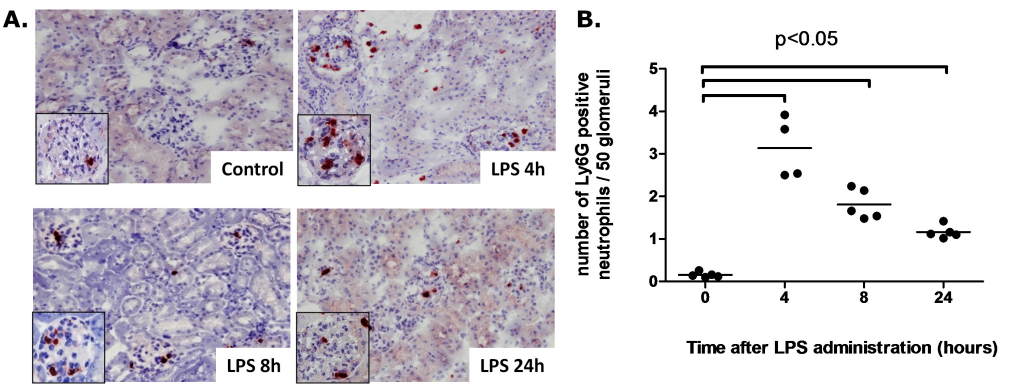


Figure 6.7. Kinetics of neutrophil influx in kidneys of mice subjected to LPS challenge.
(A) Renal infiltrating neutrophils were detected by Ly6G immunohistochemical staining in mouse kidneys at different time points after LPS administration. Original magnification 200x, inserts show glomeruli at original magnification of 400x. (B) Quantification of the extent of neutrophil influx was assessed by counting 50 randomly chosen glomeruli per biopsy at 400x magnification p<0.05.*

represent a process that is absent in the in vitro cell culture system employed. Especially in the microvasculature, leukocyte-endothelial cell interactions can be rather extensive as the diameter of the capillaries if often as small as, or even smaller than the diameter of the white blood cells passing by[30]. To examine the hypothesis that neutrophil-endothelial cell interactions contribute to the loss of renal microvascular Tie2, and that this loss is related to loss of glomerular endothelial integrity, we depleted neutrophils prior to LPS administration and studied its consequences for Tie2 expression and proteinuria. FACS analysis of whole blood of mice 24 hours after injection of NIMP-antibody demonstrated that the mice had become severely neutropenic, with only 2.1 +/- 1.5% of total white blood cell count being neutrophils versus 23.7 +/- 9.1% in mice treated with control IgG

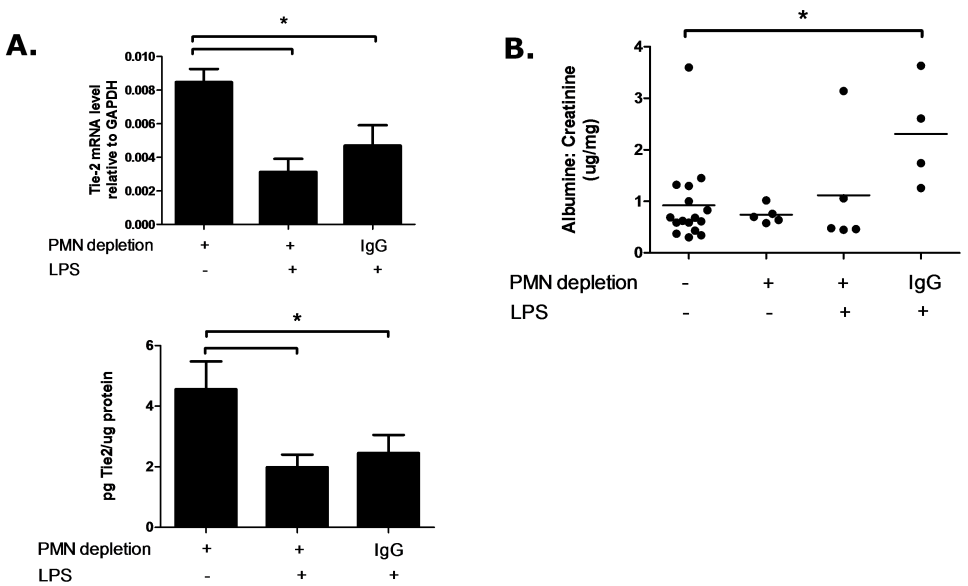
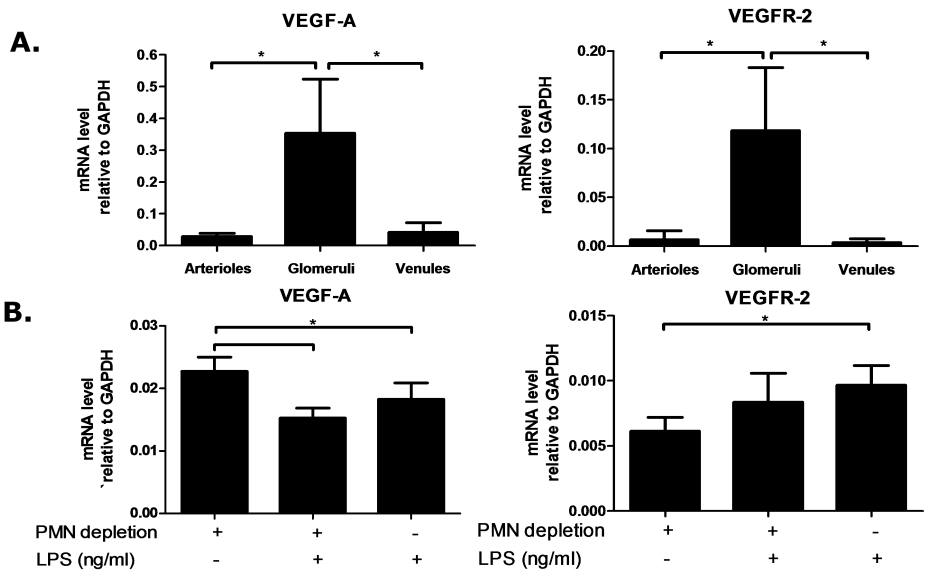


Figure 6.8. Neutrophil depletion did not affect LPS induced loss of Tie2, yet abolished loss of glomerular barrier function.

*(A) Effect of LPS on Tie2 mRNA (top) and protein (bottom) expression in neutrophil depleted mice. Mice were 24 hours prior to LPS challenge treated with anti-NIMP antibody to deplete PMN, or control IgG antibody that did not affect PMN count as assessed by flowcytometry (not shown). At t=0 mice were i.p. challenged with LPS at 0.5 mg/kg and sacrificed 8 hours later. Mean of n=5 ± SD, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferonni correction. Mean ± SD, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferonni correction. (B) Neutrophil depletion by pre-treatment of mice with anti-NIMP antibody abolished LPS induced proteinuria, while pre-treatment with control antibody did not affect this pathophysiological process. All mice were housed in a metabolic cage prior to the experiment (PMN-, LPS – group), while after the PMN of IgG and LPS injection mice were housed for 8 hours in a metabolic cage before they were sacrificed. Mean and individual values are shown, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferroni correction.*

antibody ($p < 0.001$). Interestingly, neutrophil depletion did not block the LPS induced Tie2 downregulation, neither at the mRNA nor at the protein level (figure 6.8A). VEGF-A has a role in the maintenance of glomerular endothelial integrity under physiological circumstances and VEGF signaling may play a protective role in pathophysiological stress. In control kidneys, mRNA encoding VEGF-A and VEGFR-2 were mainly localized in the glomeruli (supplement figure B-A), corroborating previous reports[31]. 8 Hours after LPS administration no differences in VEGF-A nor VEGFR-2 between the neutrophil depleted and neutrophil competent mice could been observed (supplemental figure 6B). At the same time neutrophil depletion did diminish the occurrence of proteinuria in response to LPS administration (figure 6.8B).



Supplemental figure 6B. VEGF-A and VEGFR2 are expressed in different microvascular beds in healthy mouse kidney. Their expression levels were not affected by neutrophil depletion.

*(A) Expression of VEGF-A and VEGFR-2 mRNA levels analysed by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in three renal microvascular beds obtained by laser microdissection. Mean values ± SD of 3 mice per group, * p<0.05.*

(B) Effect of LPS on VEGF-A and VEGFR-2 mRNA expression in PMN depleted versus neutrophil competent mice. Mice were 24 hours prior to LPS challenge treated with anti-NIMP antibody to deplete neutrophils, or control IgG antibody that did not affect PMN count as assessed by flow cytometry (not shown). At t=0 mice were i.p. challenged with LPS at 0.5 mg/kg and sacrificed 8 hours later. Mean of n=5 ± SD.

DISCUSSION

In various conditions of shock, the microvasculature of the kidney loses its integrity, leading to protein leakage and loss of kidney function. As the receptor tyrosine kinase Tie2 is implicated in the control of vascular integrity, we studied in mouse kidney the consequences of hemorrhagic shock and endotoxaemia on Tie2 expression in relation to proteinuria. In this study, we demonstrated for the first time that both Tie2 mRNA and protein were rapidly, and temporarily lost from the renal microvasculature in reaction to shock conditions. At the same time, the microvasculature was strongly activated leading to recruitment of neutrophils into the glomerular compartment and concurrent proteinuria. Neutrophil depletion resulted in reduction of proteinuria, which was however not accompanied by Tie2 mRNA or protein rescue, implying that Tie2 may not be a major factor controlling in the maintenance of the glomerular filtration barrier in acute shock.

Tie2 protein loss can be explained by shock induced Tie2 degradation. Bogdanovic et al elegantly showed in HUVEC cultures that in response to Ang-1, Tie2 is rapidly internalized and degraded, while Ang-2 mildly induced Tie2 degradation[27]. In healthy human volunteers subjected to LPS, a systemic increase in Ang-2 levels was observed with a maximum peak of 5 times control values at 4.5 hours after LPS challenge, while Ang-1 remained relatively unchanged[32]. Would a similar change in serum levels occur in our mouse model, one could hypothesize that in vivo a rise in Ang-2 levels may be the cause of Tie2 internalization. As at present systemic Ang-2 levels cannot be assessed in mice due to lack of proper analytical tools, the role of Ang-2 binding to Tie2 as a trigger for Tie2 protein degradation in the renal microvasculature remains speculative. The rapid and temporary loss of Tie2 mRNA can at present not be accounted for. Possibly, shear stress induced changes may acutely affect endothelial Tie2 expression, as was previously reported to be a major controlling factor for the expression of the orphan receptor Tie1[33]. If this would be the case, it could explain why a reduction in Tie2 mRNA levels was not be brought about in our static in vitro model systems. Preliminary studies on the effects of i.v. TNF-α administration on Tie2 in our laboratory revealed a direct or indirect role for NF-κB in the control of renal mRNA loss, as pretreatment of mice with an NF-κB inhibitor resulted in Tie2 mRNA rescue upon TNF-α challenge (unpublished data).

As in vitro studies could not mimic the in vivo observations, further studies on the molecular mechanisms underlying the current observations should be executed in vivo, and may need to make use of pharmacological tools, or endothelial cell specific knock outs to affect specific kinases. Whether a causal relation exists between Tie2 loss and changes in pO_2 or shear stress, immune cell expressed Tie2 – microvascular endothelial cell Angiopoietin/Tie2 interactions[34], or e.g., Interleukin-18[35], and what the functional consequences of Tie2 loss will be for the renal microvasculature, will be subject of future studies.

The fact that under acute shock conditions, the renal microvasculature temporarily loses both Tie2 protein and mRNA, but that the loss is not per se associated with major changes in glomerular barrier function, implies that other factors are likely involved in the regulation of the integrity of glomerular microvascular segments[4]. In our effort to identify these factors we demonstrated that neither VEGF-A nor its receptor VEGFR-2 were differentially affected in the neutrophil competent versus the neutrophil incompetent mice.

Moreover, in the acute shock conditions in both mice and man studied here, loss of Tie2 was not associated with increased plasma levels of sTie2, while sTie2 has previously been shown to be associated with microvascular dysfunction under pathological conditions in both mice and men[25; 36]. For example, sTie2 plasma levels are elevated in Crohn's disease[37], critical limb ischemia[38], and acute myocardial infarction[39]. Lowering of sTie2 is furthermore a prognostic marker in the treatment of renal carcinoma[40], a tumor type associated with elevated VEGF production which was identified as one of the triggers for Tie2 shedding from the endothelial membrane[28]. Proteinuria is present early in septic patients and a prognostic factor for the development of sepsis in postoperative patients[41], and we cannot rule out that in more complex situations of shock deviant Tie2 expression is a contributing factor for proteinuria, and that sTie2 levels are subject to change.

Recently, Mofarrahi and colleagues reported on the downregulation of Tie2 protein in liver, lungs and diaphragm of LPS challenged mice[42]. The kinetics of Tie2 downregulation between these organs and the kidney examined in our study, differed quite significantly. While in the liver and lung Tie2 protein levels did not normalize up to 24 hours after LPS administration, in the kidneys they do. These deviations may be explained by the fact

that effects of LPS are dosage, and LPS and mouse strain dependent[43]. Both studies used C57Bl/6 mice, yet the strains of E. Coli were different, as was the dose (serotype O55:B5 at 20 mg/kg vs 026:B6l at 5 mg/kg respectively). The considerable heterogeneity in basic microvascular endothelial cell behavior in organ specific microenvironments[44] may contribute to differences in molecular control of the observed changes. As Mofarrahi and colleagues did not relate Tie2 loss with vascular leakage, it remains to be established whether in other vascular segments in the body loss of Tie2 is associated with loss of vascular integrity.

Our mouse models to represent patients with critical illness have some shortcomings. The hemorrhagic shock models may have resemblance with patients with trauma hemorrhage, while LPS induced shock is certainly a laboratory model for human sepsis. Shock induced organ failure is a multi-step and time dependent process and in our models full development of the organ failure is not awaited. Also the influence of organ failure support, like mechanical ventilation which per se could induce multiple organ failure, is not studied in our animal models. Although there are more clinical relevant animal models for sepsis and trauma hemorrhage, we chose to use our models based on the fact that these highly standardized and frequently used single hit animal models are reproducible and make comparison with the published research possible. The lack of multiple insults in our models, which are seen in trauma hemorrhage and sepsis patients, might compromise translation of our findings. Yet it does not affect our findings per se that also Tie2 can be dynamically controlled.

To study Tie2 down regulation in kidneys of septic patients, kidney biopsies are required. Because of the risks of bleeding, it is unethical to do this for research purposes. We therefore tried to mimic the septic response in an ex vivo kidney slice model[24]. Kidney slices were incubated with different sepsis mediators, yet none of them invoked a Tie2 downregulatory response more than the downregulation already induced by the 8 hours ex .vivo incubation. Of note was, the fact that in all experiments, incubation of kidney slices per se in well oxygenated conditions induced Tie2 mRNA loss already within 4 hours while the ATP content of the slices were not compromised (data not shown). Likely, early in the ex-vivo experiments reactions in the kidney tissue are activated. As Tie2 is related to vascular integrity, it may be worthwhile to follow up this observation in the scope of organ preservation for transplantation purposes.

In summary, we observed a rapid and temporary, substantial loss of Tie2 mRNA and protein from the renal microvasculature in reaction to hemorrhagic shock and LPS mediated endotoxaemia without concurrent sTie2 level increase. Loss of Tie2 could not be directly related to the occurrence of proteinuria.

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Chapter 7

Time course of Angiopoietin-2 release during experimental human endotoxemia and sepsis

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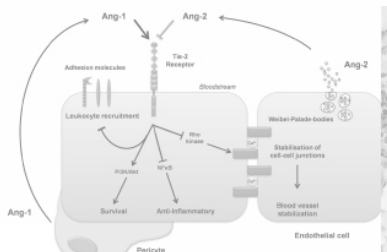

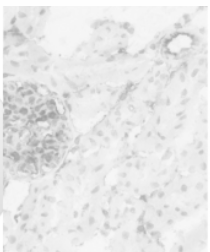
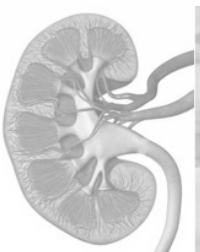


ABSTRACT

Introduction: Endothelial activation leading to vascular barrier breakdown denotes a devastating event in sepsis. Angiopoietin (Ang)-2, a circulating antagonistic ligand of the endothelial specific Tie2 receptor, is rapidly released from Weibel-Palade bodies and has been identified as a non-redundant gatekeeper of endothelial activation. We aimed to study: the time course of Ang-2 release during human experimental endotoxemia; the association of Ang-2 with soluble adhesion molecules and inflammatory cytokines; and the early time course of Ang-2 release during sepsis in critically ill patients.

Methods: In 22 healthy volunteers during a 24-hours period after a single intravenous injection of lipopolysaccharide (LPS; 4 ng/kg) the following measurement were taken by immuno luminometric assay (ILMA), ELISA, and bead-based multiplex technology: circulating Ang-1, Ang-2, soluble Tie2 receptor, the inflammatory molecules TNF-α, IL-6, IL-8 and C-reactive protein, and the soluble endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin. A single oral dose of placebo or the p38 mitogen activated protein (MAP) kinase inhibitor drug, RWJ-67657, was administered 30 minutes before the endotoxin infusion. In addition, the course of circulating Ang-2 was analyzed in 21 septic patients at intensive care unit (ICU) admission and after 24 and 72 hours, respectively.

Results: During endotoxemia, circulating Ang-2 levels were significantly elevated, reaching peak levels 4.5 hours after LPS infusion. Ang-2 exhibited a kinetic profile similar to early proinflammatory cytokines TNF-α, IL-6, and IL-8. Ang-2 levels peaked prior to soluble endothelial-specific adhesion molecules. Finally, Ang-2 correlated with TNF-α levels ($r = 0.61$, $p = 0.003$), soluble E-selectin levels ($r = 0.64$, $p<0.002$), and the heart rate/mean arterial pressure index ($r = 0.75$, $p<0.0001$). In septic patients, Ang-2 increased in non-survivors only, and was significantly higher compared with survivors at baseline, 24 hours and 72 hours.

Conclusions: LPS is a triggering factor for Ang-2 release in men. Circulating Ang-2 appears in the systemic circulation during experimental human endotoxemia in a distinctive

temporal sequence and correlates with TNF-α and E-selectin levels. In addition, not only higher baseline Ang-2 concentrations, but also a persistent increase in Ang-2 during the early course identifies septic patients with unfavorable outcome.

INTRODUCTION

Microvascular capillary leakage resulting in tissue edema, vasodilation refractory to vasopressors, and increased recruitment of leucocytes denote key features of sepsis-related endothelial-cell activation. During the course of severe sepsis and septic shock, widespread endothelial-cell activation contributes to the initiation and progression of multi-organ failure[1]. Recently, Angiopoietin-2 (Ang-2) has emerged as a key regulator of endothelial-cell activation[2]. In critically ill patients, Ang-2 increases endothelial permeability and is considered a key molecule in the pathogenesis of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[3; 4].

Angiopoietin-1 (Ang-1) and Ang-2 are antagonistic ligands, which bind to the extracellular domain of the Tie2 receptor, which is almost exclusively expressed by endothelial cells[5; 6]. Binding of the agonist Ang-1 to the endothelial Tie2 receptor maintains vessel integrity, inhibits vascular leakage, suppresses inflammatory gene expression, and prevents recruitment and transmigration of leukocytes[7; 8]. In vitro, Ang-2 simultaneously mediates disassembly of cell–cell and cell–matrix contacts, and causes active endothelial cell contraction in a Rho kinase-dependent fashion, followed by massive plasma leakage and loss of vasomotor tone[3; 9]. Furthermore, Ang-2 facilitates up-regulation of Inter-Cellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule -1 (VCAM-1), and E-selectin[3; 5; 10; 9].

In vivo, Ang-2-deficient mice do not exhibit any vascular inflammatory responses in experimental sepsis, and vessels in Ang-1-overexpressing mice are resistant to leakage to inflammatory stimuli[11; 12]. As a Weibel-Palade body-stored molecule (WPB), Ang-2 is rapidly released upon endothelial stimulation and is regarded the dynamic regulator within the Ang/Tie system[7; 11]. Consistently, exceptionally high level of circulating Ang-2 have been detected in critically ill patients with sepsis and sepsis-related organ dysfunction[13-15].

Beyond its role as a mediator, Ang-2 has been identified as a promising strong marker of endothelial activation in various diseases[16-18]. In critically ill septic patients, we recently showed that admission levels of circulating Ang-2, correlates with surrogate markers of tissue hypoxia, disease severity, and is a strong and independent predictor of mortality[19]. However, the exact time course of Ang-2 release during sepsis and the role of

Table 7.1. Characteristics of septic ICU patients on admission.

CHARACTERISTICS	VALUE
Patients, no.	21
Male	8 (38.1%)
Female	13 (61.9%)
Age (years, median (min - max)	57 (36 - 72)
Reason for medical ICU admission	
Pneumonia	12 (57.1%)
Peritonitis	4 (19.0%)
Urinary tract infection	2 (9.5%)
Systemic Mycosis	2 (9.5%)
Endocarditis	1 (4.8%)
Mediastinitis	1 (4.8%)
Mean arterial pressure (mmHg)	78 (58-108)
Heart rate (bpm)	95 (53 - 125)
Vasopressor support, no.	12 (57.1%)
Mechanically ventilated, no.	19 (90.5%)
FiO_2 (%)	40 (25 - 95)
APACHE II score	22 (12 - 48)
SOFA score	10 (3 - 19)
Mortality, no.	11 (52.4%)

inflammatory cytokines thereof remain elusive. Furthermore, the tempting sequential concept[11] of Ang-2 as a primer for excess endothelial adhesion molecule expression in sepsis (e.g. ICAM-1, VCAM-1, and E-selectin) has not been investigated in human sepsis.

To address these issues, we wanted to study (1) the time course of Ang-2 release, and (2) the association of Ang-2 with soluble adhesion molecules and inflammatory cytokines in a graded and well-defined human endotoxemia model. Therefore, we re-measured circulating Ang-2, cytokines, and adhesion molecules in blood samples from a placebo-controlled interventional trial on pharmacologic p38 Mitogen-Activated Protein (MAP) kinase inhibition during experimental human endotoxemia[20]. Furthermore, we analyzed circulating Ang-2 during a 72 hours time course after ICU admission in septic patients.

METHODS

Subjects

Twenty-one healthy male subjects, mean age 29 (range 19–44) years, were admitted to the research unit of our intensive care unit (Medical Department) at University Medical Center of Groningen, Groningen, The Netherlands. The local Medical Ethics Committee approved the study and written informed consent was obtained. A radial arterial catheter was placed for blood sampling. Thirty minutes before the infusion of LPS, the volunteers received a single oral dose of RWJ-67657 (4-[4-(Fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyrindinyl)-1H-imidazol-2-yl]-3-butyn-1-ol), supplied in an oral pharmaceutical formulation by R. W. Johnson Pharmaceutical Research Institute, Bassersdorf, Switzerland). Three dose levels were tested, placebo-controlled: placebo (*n* = 6), 350 mg (n = 5), 700 mg (*n* = 6) and 1400 mg (*n* = 4). At time point t = 0, LPS (E-Coli, batch EC-6, US Pharmacopeia, Twinbrook Parkway, Rockville, MD, USA) was administered as a 1 min infusion at a dose of 4 ng/kg body weight (10,000 LPS units/mg). Blood samples were drawn at several time points between pre-medication (t = 0) and 24 h after administration of LPS. Samples were placed on ice, centrifuged, stored at -80°C, and analyzed in a blinded fashion[20].

Patients

The time course of Ang-2 release during the early course of human sepsis was studied in 21 ICU patients (Internal Medicine Department) recruited at Hannover Medical School (tertiary care university hospital), Hannover, Germany. Patient characteristics are shown in table 7.1. Enrollment was performed after obtaining written informed consent from the patient or his/her legal representatives. If the patient was recovering and able to communicate, he/she was informed of the study purpose and consent was required to further maintain status as a study participant. Twenty-eight day survival was the primary outcome studied and was calculated from the day of ICU admission to day of death from any cause. Patients who did not suffer from death within the follow-up were censored at the date of last contact. The study was carried out in accordance with the declaration of Helsinki and was approved by the institutional review board. Serum samples were obtained at baseline (admission), 24h and 72h, placed on ice, centrifuged, stored at

-80°C, and analyzed in a blinded fashion.

Quantification of circulating Angiopoietin-1 and 2, and soluble Tie2

Ang-1 and Ang-2 were measured by in-house Immuno Luminometric Assay (ILMA), and Enzyme Linked Immuno Sorbent Assay (ELISA) as published previously in This Journal[16; 17; 19]. Soluble Tie2 was measured by commercially available ELISA kit (R&D Systems, Oxon, U.K.) *according to the manufacturers' instructions*.

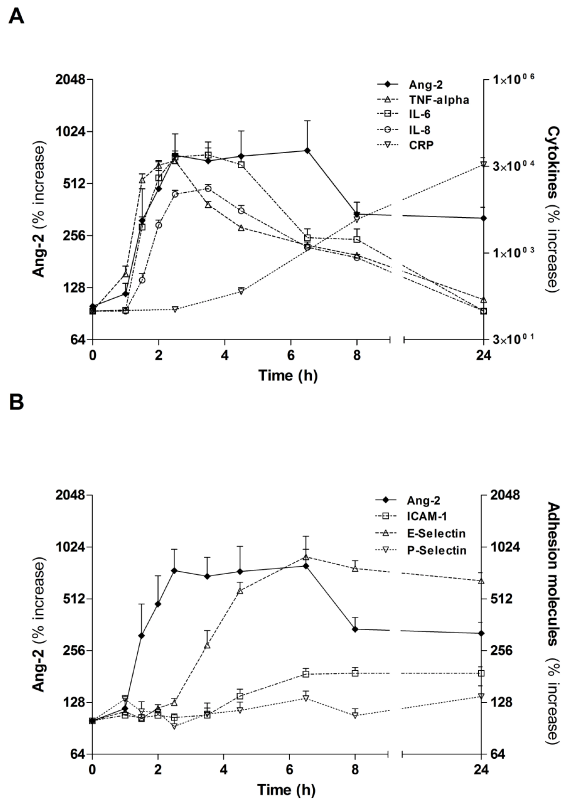

Figure 7.1. Time course of Ang-2, cytokines, and adhesion molecules after LPS challenge in healthy subjects.

(A) Concentrations of circulating Ang-2 compared to plasma levels of TNF-α. IL-6, IL-8, and C-reactive protein after LPS challenge in six healthy volunteers. (B) Concentrations of circulating Ang-2 compared to plasma levels of endothelial adhesion molecules E-selectin, P-selectin, and ICAM-1 after LPS challenge in six healthy volunteers. Non-parametric ANOVA (Friedman's test) with Dunn´s test for multiple comparison (two sided) was used to demonstrate statistical changes in Ang-2, cytokines, and adhesion molecules (y-axes denote %-increase; baseline = 100%).

Table 7.2. Time course after LPS challenge in healthy subjects

Variables	Pre-Dose	1h	1.5h	2h	2.5h	3.5h	4.5h	6.5h	8h	24h	p-value
Systolic BP (mmHg)	140 ± 14	139 ± 11	152 ± 11	158 ± 18	151 ± 18	142 ± 22	121 ± 18	107 ± 11	104 ± 11	131 ± 11	<0.0001
Diastolic BP (mmHg)	74 ± 10	74 ± 7	80 ± 7	77 ± 12	65 ± 12	61 ± 14	54 ± 11	55 ± 8	55 ± 7	66 ± 7	<0.0001
MAP (mmHg)	96 ± 11	96 ± 9	104 ±7	104 ± 13	94 ± 14	88 ± 16	77 ± 13	72 ± 8	71 ± 7	88 ± 7	<0.0001
Heart rate (bpm)	61 ± 16	59 ± 11	78 ± 19	78 ± 18	92 ± 12	98 ± 8	101 ± 8	97 ± 11	96 ± 13	81 ± 16	<0.0001
HR/MAP Index	0.64 ± 0.11	0.62 ± 0.11	0.75 ± 0.17	0.76 ± 0.22	1.01 ± 0.22	1.15 ± 0.26	1.36 ± 0.32	1.36 ± 0.27	1.4 ± 0.22	0.92 ± 0.16	<0.0001
Body temperature (°C)	35.4 ± 0.38	36.2 ± 0.54	36.4 ± 0.87	37.0 ± 1.04	37.6 ± 1.11	38.5 ± 0.66	38.9 ± 0.53	38.14 ± 0.28	37.9 ± 0.19	36.2 ± 0.29	<0.0001
White blood count (10^3/µl)	5.5 ± 0.7	-	-	-	-	-	-	-	-	10.9 ± 1.6	0.03
C-reactive protein (mg/l)	1.1 ± 2.4	0.9 ± 1.8	-	-	1.5 ± 2.4	2.2 ± 2.5	1.1 ± 2.3	1.8 ± 2.8	4.8 ± 2.0	60.0 ± 21.5	<0.0001
Ang-1 (ng/ml)	67.0 ± 20.7	58.2 ± 24.4	-	-	61.2 ± 25.0	54.3 ± 19.5	-	64.9 ± 29.1	60.3 ± 31.4	52.3 ± 21.6	0.053
Ang-2 (ng/ml)	0.57 ± 0.50	0.63 ± 0.20	1.04 ± 0.65	1.63 ± 0.89	2.33 ± 0.69	2.35 ± 1.06	2.42 ± 1.32	2.23 ± 1.18	1.61 ± 1.07	1.51 ± 1.03	<0.0001
Tie2 (ng/ml)	1.34 ± 0.31	1.23 ± 0.29	1.33 ± 0.32	1.53 ± 0.52	1.23 ± 0.20	1.3 ± 0.16	1.31 ± 0.35	1.4 ± 0.3	1.25 ± 0.32	1.43 ± 0.42	0.085

Legend: A non-parametric repeated-measures ANOVA (Friedman's test) was used to test for significant changes of variables during the time course after LPS challenge (placebo group; n=6).

Abbreviations: BP – blood pressure; HR/MAP index - heart rate/mean arterial pressure index.

Quantification of soluble endothelial-adhesion molecules and cytokines

Soluble ICAM-1, E-selectin, and P-selectin were measured using *Fluorokine® MultiAnalyte Profiling* kits and a *Luminex®* Bioanalyzer (R&D Systems, Oxon, U.K.) *according to the manufacturers' instructions*. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8) and c-reactive protein (CRP) were determined using MEDIGENIX EASIA kits from BioSource (BioSource, Nivelles, Belgium) and reported previously[20].

Statistical Analysis

The modified Kolmogorov-Smirnov test was used to test for a normal distribution of continuous variables. In the human endotoxemia model, a non-parametric ANOVA (Friedman's test) with Dunn´s test for multiple comparison (two sided) was used to demonstrate statistical changes in Ang-2, cytokines, and adhesion molecules. Correlations of Ang-2 with TNF-α, E-selectin, and the heart rate/mean arterial pressure index were calculated with Pearsons's correlation and linear regression analysis after log-transformation. Data are presented as mean ± SEM unless otherwise stated.

In patients, differences between survivors and non-survivors at baseline and during follow-up were compared by non-parametric two-sided Mann Whitney test. Receiver Operator Characteristic (ROC) procedures identified optimal cut-off values for Ang-2 to differentiate between survivors and non-survivors. Contingency table-derived data and likelihood ratios were calculated using the StatPages website. Two-sided p-values <0.05 were considered statistically significant for all statistical procedures used. All statistical analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism software (GraphPad Prism Software Inc. San Diego, California, USA).

RESULTS

Angiopoietin-2 is released in a distinctive pattern after endotoxin challenge in healthy volunteers

Normal Ang-2 concentrations (0.57 ± 0.20 ng/mL) were present at baseline in healthy volunteers (table 7.2). Ang-2 levels started to increase at 2 h, were significantly elevated from 2.5 h until 6.5 h (<0.01), reaching peak levels (2.42 ± 0.54 ng/mL) 4.5 h after LPS

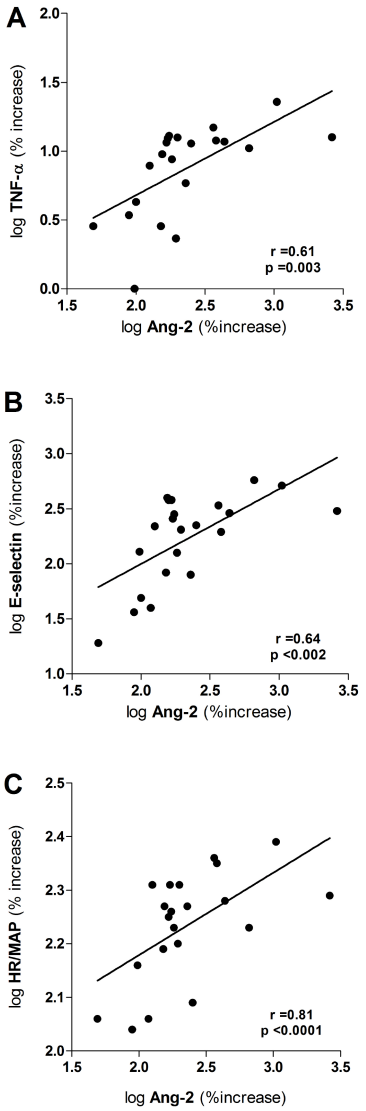

Figure 7.2. Correlation of Ang-2 with TNF-α, E-selectin and heart rate/mean arterial pressure index after LPS challenge in healthy subjects.

Dot blots showing the correlation between circulating Ang-2 and (A) plasma levels of TNF-α, (B) plasma levels of the soluble endothelial specific adhesion molecule E-selectin, and (C) the heart rate/mean arterial pressure index (HR/MAP index) at 6.5 h after LPS challenge in 21 subjects (placebo (n=6), and medication groups: 350 mg (n=5). 700 mg (n=6). and 1400 mg (n=4), respectively). Pearsons correlation coefficient was used after logarithmic transformation of variables (axes denote %-increase after logarithmic transformation; baseline = 100%).

infusion (p <0.0001) (figure 7.1) (n=6, placebo group).

In our cohort of healthy volunteers, neither endogenous sTie2, nor circulating Ang-1 concentrations changed during 24h after endotoxin challenge (table 7.2).

Angiopoietin-2 release runs in parallel with early pro-inflammatory cytokines and precedes endothelial inflammation after endotoxin challenge

Plasma levels of TNF-α were already significantly elevated at 1½ h (p<0.01) compared to baseline, and 30 minutes earlier compared to Ang-2 and IL-6 (figure 7.1A). IL-8 appeared in the circulation ~30 minutes later than Ang-2 and IL-6. Elevated Ang-2 levels declined more slowly than that of TNF-α, IL-6, and IL-8.

Soluble E-selectin appeared in the circulation later than Ang-2 and E-selectin levels were elevated from 4.5 h until 24 h (all p <0.0001). Similarly, ICAM-1 levels were elevated from 6.5 h until 24 h after LPS infusion (all p <0.0001) (figure 7.1B). However, P-selectin did not increase after endotoxin challenge in the present study (p =0.151).

Angiopoietin-2 release after endotoxin challenge is attenuated by p38 MAPK inhibition

Our previous studies have shown that inhibition of the intracellular p38 MAPK attenuated inflammatory responses during human endotoxemia[20]. Thus, we hypothesized that p38 MAPK inhibition would also have an impact on Ang-2 release. In addition to LPS treated subjects that received placebo (n=6, see above), circulating Ang-2 was

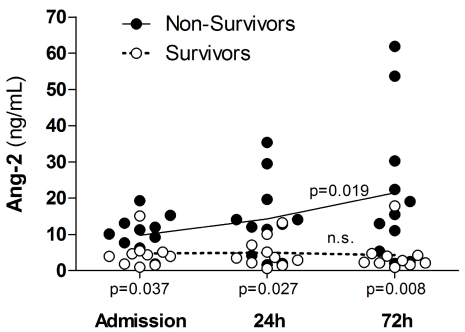

Figure 7.3. Time course of Ang-2 in critically ill patients with sepsis.
Dot blots showing the concentration of Ang-2 (ng/ml) in 21 septic on ICU admission, 24 hours and 72 hours after admission, respectively. Of note, median Ang-2 levels increased in non-survivors (p=0.019) (continuous line), but remained unchanged in survivors (p=0.83) (dotted line) during the time course. Mean Ang-2 level was higher in non-survivors (filled circles, n=11) compared to survivors (open circles, n=10) on admission (p=0.032), after 24 hours (p=0.027), and 72 hours (p=0.008) (two-sided Mann-Whitney test).

determined in LPS treated subjects that were randomized to different doses of an oral p38 MAPK inhibitor[21]. In contrast to the placebo group (LPS without p38 MAPK inhibitor), no statistically significant Ang-2 release occurred in any of the three interventional groups (i.e. 350 mg, 700 mg, or 1400 mg of RWJ-67657, respectively). However, when the areas under the curves (AUC) during the time course were calculated, a dose dependent effect of RWJ-67657 on Ang-2 release was present.

The AUC of absolute Ang-2 values (ng/ml) were 39.8, 31.0, 32.1, and 17.8 in the placebo and the three interventional groups, respectively. Correspondingly, the AUC of %-increase in Ang-2 from baseline were 9850, 4765, 3435, and 2567 in the placebo and the three interventional groups, respectively).

Circulating Angiopoietin-2 correlates with TNF-alpha levels, soluble E-selectin levels, and the heart rate/mean arterial pressure index

TNF-α levels correlated well with Ang-2 at 3.5 ($r = 0.44$, $p = 0.04$), 4.5 h ($r = 0.54$, $p = 0.012$), 6.5 h ($r = 0.61$, $p = 0.003$), and 8 h ($r = 0.49$, $p = 0.024$) (figure 7.2A). Likewise, levels of soluble E-selectin were closely associated with Ang-2 at 4.5 h ($r = 0.5$, $p = 0.005$), 6.5 h ($r = 0.64$, $p = 0.0013$), and 24 h ($r = 0.69$, $p < 0.0004$) (figure 7.2B), when all subjects in the endotoxin model were analyzed (n=21). Finally, we analyzed the increase in heart rate (HR)/mean arterial pressure (MAP) index as a dynamic surrogate marker of hemodynamic compromise. Indeed, a close correlation was found between the increase in circulating Ang-2 and the increase in HR/MAP index at 4.5 h ($r = 0.6$, $p = 0.003$), 6.5 h ($r = 0.58$, $p = 0.006$), and 8 h ($r = 0.75$, $p < 0.0001$) (figure 7.2C), when all subjects were analyzed (n=21).

Excess Ang-2 on admission and increasing Ang-2 level during the early course indicate unfavorable 28-day survival in septic patients

First, circulating Ang-2 on admission was 9.8 ± 3.2 ng/ml in septic patients (n=21). Regarding the kinetics of Ang-2 during follow-up, mean Ang-2 levels remained unchanged at 24h (14.3 ± 4.0 ng/ml) and 72h (18.2 ± 6.0 ng/ml) when all patients were analyzed (non-parametric repeated measures ANOVA (Friedman´s test); p=0.146) (figure 7.3). Second, when analyzed separately, non-survivors (n=11) had higher Ang-2 levels compared to survivors (n=10) on admission (9.7 ± 1.6 ng/ml vs. 4.7 ± 1.3 ng/ml; p=0.032), after 24h

(13.3 ± 3.2 ng/ml vs. 5.0 ± 1.3 ng/ml; p=0.027) and 72h (21.5 ± 6.0 ng/ml vs. 4.3 ± 1.6 ng/ml; p=0.008). In non-survivors, Ang-2 levels were significantly increased after 72h (9.7 ± 1.6 ng/ml vs. 21.5 ± 6.0 ng/ml; p=0.019). In contrast, no increase in Ang-2 level was detected in survivors during follow-up (4.7 ± 1.3 ng/ml vs. 4.3 ± 1.6 ng/ml; p=0.83) (figure 7.3). Last, we calculated sensitivity, specificity and predictive values by 2 x 2 tables including all patients (n=21) to compare the predictive value between (i) absolute Ang-2 at baseline, (ii) absolute Ang-2 at 72h, and (iii) the decrease/increase of Ang-2 between baseline and during 72h, respectively. At baseline (admission), a ROC-optimized Ang-2 cut-off value >5.9 ng/ml best identified non-survivors with 90% specificity and 81% sensitivity. The positive predictive value was 90% and the negative predictive value 81%. In patients with Ang-2 values >5.9 ng/ml, the odds ratio (OR) was 40.5 (95% CI 3.7-398.1) for death during 28-day follow-up (Fisher exact test p=0.002). Essentially the same results were obtained at 72h when a ROC-optimized Ang-2 cut-off value >5.0 ng/ml was used (Fisher exact test p=0.002). In a similar fashion, albeit with a lower statistical significance, the Ang-2 time course (as a categorical variable: increase vs. non-increase in Ang-2 during 72h) identified non-survivors with 81% specificity and 80% sensitivity. The positive predictive value was 81% and the negative predictive value 80%. In patients with increasing Ang-2 values (during 72h), the OR was 18.0 (95% CI 2.2-144.6) for death during 28-day follow-up (Fisher exact test p=0.009).

Discussion

The present study dissects the time course of Ang-2 release after experimental LPS administration in healthy subjects. The decisive results are: (1) LPS (4 ng/ml) is a triggering factor for Ang-2 release in vivo; (2) circulating Ang-2 reached peak levels 4 ½ h after LPS infusion; (3) Ang-2 exhibited a kinetic profile similar to that of TNF-α, IL-6, and IL-8, peaked explicitly prior to soluble endothelial-specific adhesion molecules, and correlated with TNF-α levels, soluble E-selectin levels, and the HR/MAP index; (4) In septic patients, not only higher baseline Ang-2 but also a persistent increase in Ang-2 predicts unfavorable 28-day survival.

Clinical studies of pathophysiological changes during sepsis are potentially confounded by the absence of a well-defined onset time of inflammation, by significant co-morbid

conditions, as well as by considerable delays from the presumed initiation of inflammation until study inclusion. Animal studies, although indispensable for investigating early and late events during systemic inflammation, are potentially confounded by major inter-species differences in the sensitivity and immune response to various types of inflammatory stimuli[22; 23]. As already indicated, the present study is a re-analysis of blood samples from a placebo-controlled interventional trial on pharmacologic p38 MAP kinase inhibition in endotoxemia. The design of this trial enabled us to investigate the time course of Ang-2 release in humans in a highly standardized experimental model with a graded inflammatory response[20; 21].

After LPS infusion, peak Ang-2 levels (2.4 ± 0.5 ng/ml) are 4-fold lower than Ang-2 levels in critically ill patients at the ICU (9.8 ± 3.2 ng/ml). Emerging data from our group, as well as a recent study by Siner et. al[24] suggest the notion that survival is good in critically ill patients with low Ang-2 (< 7-8 ng/ml), whereas outcome is explicitly worse above this threshold. Indeed, septic patients with circulating Ang-2 levels below 5.9 and 5.0 ng/ml (admission and 72h) identified patients with good 28-day survival in the present study. Compared to the experimental endotoxemia model (single dose of LPS), the inflammatory stimuli in critically illness are probably more intense, often persistent, and multiple in nature. Thus, a rather low but significant Ang-2 peak level of 2.4 ng/ml (4-fold vs. baseline) during experimental endotoxemia is probably adequate and well in line with the aforementioned data. Although we cannot rule out loss of Ang-2 immunoreactivity due to deep-freeze storage for several years, in our experience this phenomenon is negligible[25].

Ang-2 exhibited a kinetic profile that is similar to the early pro-inflammatory cytokines TNF-α, IL-6, and IL-8. In the present study, TNF-α level increased somewhat earlier than Ang-2 levels did. As previously shown, the release of TNF-α and several other cytokines during human endotoxemia is blocked by p38 MAPK inhibition in a dose dependent manner[21]. In the present study, p38 MAPK inhibition blocked Ang-2 release in a similar fashion. In addition, Ang-2 correlated well with TNF-α throughout the time course after LPS infusion. This implies that either the Ang-2 release is mediated by TNF-α, or that a p38-MAP kinase dependent upstream signaling pathway controls both, TNF-α and Ang-2 release. Well in line with this data, Orfanos et al. reported a strong relationship of Ang-2 with TNF-α in critically ill patients, suggesting that the latter may participate in the

regulation of Ang-2 production in sepsis[26]. In contrast, Fiedler et al. showed that even high concentrations of TNF-α are not sufficient to induce Ang-2 release from Human Umbilical Vein Cells (HUVECs) in vitro[27]. However, we cannot exclude that there is an independent route with a slower signaling pathway, and the fact that TNF-α preceded Ang-2 release does not prove causality.

Expression of endothelial adhesion molecules such as E-selectin, VCAM-1, and ICAM-1, are a consistent feature of sepsis[28; 29]. As a functional antagonist of Ang-1/Tie2 signaling, Ang-2 promotes up-regulation of endothelial adhesion molecules (i.e. endothelial activation), by sensitizing endothelial cells toward cytokine-induced adhesion molecule expression. Consistently, firm leukocyte adhesion and subsequent transmigration is almost absent during experimental sepsis in Ang-2 deficient animals[3; 7; 11]. In the present study, Ang-2 levels increased and peaked explicitly prior to soluble endothelial-specific adhesion molecules E-selectin and ICAM-1. Furthermore, soluble E-selectin correlated well with circulating Ang-2 throughout the time course. This temporal sequence is in line with the concept proposed by Fiedler et al[7] that EC activation might indeed represent a predominantly Ang-2 driven process in vivo.

Although (circulating) Ang-2 has a significant adverse effect on pulmonary vascular barrier properties in sepsis[3; 4] its role in extra-pulmonary endothelial activation and systemic loss of barrier function is less well defined. However, Ang-1 increases arteriolar vasoconstriction to phenylephrine in the presence of LPS in vitro[30] and preserves blood pressure and cardiac output in septic rats in vivo[9]. Indeed, we found a close correlation between circulating Ang-2 and the HR/MAP as a surrogate marker for hemodynamic compromise in the present study. Although this observation does not prove causality, it is well in line with a significant association of circulating Ang-2 levels with MAP, and vasopressor requirement in a large cohort of critically ill patients (Kümpers et al. submitted).

Over the past few years, it has been appreciated that multiple components of WBP, such as Ang-2, P-selectin, and IL-8, are co-stored with von vWF, the major constituent of WPBs[7]. It has been shown that storage of Ang-2 and P-selectin in WPB is mutually exclusive. Interestingly, we detected a selective release of the aforementioned WPB stored mediators: Ang-2 is released first, then IL-8, and P-selectin is not released at all. The phenomenon that some components are selectively released from WPB has already

been show in vitro[31] and deserves further attention in in vitro studies of differential regulation of WPB exocytosis.

As a prepackaged constituent of WPB, it is not surprising that Ang-2 levels on admission are increased in response to early endothelial activation in critically ill patients[14; 24; 19]. Well in line, high Ang-2 levels on admission are associated with unfavorable 28-day survival. However, it still remained unanswered whether Ang-2 levels either decline, or even increase during the course of sepsis, as recently summarized by Giuliano and Wheeler in this Journal[32].

In vitro, intracellular Ang-2 pools are rapidly replenished after stimulated depletion with a PKC activator (phorbol 12-myristate 13-acetate)[27]. Furthermore, LPS administration has been shown to increase Ang-2 in a murine sepsis model[33]. Based on available data we hypothesized that levels of circulating Ang-2 might even increase in patients with persistent inflammation and/or clinical deterioration. Indeed, in our cohort of septic patients, non-survivors, not only presented with higher admission Ang-2 levels but also showed a significant increase in Ang-2 during a period of 72h. It is tempting to speculate that LPS regulates both, the release of Ang-2 from WPB and the transcriptional induction at the same time. However, additional pre-clinical but also clinical studies are urgently needed to clarify this issue.

Our study has several limitations. The human endotoxin model carries a risk of inappropriate extrapolation from experimental findings to the clinical setting. However, this is the only model that renders an opportunity to study the early mechanisms of endothelial activation during a time course in human subjects. Since von Willebrand Factor (vWF) (as the major constituent of WPBs) was not determined in this study, and citrated plasma samples from the original trial were not available for re-evaluation, we cannot exclude that Ang-2 might have derived from endothelial cells exclusively. At least murine macrophages seem to express smaller quantities of Ang-2 as well, but this has not been tested in experimental sepsis[34]. However, the time course of Ang-2 release in the present study was well in line with that of vWF release during endotoxemia[35].

Further, the sample size of the septic cohort was small. Thus, ROC procedures and 2 x 2 tables should be interpreted with caution. However, Fisher´s exact test (still appropriate even with small sample size) confirmed the significance of our findings. Finally, elevated circulating Ang-2 is not an exclusive feature of endotoxemia and sepsis, but rather

reflects endothelial activation and vascular damage in diseases that share a significant inflammatory endothelial phenotype[16; 17; 36].

Conclusions

We could show for the first time that LPS administration is a triggering factor for Ang-2 release in men. Circulating Ang-2 appears in the systemic circulation during experimental human endotoxemia in a distinctive temporal sequence and correlates with TNF-α and E-selectin levels. In addition, not only higher baseline Ang-2 concentrations, but also a persistent increase in Ang-2 during the early course identifies septic patients with unfavorable outcome.

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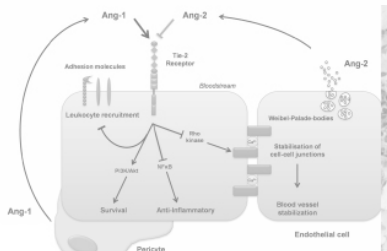
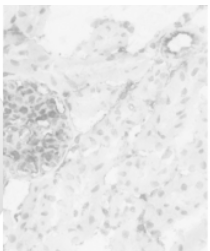
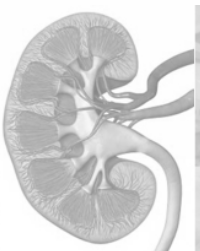
Chapter 8

Summary & Future perspectives




8.1 SUMMARY: THE MICROVASCULAR ENDOTHELIAL CELL IN SHOCK

Multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic (HS)- and septic shock and related to high morbidity and mortality. Interaction between activated neutrophils and activated endothelial cells is considered to play a prominent role in the pathophysiology of MODS (as introduced in **CHAPTER 1**). Insight in the nature and molecular basis of endothelial cell activation during shock can assist in identifying new rational targets for early therapeutic intervention and in evaluating frequently employed interventions in shock. In this thesis we used models of hemorrhagic- and septic shock to study microvascular endothelial responses in shock. We hypothesized that shock induced stress (being it sepsis or hemorrhage induced) activates microvascular endothelial cells to a pro-inflammatory state, and that microvascular beds in different organs would respond differently to the same stress. This pro-inflammatory endothelial activation can attract leukocytes to the organs, leading to organ damage. Understanding the nature, the molecular control, and the microvascular bed specific differences of these processes may allow us to identify targets for therapeutic intervention.

In **CHAPTER 2**, we examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS. Anesthetized mice were subjected to controlled hemorrhage to a Mean Arterial Pressure (MAP) of 30 mmHg. Mice were sacrificed after 15, 30, 60 or 90 minutes of HS. After 90 minutes of hemorrhagic shock, a group of mice was resuscitated with 6% hydroxyethyl starch. Untreated mice and sham shock mice that underwent instrumentation and 90 minutes of anesthesia without shock served as controls. Gene expression in kidney, liver, lung, brain, and heart tissue was studied. Induction of inflammatory genes occurred early during HS and already before resuscitation. Expression of adhesion molecules (P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1)) was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial genes CD31 and VE-cadherin, which function in endothelial cell homeostasis and integrity, were not significantly affected during the shock phase. The rapid inflammatory activation was not paralleled by induction of hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α). This study demonstrated the occurrence of early and organ-specific endothelial cell activation during hemorrhagic shock.

Mechanical ventilation (MV) is frequently employed in patients with HS. Intubation and MV protects organs from hypoxia and hypercapnia, on the other hand MV itself may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. So in **CHAPTER 3**, our aim was to investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lung and kidney, as well as the role of systemic hypoxia in EC (endothelial cell) activation in these organs. As already evident from chapter 2, 90 minutes after shock induction a vascular bed specific, heterogeneous pro-inflammatory endothelial activation represented by E-selectin, VCAM-1 and ICAM-1 expression was seen in kidney and lung. No differences in endothelial adhesion molecules between the spontaneous breathing and mechanically ventilated mice were found. In the presence of HS, no differences in organ mRNA levels for endothelial pro-inflammatory cytokines (TNF-α, IL-6, MCP-1) were found between the spontaneous breathing and mechanically ventilated mice. In plasma, CXCL-1 and IL-6 were increased 90 minutes after shock induction, with no differences between HS alone and HS combined with mechanical ventilation During HS, HIF-1α mRNA was not induced in the kidney, while in the lung HS led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation. To determine the contribution of tissue hypoxia to the observed changes due to decreased oxygen delivery to the pro-inflammatory endothelial activation during the shock period, we subsequently studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. 2 hours of 6% hypoxia did not induce expression of E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice. In this chapter we concluded that HS induced endothelial activation is not augmented nor prevented by mechanical ventilation during the shock phase, and that 2 hours of hypoxia alone does not lead to the endothelial activation observed in the HS model.

Adiponectin is an adipocyte-derived anti-inflammatory cytokine that has been shown to attenuate endothelial activation. Previous studies have demonstrated that sepsis is associated with reduced circulating levels of adiponectin. Thus, we hypothesized that sepsis-mediated adiponectin deficiency results in accentuated endothelial activation and secondary multi-organ dysfunction. In **CHAPTER 4**, we show that circulating levels of adiponectin are reduced in endotoxemia, but increased in cecal ligation puncture (CLP) sepsis models. Quantitative RT-PCR for adiponectin and its receptors revealed no

changes respectively significant reduction in gene expression in either model of sepsis, the pattern of response being model and organ specific. Adiponectin deficiency resulted in increased expression of endothelial adhesion and coagulation molecules in the lung, liver and kidney during sepsis, increased macrophage and neutrophil infiltration, and vascular leakage in the liver and kidney during experimental sepsis. This was accompanied by impaired survival following CLP, and soluble endothelial adhesion molecules sE-selectin and sICAM-1. These data suggest a protective role of adiponectin in diminishing endothelial dysfunction during sepsis.

In **CHAPTER 5** we reviewed the Angiopoietin/Tie2 system, which is an endothelial receptor system which influences endothelial integrity and vascular leakage, and engagement in inflammation and leukocyte recruitment (figure 8.1). The Ang/Tie system consists of the ligands Ang-1 and Ang-2, and the receptors Tie1 and Tie2. Ang-1 and Ang-2 bind to Tie2, and while Ang-1 induces Tie2 phosphorylation, Ang-2 binding competes for this binding and inhibits its phosphorylation. The receptor Tie2 is a 140 kD tyrosine

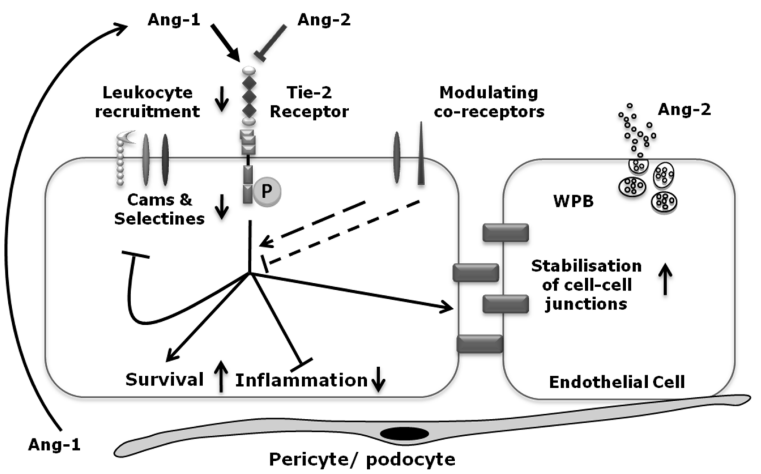


Figure 8.1. A schematic model of the Angiopoietin-Tie2(Ang-Tie2) ligand-receptor system.
Quiescent endothelial cells are interact with pericytes that constitutively produce Ang-1. As a vascular maintenance factor, Ang-1 reacts with the endothelial tyrosine kinase receptor Tie2. Ligand binding to the extracellular domain of Tie2 results in receptor autophosphorylation, docking of adaptors and coupling to intracellular signaling pathways. Signal transduction by Tie2 leads to vascular stabilization. Tie2 activation also inhibits adhesion molecules (for example, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin). Ang-2 is rapidly released from Weibel-Palade bodies (WPB) upon stimulation by various inflammatory agents. Ang-2 acts as a partial antagonist of Ang-1, and reduces Tie2 signaling, causing disassembly of cell-cell junctions. In inflammation, this process causes capillary leakage and facilitates transmigration of leukocytes.

kinase receptor, which is shed from the EC and this sTie2 may be involved in ligand scavenging. In summary, Ang-1 can be viewed as a stabilizing messenger by continuous low level Tie2 phosphorylation and Ang-2 as a destabilizing messenger preparing the endothelium for action. The changes observed in the Ang/Tie2 system in murine and human shock and sepsis might all play a role in MODS symptoms.

One of the important organs that fail during MODS is the kidney. Both hemorrhagic shock and endotoxemia induce pronounced vascular activation in the kidney which coincides with albuminuria and glomerular barrier dysfunction. In **CHAPTER 6** we hypothesized that changes in the above mentioned Angiopoietin/Tie2 system underlie this loss of glomerular barrier function. In healthy murine and human kidney, Tie2 is heterogeneously expressed in microvascular beds. In mice subjected to hemorrhagic and septic shock, Tie2 mRNA and protein were rapidly, and temporarily, lost from the renal microvasculature, and normalized within 24 hours after initiation of the shock insult. The loss of Tie2 protein could not be attributed to shedding as both in mice and healthy volunteers subjected to endotoxemia, sTie2 levels in the systemic circulation did not change. In an attempt to identify the molecular control of Tie2, we activated glomerular endothelial cell cultures and human kidney slices *in vitro* with LPS or TNF-α, but did not observe any change in Tie2 mRNA levels. In parallel to the loss of Tie2 *in vivo*, an overt influx of neutrophils in the glomerular compartment was seen which coincided with proteinuria. As neutrophil-endothelial cell interactions may play a role in endothelial adaptation to shock, and these effects cannot be easily mimicked *in vitro*, we depleted neutrophils before shock induction *in vivo*. While this neutrophil depletion abolished proteinuria, Tie2 was not rescued, implying that Tie2 may not be a major factor controlling maintenance of the glomerular filtration barrier in this sepsis model.

To bring our pre-clinical observations to the patient we next studied the time course of Ang-2 release during human experimental endotoxemia, possible association of Ang-2 levels with the levels of soluble adhesion molecules and inflammatory cytokines, and the early time course of Ang-2 release during sepsis in critically ill patients. In **CHAPTER 7** circulating Ang-1, Ang-2, soluble Tie2 receptor, the inflammatory molecules TNF-α, IL-6, IL-8 and C-reactive protein, and the soluble endothelial adhesion molecules P-selectin, E-selectin, and ICAM-1 were measured in healthy volunteers during a 24-hour period after a single intravenous injection of LPS (4 ng/kg). In addition, the course of circulating Ang-2

was analyzed in septic patients at ICU admission and after 24 and 72 hours, respectively. During human endotoxemia, circulating Ang-2 levels were elevated, reaching peak levels 4.5 hours after LPS infusion. Ang-2 exhibited a kinetic profile similar to that of the early proinflammatory cytokines TNF-α, IL-6, and IL-8. Ang-2 levels peaked prior to the soluble endothelial-specific adhesion molecules. The increase of Ang-2 correlated with the increase of TNF-α levels, and the increase of soluble E-selectin levels. In septic patients, Ang-2 increased in non-survivors only, and was significantly higher compared with levels in survivors at baseline as well as at 24 hours and 72 hours. From this research we concluded that LPS is a triggering factor for Ang-2 release in man. A persistent increase in Ang-2 during the early course identifies septic patients with unfavorable outcome.

In summary, several studies in this thesis performed in models of hemorrhagic- and septic shock show that microvascular endothelial cells respond early in shock with a proinflammatory phenotype and that these responses are organ and vascular bed specific. This proinflammatory endothelial activation is accompanied by leukocyte recruitment and organ dysfunction. The Angiopoietin/Tie2 system is in disbalance during shock, although there is still insufficient molecular knowledge of the Angiopoietin/Tie2 system in shock states to rationally support therapeutic intervention in this system in critically ill patients (*vide infra*).

8.2 Future perspectives

Ever since the description of multi-organ dysfunction syndrome (MODS), patients admitted to the ICU with MODS have a high mortality irrespective whether the underlying condition is sepsis, trauma, pancreatitis, or any other major insult[1]. Therapy has been directed at maintaining physiological parameters such as blood pressure, tissue oxygenation, glucose, and electrolytes within certain boundaries. The maintenance of physiological parameters does not treat the cause of MODS, it is merely a supportive measure.

A major setback in the treatment of MODS patients is that we do not completely understand the mechanisms underlying MODS. Immunity, inflammation, coagulation, cell death and survival, cell metabolism, vascular leakage, tissue hypoxia, and altered cell trafficking all have been connected to MODS, and all share components and represent important physiological systems, giving it the aspect of an almost intractable problem[2-5]. In infection related MODS enormous efforts have been made to unravel and to intervene in the cascade of events leading from infection via MODS to death. Therapies directed at crucial points in the inflammatory and coagulation cascades have, until now, not resulted in therapeutic options that are effective in every day clinical reality[3; 4]. Whether interventions failed because they are not effective or because the way these drugs are tested is inadequate, is not clear.

With the advent of promising targets for therapy aimed at microvascular dysfunction to diminish early EC activation and influencing the Angiopoietin/Tie2 signaling pathway, we have to keep the failures of the past in mind. In this chapter, we describe the route to the clinic and the hurdles that have to be overcome by focusing on intervening in the Ang/Tie2 system in shock states.

The reductionist approach and the way interventions in patients are tested

The reductionist approach attempts to describe and understand biological systems in terms of simple components and so far has revealed a multitude of important mediators of MODS[6]. In modern drug development, compounds to influence these mediators are easily developed, and many drugs have been tested in the last decade but failed the ultimate phase III randomized controlled trials. As an example of a mediator one could

look at TNF-α. Cachexin, later called TNF-α, has long been recognized as an early response molecule in inflammation. With monoclonal antibodies and soluble TNF-α receptors this pro-inflammatory cytokine can be neutralized. In *in vitro* studies the effects created were beneficial, and in animal models of sepsis including endotoxaemia or caecal ligation and puncture (CLP), improved animal survival was observed upon TNF-α blockade. In patients, a phase II study did not show harm, and suggested a good survival with improvement of clinical chemistry supposed to predict survival. However, the ultimate test of a phase III study on anti-TNF-α antibodies did not decrease mortality[7]. All tests prior to the randomised controlled trial (RCT) supported the fact that the drug was effective in the sense that it influenced the mediator. The reductionist approach may have overlooked important redundant systems and the complexity of the disease in a real life situation. Another possibility could be that the mediator has also beneficial functions in patients or that timing and model differences between patient and animal models can explain the failure. The functions of the mediator involved may even change during the course of the illness, for example from being vital in the initial period to detrimental later on.

The reductionist approach does not seem to be the road to take in the multi-causal, multifactorial diseases that underlie MODS[8]. Instead a holistic or integrated perspective is needed, and as such, an RCT might not be the best way to test interventions in MODS[9; 10]. MODS is a syndrome instead of a disease. Trauma, hemorrhagic shock followed by an infection and insults imposed by mechanical ventilation is a usual sequence. These different insults have specific and common responses. For instance, in rat models of endotoxaemia, hemorrhagic shock and ventilator induced lung injury, these differences were elegantly unmasked by genome wide expression analysis in the injured lungs[11]. From the more than 30,000 expressed sequences on the Affymetrix® chip, 885 genes were LPS challenge specific, 232 genes were ventilator induced lung injury specific and 301 genes were hemorrhagic shock specific, while 147 genes showed overlap between the three insults[11]. In RCTs these insults are not standardized and in human sepsis all patients will be in a different time point in their disease. As long as we are not able in sepsis to quantify and qualify the infection and the host response, as nowadays in oncology treatment, we will not be able adequately treat these insults. In oncology for instance, gene-expression signatures are used to empower the identification of prognostic subclasses in tumor types. In early stage breast cancer the risk of relapse can be predicted by tumor gene

expression arrays. These profiles which are obtained with the use of tumor- derived mRNA assayed on microarrays are being used in clinical practice to predict prognosis and hence to influence therapeutic intervention[12]. The holistic overview, taking into account interactions of different genes in damage and repair systems in MODS patients, might help to select specific subgroups of patients with this complex multifactorial syndrome, in which specific therapies are beneficial.

Shock research: heterogenic diseases and homogeneous animal models

Not only the sequence of insults differs, patients also differ tremendously. Patients have different life and medical histories, in addition to age, sex, and genetic background. To find out the heterogeneity of HS insults in patients treated in the University Medical Center Groningen (Groningen, The Netherlands) we performed an inventory of the different diagnoses for patients with HS (van Meurs, Nijsten et al, unpublished). We defined massive transfusion as a hallmark of HS and based on this all patients were selected who obtained a transfusion of 10 or more units of red blood cell concentrates (RBC) on a single day. The leukocytes transfused with the RBC are considered to mediate adverse effects, we therefore differentiated between patients that received leukocyte-depleted RBC (ld-RBC) and conventional RBC (c-RBC). All patients between 1-1-2000 and 31-12-2006 were identified using the local hospital and local blood bank databases. The underlying disease, the indication for transfusion, the hospital mortality, the 30 day mortality and the 1 year mortality were recorded. 510 patients received 10 or more RBCs on a single day of which 122 patients received c-RBCs and 388 received ld-RBCs. The patient categories are highly variable (table 8.1). Massive transfusion was performed during acute or elective surgery in most patients: 84% in the c-RBC group and 83% in the ld-RBC group. Within the surgical population 39% of the patients received a massive blood transfusion because of liver transplantation or cardiac surgery. The in-hospital mortality was high, 36% in the c-RBC and 28% in the ld-RBC group, and not significantly different. The challenge that we are facing is that it is highly unlikely that MODS in a liver transplant patient with HS has the same etiology and therefore similar therapeutic needs as an otherwise young and healthy poly trauma victim. Studies for markers of endothelial activation and the Ang/Tie-2 system in these patients will be hampered by these large patient differences, while patient groups are too small to select one patient

Table 8.1. Patient characteristics and mortality rates of patients transfused 10 or more conventional (c-) RBC and leukocyte-depleted (ld-) RBC units on a single day.

	c-RBC	**ld-RBC**
Number of patients	122 (% of c-RBC group)	388 (% of ld-RBC group)
Clinical context of transfusion		
Elective surgery	3 (3%)	14 (4%)
Abdominal surgery	23 (19%)	43 (11%)
Vascular surgery	16 (13%)	40 (10%)
Liver transplantation	17 (14%)	57 15%)
Lung transplantation	2 (2%)	30 (8%)
Cardiac surgery	29 (24%)	68 (18%)
Trauma	13 (11%)	71 (18%)
Elective medical	11 (9%)	34 (9%)
Emergency medical	8 (7%)	31(8%)
Number of RBCs (SD)	15.0 (6.9)	14.3 (5.7)
In-hospital-mortality (%)	44 (36%)	111 (28%)
30-day mortality (%)	48 (39%)	124 (31%)
1-year mortality (%)	61 (50%)	199 (51%)
Age (years, SD)	57 (18)	53 (20)
Gender (percentage male)	66 %	60 %

Clinical context of transfusion, age and sex of the patients and the in-hospital, 30 day – and 1 year mortality of the patients are shown in the c-RBC versus the ld-RBC group.

category with less variation.

To study the effects of therapeutic interventions, RCTs are performed. In an RCT researchers stratify for known predictors of outcome. In sepsis research, researchers stratify for APACHE score and exclude patients with certain co-morbidity. For all unknown outcome determining factors, randomization is performed because of the premise that this will randomly mix these unknown factors between the groups, and therapy is the only outcome modifying factor. In MODS RCTs usually 500-3,000 patients are randomized. This

number is determined by the large background variation in mortality and by the relatively small effect of the intervention of interest. In these large populations, patients with very different characteristics will be included due to our limited understanding of MODS. Both beneficial and detrimental outcomes of an intervention can be balanced in the study group without noticing. Post-hoc analyses can only be hypothesis generating and should always be confirmed by prospective testing[13; 14]. Extensive pre-study translational reasoning and communication with medical doctors and pre-clinical researchers in the field might solve part of the problem[14]. As an example of where translational reasoning could influence intervention studies in MODS, one can look at a study in which the authors demonstrated that phospho-specific whole blood flow cytometry could be used to assess activated signaling pathways in leukocytes isolated from pancreatitis patients[15]. In this study pancreatitis patients showed decreased NF-κB phosphorylation in TNF-α stimulated lymphocytes, whereas phospho-p38 MAPK was increased in TNF-α stimulated lymphocytes of pancreatitis patients compared to healthy controls[15]. These results from whole blood phospho-specific flow cytometry may allow for determination of immune status and provide a rational basis for timing and intensity of immunomodulatory therapies in MODS[16]. In this translational way researchers and clinicians might become better in stratifying patients and diminish large inter-group variability in MODS studies. The fact that ex-vivo approaches cannot determine the microvascular endothelial cell inflammation status, as these responses are highly heterogeneous, hampers the use of this technique to stratify for endothelial inflammation.

Furthermore, RCTs in MODS patients might not be capable in identifying useful therapies and may represent a very inefficient way to try this[17; 18]. In observational research, when diseases are discovered and explained, case control studies and retrospective follow-up studies might even be more valuable than RCTs[19]. Cohort studies can help to define dose and timing if we can identify clinically relevant parameters for outcome[4; 10].

Some of this patient heterogeneity can be taken into account when developing animal models for shock and MODS. Some heterogeneity is the result of co-morbidities and reduced physiological reserve. In the absence of significant co-morbidities, 'healthy' ageing still seems to be associated with increased vulnerability to insults associated with systemic inflammation, resulting in significantly worse outcomes in terms of morbidity and mortality from these insults. In our lab, for instance, Francis M. Wulfert, MD,

performed experiments in which she compared 'healthy' 18 months old mice with 3 months old young mice with regard to responsiveness to LPS administration (Wulfert et al, submitted). These experiments revealed that upon endotoxaemia the endothelium in aged mice responded by a more extensive increase in expression of P-selectin and E-selectin in comparison with young mice. Moreover, in aged mice circulating PMN count increased significantly upon LPS exposure, which was paralleled by an increase in PMN influx in the kidney. In the research group of Dr. I. H. Chaudry (Center for Surgical Research, University of Alabama, Birmingham, USA) the focus has been, amongst others, on sex specific differences in the immune response after major hemorrhage[20]. In their rodent models female mice are protected against the detrimental effects of hemorrhagic shock. In the laboratory of our collaborator Dr. Kiichiro Yano (Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA) the focus is on adiposity and western style diet in relation to endothelial activation in sepsis, as previous studies suggest that obesity is associated with inferior outcome in septic patients[21]. The hypothesis is that cross talk between adipoyctes and endothelial cells are an important determinant of endothelial behavior in sepsis. In a mouse LPS injection model, it was found that obesity increases sepsis morbidity and mortality (figure 8.2). This increase was associated with increased endothelial activation in kidney and lung (figure 8.3).

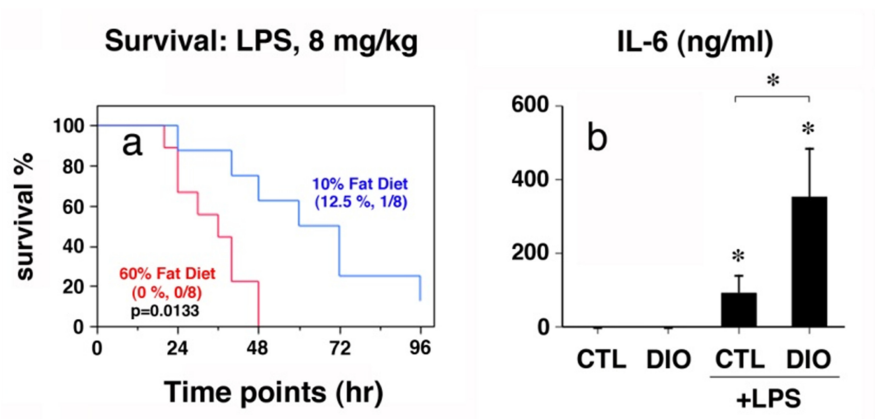


Figure 8.2. Effects of obesity on IL-6 plasma levels and mortality in endotoxemic mice.
*Control mice (CTL) (10% fatty diet fed), DIO (60% fatty diet fed) mice, were injected i.p. with 8 mg/kg LPS or with NaCl 0.9%. (A) Survival was monitored up to 96 hrs. (B) Plasma samples were obtained at 24 hrs after ip injection and IL-6 was analyzed by ELISA. All data are expressed as mean + S.D. of three independent experiments. *: p < 0.05 (van Meurs and Yano et al, unpublished).*

These observed differences between mice of different sex, age and obesity will be taken into account in future studies in hemorrhagic and septic shock. Translational approaches can be used to find subgroups of patients that benefit from certain therapies, which can be researched in animal models specifically developed for this subgroup of patients.

The road to the clinic for Ang/Tie2 based therapies

Based on data in chapter 5, 6, and 7 one can hypothesize that the Ang/Tie2 signaling system may play a crucial role in the processes that underlie the symptoms of MODS. Since therapeutic intervention in this system has successfully passed several pre-clinical tests (summarized in chapter 5) and based on our own data from animal and human shock models (chapter 6 and 7) it is timely to start drawing a road map for further preclinical and clinical development.

To prevent the development of MODS or treat it when it has occurred, one of the key issues is to bring the endothelial cells back to a quiescent, non leakage state (chapter 5). From the various pre-clinical studies we can speculate that an increase in Tie2 phosphorylation leads to diminished inflammation and vascular leakage. This increase in Tie2 phosphorylation could be achieved by several means. First, Ang-1 and its modified products could be administered systemically, also gene therapy aimed at increasing systemic Ang-1 protein levels could be a future option[22-25]. Experimental studies with

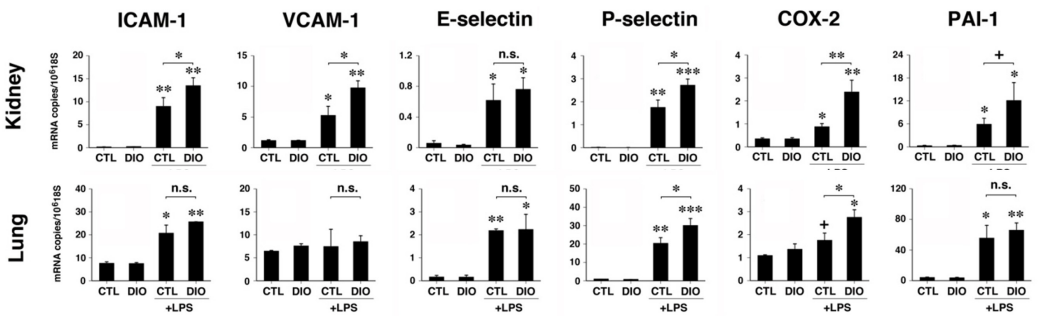


Figure 8.3. Effects of obesity on tissue mRNA levels of inflammatory and coagulation molecules in endotoxemic mice.

*Control mice (CTL) (10% fatty diet fed), DIO (60% fatty diet fed) mice, were injected i.p. with or without 8 mg/kg LPS. The results of quantitative real time PCR analyses of ICAM-1, VCAM-1, E-selectin, P-selectin, COX-2 and PAI-1 in the kidney, and lung of control treated and DIO mice. at 24 hr after LPS administration. All data was expressed as mean + S.D. of three independent experiments. *: p<0.05, **: p<0.001, ***: p<0.0001 (van Meurs and Yano et al, unpublished).*

recombinant human Ang-1 in sepsis induced MODS in mice show that rhAng-1 prevents pulmonary vascular leakage, attenuates neutrophil accumulation in lung tissue, and prevents acute kidney injury (David and Kümpers, personal communication). Experimental studies with recombinant human Ang-1 treatment of ventilator induced lung injury diminished pulmonary inflammation, VEGF, and Ang-2 expression, yet did not protect against pulmonary vascular leakage[26]. As the endothelium is heterogenic between different organs and vascular beds, the effects of systemic Ang-1 administration on non leaky vessels in sepsis remain to be determined. Furthermore, this heterogeneity makes that the effects of systemic Ang-1 administration on different microvascular diseased, leaky vessels, will probably also be not homogeneous. Not only recombinant proteins can be used, also Vasculotide, a novel Tie2 agonist, is available for animal research (Dr. Dan Dumont, Sunnybrook Research Institute (SRI), University of Toronto, Canada)[27]. Second, Ang-2 levels can be inhibited by humanized antibodies or fragments thereof that neutralize the activity of Ang-2. As Ang-2 is the more dynamic factor compared to Ang-1, also therapies aimed at removal of Ang-2 for instance with plasmapheresis could be effective[28]. Third, modulation of co-receptors could be used to activate the phosphorylation status of Tie2, as they can influence the binding of Angiopoietins to the Tie2 receptor. Cleavage of the Tie1 receptor from endothelial cells by VEGF-A makes that Ang-1 binds with increased affinity to the Tie2 receptor[29]. Furthermore, VEGF can also activate Tie2 directly, via a mechanism involving proteolytic cleavage of the associated tyrosine kinase Tie1 leading to phosphorylation of Tie2[30]. Lastly, existing therapies applied to septic patients might partly exert these effects because they influence the Ang/Tie2 system. Pre-clinical data show that activated protein C induces Tie2 mRNA and protein *in vitro*, and shifts the Ang-1/Ang-2 mRNA balance to favor endothelial integrity and diminish vascular leakage[31]. These translational experiments might give clues for better patient selection for activated protein C therapy in sepsis, as especially subpopulations with high Ang-2 levels might benefit from therapy. To examine the effect of activated protein C in patients with the highest Ang-2 levels, the plasma from septic patients collected during the PROWESS trial (activated protein C therapy in sepsis)[13], could be analyzed for Ang-2 levels and other markers of endothelial activation, and related to outcome parameters.

Which molecular data are missing from animal models to understand the Ang/Tie2 system in shock and MODS ?

There is still insufficient molecular knowledge of the Ang/Tie2 system to rationally support therapeutic intervention in this system in critically ill patients. There are several issues at a molecular level that have to be solved, and include:

1. The Tie2 and p-Tie2 distribution in the vasculature.
2. The local Ang-1/Ang-2 ratios in microvascular beds.
3. The dual agonist-antagonist function of Ang-2 *in vivo*.
4. The role and levels of soluble Tie2.
5. The effect of co-receptor stimulation on Tie2 phosphorylation status *in vivo*.
6. The effect of influencing the Ang/Tie2 system on angiogenesis and other normal physiological responses.
7. The meaning of Ang-1 levels, Ang-2 levels and sTie2 levels and their relation with p-Tie2 status in organs.

First, the receptor Tie2 itself is not equally distributed among the vascular tree. For instance, in the kidney most mRNA for Tie2 has been found in the glomerulus, while least is found in the venules (figure 6.1)[32]. Wong et al were one of the first to describe that Tie2 phosphorylation (p-Tie2), is present in all tissues using Western Blot on whole organ protein isolates[33]. Yet, until now specific antibodies for phosphorylated Tie2 are not suitable for immunohistochemistry to study whether this p-Tie2 status is evenly distributed throughout the vasculature. Moreover, the fact that Tie2 is downregulated during shock states as presented in chapter 6, makes therapy aimed at increasing Tie2 phosphorylation status even more demanding or even impossible, as the total amount of Tie2 for phosphorylation is diminished (figure 6.2)[32]. In this thesis we tried to mimic the *in vivo* situation by *ex-vivo* incubating human kidney slices (figure 6.5) with or without sepsis mediators. Unfortunately, Tie2 was lost rapidly from the vasculature *ex vivo*, also when incubated without sepsis mediators. Furthermore HUVEC lost Tie2 receptor rapidly *in vitro*, after 3 passages the mRNA levels were already 6 fold reduced (Kurniati, Molema et al, unpublished). Also primary endothelial cells harvested from mouse kidney lost their Tie2 receptor rapidly within the first days of culture (Molema, Aird et al,

unpublished). Before we start influencing Ang/Tie2 balances in the whole body, animal studies focusing on pTie2 levels in different organs have to be performed to understand this system on an organ and vascular bed specific level in health and disease[34].

Second, it is currently believed that a relative surplus of Ang-1 drives Tie2 phosphorylation, but recent experimental evidence from our own laboratory suggests that within the kidney, different Ang-1/Ang-2 mRNA ratios prevail in different microvascular segments (unpublished observations). Differences in Ang-1/Ang-2 balances cannot yet be studied on a protein level due to lack of analytical tools for quantifying mouse Ang-1 and Ang-2 protein. If the differences in mRNA reflect local protein differences, these observations imply that a resting status of the (micro)vasculature brought about by the Ang/Tie2 system is brought about by different molecular balances that depend on local conditions, and that systemic interference with the system can have spatiotemporally controlled differential effects on the microvasculature. To further understand these differences, experiments in the whole organism have to be performed, and while interfering with the Ang/Tie2 system different organ responses have to be examined. To study organ related heterogeneity, the leakage in the lungs, measured with broncho alveolar lavage, and the leakage in the kidney, measured by albuminuria, could be compared[35].

Third, recent data in HUVEC clearly show that also Ang-2 can phosphorylate Tie2 *in vitro*[36], suggesting that Ang-2 is a partial agonist/antagonist of Tie2 signaling[37], yet we have no knowledge of the occurrence of Ang-2 mediated Tie2 phosphorylation *in vivo*. We also do not know the protein levels that are present in the different microvascular segments of the circulation and thereby we can not extrapolate these *in vitro* data to the *in vivo* situation. These observations warrant new studies on the effect of local Ang-1/Ang-2 balances in organs and in microvascular beds on phosphorylation status of Tie2 where neither *ex vivo* nor *in vitro* experiments are suitable to answer these important questions.

Fourth, sTie2 functions as a scavenger receptor for Ang-1 and Ang-2 *in vitro*, and is able to decrease p-Tie2 status in HUVEC[37]. The role of sTie2 as a Ang-2 scavenger is not totally clear in sepsis. Recently the systemic levels of sTie2 were determined in sepsis and found to be 7.43 ng/ml whereas in post operative mostly cardiac surgical patients the levels were 5.03 ng/ml[38; 39]. We should be cautious about interpreting these data in a

way as sTie2 being increased in sepsis, as the levels of sTie2 could be increased in sepsis or decreased post cardiopulmonary bypass, as found recently in our own preliminary data analysis in a large cohort of post cardiosurgical patients (van Meurs, de Vries et al, unpublished).

Fifth, there are other receptors and effector molecules that control endothelial inflammation and vascular leakage. The balances and influences of, for instance, the VEGF system and the VE-cadherin system, on the Ang/Tie2 system are not completely understood.

Sixth, the Ang/Tie2 system is involved in the control of angiogenesis and vascular stabilization. These processes are important with respect to the long term effects of intervening in the Ang/Tie2 system in sepsis. What the effect of intervention will be in patients with trauma or other diseases requiring blood vessel formation or repair deserves further attention.

Seventh, we still do not completely understand what the changes in the levels of soluble markers of the Ang/Tie2 system mean in sepsis in patients. These functions should be examined in Ang-1, Ang-2, and Tie2 conditional knock-out (k.o.) and knock-in (k.i.) mouse models. Complete knockout of Ang-1 results in lethality at embryonic day 11-12.5[40], and complete k.o. of Ang-2 results in lethality 14 days after birth in mice with a 129/J background, while in mice with a C57/Bl6 background Ang-2 k.o. leads to 10% lethality[41]. Cre-Lox recombination is commonly used to generate a conditional knockout animal, in which a gene is only knocked out in a specific tissue and at a specific time, which circumvents k.o. related embryonic and early mortality. Furthermore, Cre-Lox conditional knockout mice have the advantage that redundancy pathways have less time to compensate for the functional loss of the k.o. gene than in the non conditionally k.o. animals.

Summarizing, it is still largely unknown how the Ang/Tie2 system molecularly controls microvascular function *in vivo* in the local environment within the organs, and how it changes at a molecular level in critically ill patients, and what the consequences of these changes are for local microvascular behavior. Extensive research is needed to translate preclinical models to patient care. Some of the research possibilities and caveats in patient studies, LPS challenged healthy volunteers, animals and cells are discussed in

this thesis. Before therapeutic intervention in patients is initiated, the spatiotemporal changes in expression of the Ang/Tie2 members, their effects on phosphorylation of Tie2 and downstream consequences in microvascular beds have to be known.

Future outlook

Optimize animal models, select organs and time points for intervention and optimize research in patients.

Differences and similarities between the animal models used and the patients presenting in the clinic have to be firmly established. Animal models have to be adapted to the clinical situation as good as possible. An excellent example of how this can be approached was given by studies in the early 2000s in the laboratory of Dr. John M. Harlan on anti-endothelial adhesion therapy. In animal models of different inflammatory diseases and shock, therapy with anti-CD18 antibodies that block leukocyte-endothelial cell interaction showed protective effects[42]. The clinical disappointment was extensive when in hemorrhagic shock the blockade of CD18, a part of LFA-1 complex on leukocytes that affects binding to endothelial adhesion molecules ICAM-1 and VCAM-1 (see introduction), was not protective[43]. Harlan's lab next compared the ischemia times of Ischemia/Reperfusion (I/R) animal models and those generally observed in the clinic, and found the latter to be generally longer than in the animal models. Increasing the I/R time in an animal model in which muscle injury was induced by aortic clamping demonstrated a shift from a CD18-dependent injury at short I/R times to a CD18-independent mechanism at longer I/R times[44]. This example shows that it is of the utmost importance to develop models that mimic the clinical time frame of diseases as well as that of therapeutic intervention. Furthermore, we study our interventions in young healthy male mice while intensivists are faced with older, often female, patients with multiple co-morbid diseases. These factors are known to have its effects on MODS complexity, yet only have been sparsely studied so far in mouse models[45; 46]. We might try to optimize our animal models to bring sex, age and comordidity differences into account in these models[47]. If pre-clinical models are optimized for therapeutic results rather than on mimicking the clinical situation, we will end up with state-of-the-art models that show impressive effects in treatment groups that have no predictive value for the human pathology.

In the case of the Ang/Tie2 system, it needs to be studied whether the components of the system in animal shock models act similar to those critically ill patients. When deviations are discovered, they have to be properly defined as well as their consequences for general physiology, pathology and pathophysiology. As it is - in general - not possible to sample human/patient organs at various time points, researchers and clinicians are dependent on measuring the soluble factors Ang-1, Ang-2 and sTie2, for instance in urine, sputum and blood in humans and in mice in healthy and diseased conditions, and relate this to organ specific changes observed in mice. The organ specific endothelial response pattern in E-selectin, P-selectin and VCAM-1, ICAM-1 expression seen in sepsis models cannot be unmasked by measuring soluble endothelial adhesion molecules, nor using skin biopsies[48], so the translation of soluble markers and organ function in patients to organ related differences in mice is of the utmost importance. Most medical therapies were initially developed in animal research, but only about one third of highly cited animal research from top scientific journals were successfully translated and replicated in human randomised trials[49].

Observations of changes in the Ang/Tie2 system have to be dissected at multiple levels, i.c., at the level of the organs and time frame of the pathology related cell dysfunction. It could very well be that the Ang/Tie2 molecular system is protective in one organ system, while it is detrimental in another one, or protective but at another time point during disease development. Focusing on the lung and the kidney, two major organs involved in MODS, may prove worthwhile as it is relatively easy to get close to the endothelium by analyzing urine and sputum both in mice and men. Moreover, technically it would be possible for these two organs to deliver drugs selectively for example via catheters in local feeding arteries. The initial step in selective drug delivery to the injured kidney in AKI have been made in rats[50]. An experiment of mind might be to deliver Ang-1 selectively to the kidneys early in critical illness by arterial catheterization. The effect can then be monitored by urine output, proteinuria, and other markers of acute kidney injury, besides monitoring Ang/Tie2 protein accumulation, or lack thereof, in the urine[51]. The purpose can be either protection of an organ not yet involved or repair of an organ with a dysbalanced Ang/Tie2 system and concurrent pathophysiology. This local delivery strategy could be beneficial when organs are in a different phase of dysfunction.

Furthermore, large standardized insults to patients could be used to study the Ang/

Tie2 system in patients. Insults like open heart surgery or oesophagus resection could be used to study the levels of circulating Ang-1, Ang-2, and soluble Tie2 in time and relate these levels to proteinuria, kidney failure and other mediators and markers of systemic inflammation. When we use large and well documented cohorts of patients, these analyses can be done in patients with organs at risk, for instance by using RIFLE criteria for kidney injury and match other patients in this large cohort with the same risk factors but no kidney failure.

The massive amount of data, already know for many years as well as those newly generated, probably cannot be handled with the statistics that are used nowadays. Maybe even methods from astrophysics, social studies, or other disciplines that are used to handle large multi-dimensional databases have to be borrowed[52].

Conclusion

Endothelial cell function is severely altered in a spatial-temporal pattern in shock states and in MODS. There is an early EC activation in brain, lung, heart, liver and kidney, with organ and vascular bed specific differences in HS and sepsis. Amongst others the Ang/Tie2 system changes its expression pattern. Intervening in the Ang/Tie2 system bears a promise for future therapy of MODS, but we need to create a better understanding of what happens with this system at the molecular, cellular, organ and whole patient level, before starting to test therapeutic interventions. Animal models have to be optimized, the way we test interventions in critically ill patients have to be redefined, and the effects we aim for in patients and animal models have to be defined. Only then can we hit this long and winding road where intensive and close communication between researchers and clinicians will help us examine the multiple levels of the complex interplay drawn above at the same time. Early translational approaches will be needed to prevent therapeutic failure in a late phase of research. Although therapeutic success of Ang/Tie2 modulating therapies is not the only imaginable benefit of this research, we will certainly learn and understand beyond this scope, about the devastating aetiology and the vascular consequences of MODS in critically ill patients. This in depth knowledge will certainly be valuable for the design of new treatment options for critically ill patients in the (near) future.

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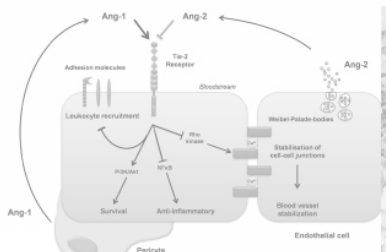
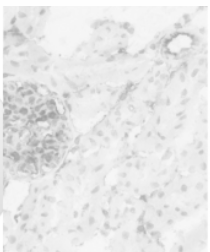
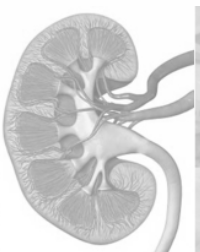
Chapter 9

Nederlandse samenvatting: De microvasculaire endotheelcel in shock




NEDERLANDSE SAMENVATTING

Op intensive care's worden patiënten behandeld die zonder inzet van intensieve zorg door verpleegkundigen en dokters en orgaanfunctie vervangende therapie, waarschijnlijk op korte termijn zouden overlijden. Therapie op de intensive care (IC) is erop gericht snel een diagnose te stellen terwijl ondertussen behandeling van herstelbare oorzaken en gevolgen van kritische ziekte wordt ingezet. Een belangrijk syndroom waar veel IC-patiënten mee te maken hebben is het tegelijkertijd falen van meerdere organen. In het Engels wordt dit multiple organ dysfunction syndrome (MODS) genoemd. Vaak wordt MODS voorafgegaan door een periode van shock, dat wil zeggen een periode waarin de circulatie van de patiënt onvoldoende in staat is zuurstof en voedingsstoffen naar de weefsels te transporteren. Deze shock gaat gepaard met een verlaagde bloeddruk, het lekken van vocht door de vaatwanden en migratie van ontstekingscellen naar organen.

Een groot probleem in de behandeling van multi orgaan falen is dat we het onderliggende ziekmakende mechanisme niet goed begrijpen. In het verleden werd aangenomen dat een afgenomen aanbod van zuurstof en voedingsstoffen aan de weefsels de belangrijkste oorzaak was, maar onderzoek heeft laten zien dat er veel meer oorzaken zijn aan te wijzen. Eén van die oorzaken is de (ongecontroleerde) reactie van bloedvatwandcellen bij kritisch zieke patiënten. Deze bloedvatwandcellen, de zogenaamde endotheelcellen, bekleden alle bloedvaten van ons lichaam en spelen een belangrijke rol in het handhaven van bloedstolling en antistolling, het reguleren van ontstekingscellen in hun reis door het lichaam, het lekken van vocht door de bloedvatwand, het regelen van de bloeddruk en nog vele andere processen. Al deze functies van endotheelcellen kunnen gestoord zijn in shock en MODS.

De interesse in dit proefschrift ging heel specifiek uit naar de endotheelcellen van de allerkleinste bloedvaatjes in shock, de capillairen en de zogenoemde postcapillaire venules omdat daar ontstekingscellen uittreden en vaatwandlekkage optreedt. Het grootste deel van het onderzoek is uitgevoerd in muizenmodellen met deels een validatie van waarnemingen in bloed van patiënten. Alle studies werden verricht vanuit de veronderstelling dat succesvolle behandeling van MODS gericht op microvasculaire endotheelcellen begint bij het begrijpen van het gedrag van deze endotheelcellen op hun *eigen* plek in het lichaam.

Endotheelcellen zijn lastig te bestuderen omdat ze in bloedvaten in organen zitten en daarmee niet erg toegankelijk zijn voor klassieke onderzoekstechnieken van artsen, zoals kijken, luisteren en voelen. Ook het laboratoriumonderzoek is gecompliceerd omdat endotheelcellen weliswaar stoffen uitscheiden die we kunnen meten in het bloed, maar dat niet allemaal op een zelfde manier op hetzelfde moment doen. Endotheelcellen op verschillende plekken in het lichaam kunnen namelijk heel verschillend reageren. Bijvoorbeeld de endotheelcel in de glomerulus (een onderdeel van de nier waar het bloed gezuiverd wordt) gedraagt zich heel anders dan die in de long waar zuurstof wordt opgenomen en CO_2 uitgeblazen. In het bloed kunnen we niet zien welke endotheelcellen zijn aangedaan omdat we alleen de respons in het totale lichaam kunnen meten. Geïsoleerde endotheelcellen buiten het lichaam zijn maar in beperkte mate te gebruiken voor het meten van een respons omdat het gedrag van een endotheelcel sterk wordt bepaald door zijn omgeving. En die gaat verloren in een kweekvaatje. Verder zijn afbeeldingtechnieken met röntgenstraling of ultrageluid te weinig gevoelig om endotheelcellen per cel in een orgaan af te beelden.

Alle voorgaande afwegingen overziend kwamen we tot de conclusie dat we genoodzaakt waren modelsystemen in proefdieren te gebruiken om endotheelcellen tijdens shock te onderzoeken. In **HOOFDSTUK 2** hebben we daartoe eerst een model opgezet voor verbloedingsshock gevolgd door behandeling met vocht, zoals patiënten op operatiekamers tijdens chirurgie of tengevolge van een ernstig ongeval ondergaan. In dit model konden we laten zien dat endotheelcellen hun gedrag veranderden onder invloed van shock, de zogenoemde endotheelcelactivatie, en dat dit ook na de toediening van vocht nog doorging. Deze reacties waren voor verschillende organen niet gelijk. Dit leidde tot de conclusie dat het patroon in het ene vaatbed niet voorspellend is voor het patroon in het andere vaatbed. In **HOOFDSTUK 3** hebben we daarna gekeken welke invloed beademen, een veel gebruikte behandeling door artsen die voor shockpatiënten zorgen, heeft op het gedrag van endotheelcellen in verbloedingsshock. Beademing wordt verricht om behandelingen en diagnostiek mogelijk te maken en om voldoende zuurstof in het bloed te handhaven en het koolzuurgas op normale waarde te houden. Dit zou gunstig zijn, maar we weten ook dat beademing zelf orgaanfunctiestoornissen kan opwekken. In dit hoofdstuk zagen we dat beademen geen invloed had op de endotheelcelactivatie en dat alleen een zuurstoftekort in de weefsels onvoldoende was

om endotheelcellen te activeren.

In **HOOFDSTUK 4** gebruikten we muizenmodellen voor bloedvergiftiging, ook wel sepsis genoemd, een andere vorm van shock die in de kliniek ook tot MODS leidt. In dit hoofdstuk waren we geïnteresseerd in de effecten van adiponectine, een in vetcellen geproduceerde stof, op het gedrag van endotheelcellen in organen tijdens de ontwikkeling van MODS. De effecten van adiponectine staan in toenemende mate in de belangstelling omdat IC's steeds vaker te maken krijgen met patiënten met vetzucht. Middels speciaal voor dit doel gefokte muizen die geen adiponectine maken (de zogenaamde adiponectine knock out muizen) konden we laten zien dat het niet hebben van adiponectine de muizen gevoeliger maakte voor een aantal bloedvatwandafwijkingen tijdens de bloedvergiftiging. Ook patienten met vetzucht hebben lagere adiponectine spiegels, dat zou kunnen bijdragen aan een slechter herstel van MODS.

Het Angiopoietine/Tie2 systeem is een moleculair endotheelcel-receptor systeem dat het gedrag van endotheelcellen beïnvloedt (**HOOFDSTUK 5**). Activatie van Tie2 door Angiopoietine-1, het eiwit dat aan deze receptor bindt, houdt endotheelcellen in een rustige, niet ontstoken, toestand. In **HOOFDSTUK 6** laten we zien dat in verbloedingsshock en bloedvergiftiging het molecuul Tie2, die dient als receptor voor Angiopoietines, omlaag gaat in de nier, een effect dat niet te onderzoeken is in geïsoleerde endotheelcellen buiten het proefdier. Het omlaag gaan van Tie2 heeft belangrijke implicaties voor therapie gericht op het toedienen van één van zijn liganden, de agonist Angiopoietine-1, dat in de literatuur worden voorgesteld voor de behandeling van MODS. In **HOOFDSTUK 7** hebben we vervolgens geprobeerd deze bevindingen terug te brengen naar de "rand van het patiëntenbed". We hebben gekeken naar het andere ligand van Tie2, namelijk Angiopoietine-2 (Ang-2), een zogenaamde competitieve antagonist voor Tie2. Van Angiopoietine-2 is bekend dat het een ontsteking bevorderende invloed heeft op endotheelcellen en het is verhoogd in patiënten met ernstige bloedvergiftiging. In een model waarbij gezonde vrijwilligers een bestanddeel van bacteriën krijgen ingespoten, bleken Ang-2 spiegels in de bloedbaan snel te stijgen, tegelijkertijd met andere vroege ontstekingonderhoudende eiwitten. Verder bleek dat bij patiënten op de IC met bloedvergiftiging Ang-2 bloedspiegels hoger waren als zij uiteindelijk aan hun aandoening overleden, terwijl patiënten die wel reageerden op de ingezette therapie en zouden overleven, een normalisering van Ang-2 bloedspiegels lieten zien. Deze studie

laat zien dat het inspuiten van een bacterieel product voldoende is om het Angiopoietine systeem te ontregelen.

Samenvattend tonen de in dit proefschrift beschreven experimenten aan dat microvasculaire endotheelcellen hun gedrag sterk veranderen onder invloed van verschillende soorten shock en MODS. Deze veranderingen zijn orgaan- en vaatbed specifiek. Ook het Angiopoietine/Tie2 systeem is gestoord in shock, maar er is nog onvoldoende kennis over de consequenties hiervan om te pleiten voor het beïnvloeden van dit systeem met als doel MODS te verminderen en patiëntuitkomsten te verbeteren. In de discussie van dit proefschrift (**HOOFDSTUK 8**) wordt een aantal voorstellen gedaan die er toe zouden kunnen leiden dat we het Angiopoietine/Tie2 systeem beter leren begrijpen. Interventie studies ter vermindering van MODS gericht op dit systeem kunnen in de toekomst hopelijk leiden tot verbeteren van de conditie van kritisch zieke patiënten.

Appendices

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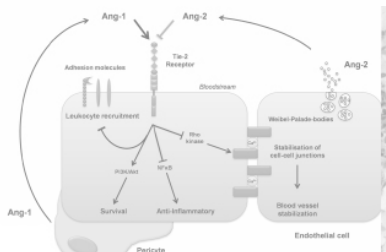

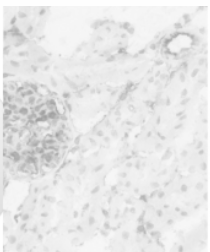
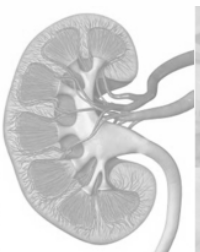


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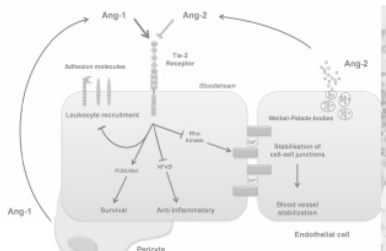
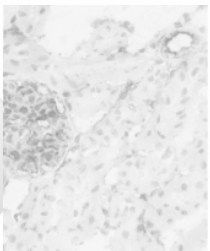
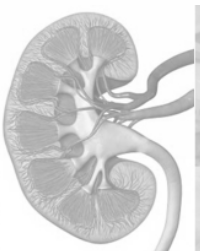
Appendices

Curriculum Vitae & List of publications




CURRICULUM VITAE

Personal

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Place of birth:	Utrecht
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Education

1986 - 1992	Atheneum Sint Maartens College, Haren (Groningen).
1992 - 1997	Doctoral exam Medical Sciences, University of Groningen (RUG), The Netherlands.
1996	Science project, 'Intubating conditions after Org 9487 in comparison to suxamethonium', Klinik für Anaesthesie und Allgemeine Intensivmedizin, Leopold-Franzens-Universitaet, Innsbruck, Austria, supervision Univ. Doz. H.J. Sparr en Prof. Dr. J.M.K.H. Wierda.

1997 -1999	Medical training (co-assistentschappen), Stichting Deventer Ziekenhuizen, Deventer, The Netherlands.
1999	MD, Medical Sciences, University Medical Center Groningen, University of Groningen (RUG), The Netherlands (*cum laude*).
2005	Start PhD project "The microvascular endothelial cell in shock" under the supervision of Prof. Dr. G. Molema.
2006	Registration as an anesthesiologist (MSRC). European Certificate in Intensive Care Medicine (ESICM).

Professional appointments

1999 – 2000	Resident in Intensive Care, Intensive Care, Isala Klinieken, Weezenlanden, Zwolle, The Netherlands.
2000 – 2006	Resident in training in Anesthesiology, University Medical Center Groningen, Groningen, The Netherlands, instructor Dr. K. Kuizenga MD. PhD.
2005 – 2006	Fellow in training in Intensive Care Medicine, University Medical Center Groningen, Groningen, The Netherlands, instructor Prof. Dr. L.P.H.J. Aarts MD PhD, Prof. Dr. J.E. Tulleken MD. PhD.
Okt 2006 –	Anesthesiologist-Intensivist, Department of Critical Care, University Medical Center Groningen, Groningen, The Netherlands.
Feb 2010- Aug 2010	Research Fellow in Medicine, Department of Emergency Medicine, Harvard Medical School, Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Boston, USA. Under the supervision of Dr. Kiichiro Yano: "Endothelial responses in sepsis, the role of fat derived hormones".

Professional associated qualifications

MD:

2004	Advanced Trauma Life Support Certificate (ATLS).
2005	Advanced Pediatric Life Support Certificate (SSHK).
2008	Teach the Teacher course I (teaching medical professionals).

Research:

2007	Working with laboratory animals (art. 9 certificate)
2007	Safe Microbiological Techniques (VMT)
2010	Collaborative Institutional Training Initiative (CITI)

1. Working with Mice in Research Settings
2. Reducing Pain and Distress in Laboratory Mice and Rats
3. Working with the IACUC

List of publications

Full papers:

1. **van Meurs M**, van der Starre P.J. Groene urine. Nederlands Tijdschrift voor Anesthesiologie 2000;(13):87-9.
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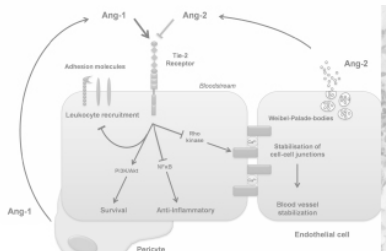
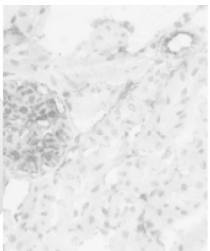
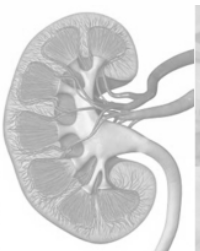
Appendices

Dankwoord




DANKWOORD

Na mijn opleiding tot anesthesioloog wilde ik in het UMCG blijven werken. Tijdens mijn sollicitatie gesprek kwam de vraag ter tafel of ik interesse had in het doen van promotie onderzoek. Het betrof een translationeel onderzoek naar vaatschade in shock onder de begeleiding van een pre klinische en een klinische hoogleraar. Ik had toen eigenlijk geen idee waar ik impulsief "ja" op zei. De resultaten van dit project zijn in dit proefschrift beschreven. Het werd een bijzonder leuk en uitdagend project. Het leukste was dat ik met zoveel verschillende mensen mocht samenwerken en van hen mocht leren. Graag wil ik een aantal van hen bij naam noemen.

De promotoren: Professor dr. G. Molema, beste Ingrid, vanzelfsprekend ben jij de eerste, en allerbelangrijkste in dit rijtje. Jij hebt voor mij de mogelijkheid gecreëerd om binnen de Medische Biologie als klinisch fellow onderzoek te doen. Het is dankzij jou dat het proefschrift z'n uiteindelijke vorm heeft gekregen. Jij hebt mij in staat gesteld om stap voor stap de basale wetenschap te leren kennen en me daarin geschoold. Je hebt de gave mensen uit te dagen iets verder te springen dan ze zelf voor mogelijk hielden, maar je hebt er ook begrip voor als ze soms wat eerder landen. Je bezieling voor wetenschappelijk onderzoek is groot en je enthousiasme is bekend tot ver buiten de EBVDT groep en de landsgrenzen.Dat maakt je tot een fantastisch wetenschappelijke docent. Wat dat betreft had mijn promotie nog best een beetje langer mogen duren. Ik hoop in de toekomst samen met jou te blijven werken aan onderwerpen die ons beide interesseren en die meer inzicht brengen in het ontstaan en de behandeling van MODS.

Professor dr. ing. J. G. Zijlstra, beste Jan, je bent het boegbeeld van de IC in Groningen. In 2006 ben je betrokken geraakt bij het pre-klinische endotheel onderzoek en na het vertrek van Leon Aarts, heb je de zorg over klinische promovendi als vanzelf van hem overgenomen. Gelukkig ben je ook medisch manager van de CHIC geworden, zodat ik niet alleen op wetenschappelijk gebied, maar ook op klinisch gebied veel van je heb kunnen leren. Ik ben blij dat het endotheel onderzoek nu op een groot aantal stapeltjes in jouw boekenkast ligt. Als ik vond dat alles nog mooier en uitgebreider moest, was jij, met je nuchtere kijk op bijna alles, mijn natuurlijk tegenwicht en daarmee heb je me geholpen om alles in perspectief te plaatsen. Ik kijk ernaar uit om samen de volgende

stappen in het translationele ICV onderzoek te zetten..

Professor dr. L. P. H. J. Aarts, beste Leon, jouw komst naar de anesthesiologie in Groningen was één van de redenen dat ik graag in het UMCG bleef werken. Jij had de visie en het geduld om te beginnen met een pre-klinische onderzoeksgroep. Verder heb je me laten zien wat de rol van een anesthesioloog-intensivist in een ziekenhuis kan (en moet) zijn. Ook na je vertrek naar Leiden kijk ik met heel veel plezier terug op onze samenwerking.

De leden van de beoordelingscommissie: Professor dr. W. C. Aird, Dear Bill, when I started this project in 2005 the first article that I read about the endothelium was your review in Blood, "The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome" (PMID 12543869). You are one of the founding fathers of endothelial biomedicine and your articles and your book 'Endothelial Biomedicine' (Aird, WC: Endothelial Biomedicine. Boston, Cambridge University Press, 2007) are the best advertisement that I ever saw for this field. I would like to thank you for judging my thesis manuscript and for the possibility to work in the CVBR. Professor dr. V. W. M. van Hinsbergh, beste professor van Hinsbergh, ik ben trots dat ik ook een Nederlandse "founding father" van de "endothelial biomedicine" mag danken voor het accuraat beoordelen van mijn proefschrift. Professor dr. M. M. R. F. Struys, beste Michel, dank voor het beoordelen van mijn proefschrift. Ook dank ik jou voor je inzet voor de shock groep. Ik hoop dat binnen de shock groep, verschillende klinische en pre-klinische afdelingen van binnen en buiten het UMCG blijven samenwerken om tot vernieuwend en verrassend onderzoek te komen. Dit onderzoek zal als basis kunnen dienen voor de zorg van de aan ons toevertrouwde ernstig zieke, vitaal bedreigde patiënten van morgen (overmorgen of volgend jaar).

Mijn paranimfen: Rianne Jongman, lieve Rianne, nadat jij de shock groep kwam versterken zijn we echt serieus aan het werk gegaan met het Ang/Tie2 onderzoek. Je staat midden in de shock groep en ondersteunt niet alleen Francis, Neng en mij, maar ook studenten en vele andere binnen en buiten de EBVDT groep. Je hebt me heel veel labvaardigheden geleerd en eigenlijk zou daarom je naam boven alle hoofdstukken kunnen staan. Ook tijdens mijn verblijf in Boston hebben onze Skype afspraken me een

schat aan waardevolle labadviezen opgeleverd, die ik daar weer dapper in de praktijk bracht. In ons translationele onderzoek gaat niets boven een goede en adequate research analist. Ook de opmaak van dit proefschrift was bij jou in goede handen. Ik hoop nog heel lang met je te mogen samenwerken.

Francis Wulfert, lieve Francis, wij begonnen bijna tegelijkertijd aan ons shock onderzoek. Jij als AGIO Anesthesiologie en ik als "AGIO" intensive care. Je optimisme, vrolijkheid en inzet tijdens de tijd dat we samen op het lab zaten zijn me altijd tot voorbeeld geweest, daarmee is dit proefschrift is voor een belangrijk deel ook jouw verdienste. Na 1 juni gaan we op naar jouw verdediging. Het lijkt me leuk om daarna weer met onze resultaten "de boer op" te gaan (Nijmegen, Keulen enz ...) Ik heb er zin in.

Al mijn pre-klinische collega's van Endothelial Biomedicine & Vascular Drug Targeting: dank! Peter Zwiers is de eerste die me ooit met een pipet heeft leren werken en hij was het die mijn jeugdig overmoedige brein heeft aangespoord meer van qRT-PCR te leren. Hopelijk ben jij in de toekomst de koning van de CLP operatie :-) Henk Moorlag de tweede die mij ooit met een pipet heeft laten werken en ondertussen HUVEC met een voor mij heel bijzondere donor in kweek nam. Sigga Ásgeirsdóttir dank ik voor het uitleggen van moeilijke moleculair biologische procedures en de hulp bij de *ex vivo* experimenten. Neng Fisheri Kurniati is de derde AIO in de shock groep en ondanks een gezin op afstand, vaak de vrolijkste. Hoogleraar Peter Heeringa is als nierexpert met een nuchtere kijk op de nier, de wetenschap en het leven heel belangrijk voor de shock groep. Jan Kamps, Piotr Kowalski en Niek Leus, dank ik voor de broodnodige mannelijke versterking van de EBVDT groep, de humor, het bier en de bitterballen tijdens de EBVDT borrels. Elise Langenkamp dank ik voor haar uitleg, precisie, vriendelijkheid en bezoek aan ons tijdens ons verblijf in Boston. Henriëtte Morselt en Martin Schipper dank voor de frequente hulp. Mirjan van Timmeren en Betty van der Veen, dank voor hulp en uitleg over de LPS studies. Gesiena van der Wal en Gopala Krishna dank voor jullie vrolijkheid en enthousiasme in de AIO kamer. Titia Vrenken en Nikola Lepse dank voor jullie nieuwe input. De "shock groep studenten": Ingeborg Noppers, Eva-Maria Kuschny, Berend Tillema, Nynke Dragt en Anne van Zoonen, dank voor jullie inzet, gezelligheid en kritische vragen. De vroegere medewerkers van de EBVDT Ageeth Knol, jij hebt als eerste analist van de shock groep aan de wieg gestaan van al de resultaten die in latere jaren uit het

onderzoek naar boven borrelden. Dank voor al je inzet en doorzettingsvermogen in de soms nog wat onduidelijke beginperiode. Ann de Haes, niet alleen labmaatje maar ook heel vriendelijke collega anesthesioloog en belangrijk voor het allereerste manuscript van de shock groep. Joanna Kuldo, Joanna Adrian, toen ik in het lab kwam, waren jullie ervaren PhD studenten , dank voor jullie hulp. Ook alle andere collega's van het U-lab en Z-lab van de Medische Biologie, bedankt .

Mijn klinische en pre-klinische collega's: Martin Houwertjes hielp mij om de drempel van het proefdieronderzoek over te stappen. Het zijn jouw "gouden" handen en inventiviteit die de mechanische ventilatie in HS studies mogelijk maakten. Inge de Graaf en Johan Bijzet, bedankt voor jullie hulp en uitleg bij verschillende studies. De oncologisch urologen I. Jan de Jong en Annemarie Leliveld-Kors droegen, zowel door patiëntenzorg als in het onderzoek (dank voor de stukjes humane nier), bij aan mijn welbevinden. De anesthesiologen Bert Ballast, Mark Wierda, Peter van der Starre (Stanford University, Department of Anesthesiology, Palo Alto, United States) en Harald Sparr (Krankenhaus Dornbirn, Anästhesie und Intensivmedizin, Dornbirn, Österreich) hebben mij op verschillende momenten in mijn opleiding enthousiast gemaakt voor het doen van onderzoek en het schrijven van stukjes.

Mijn collega intensivisten van de CHIC Marije Smit (omdat je nog nooit in een dankwoord hebt gestaan, omdat je me helpt kijken met een chirurgische blik en omdat ik blij ben dat je in het CHIC team zit), Hans Delwig (IC nestor, fotograaf van een deel van de omslag en raadsman over alles waar een stekker aanzit), Michael Rodgers (voorbeeld in arbeids ethos). Esther van Bockel en Marjon Dijkema (de langstzittende vrouw van de ICV staf!) dank ik heel hartelijk voor het goede en collegiale werkklimaat en voor het feit dat jullie mij de mogelijkheid hebben geboden naar de VS te gaan, en daarbij mijn kliniektaken hebben overgenomen. Olga Peters, Jack Ligtenberg (die ik gewoon in dit rijtje laat staan!), Farouq Ismael, Maurits Renes, Heleen Aardema, Adnan Aslan, Douwe Douma, Joep Droogh, Joost van der Maaten, John Meertens, Maarten Nijsten, Annemieke Oude Lansink, Joost Regtien, Jaap Tulleken, Hans de Vries, de fellows in opleiding tot intensivisten en de arts assistenten van de ICU, en het dagelijks bestuur van de ICV Maarten Slooff en Tanja Lips maken dat de ICV een leuke en uitdagende plek

is om te werken. Jullie prikkelende opmerkingen “of de muizen nog leefden” zetten me aan tot voortmaken.

Het secretariaat van de ICV bestaande uit Jannie Wolterman-Hovenga, Alice van Iersel en Thea Elzinga samen met Maaike Veldman hebben me op verschillende momenten uitgebreid geholpen, met name toen ik in Amerika verbleef. Hetzelfde geldt ook voor Annet Bouman-Van der Jagt. Dank!

De verpleegkundigen van de CHIC, die met hun inzet en enthousiasme, het zo leuk maken om patiëntenzorg te verrichten dat ik ook tijdens de wetenschap vaak op zaal te vinden was!. Zij vormen met z’n allen een enthousiast, motiverend en hecht team. Dick Kleijer, Tjerk Alberda, Dinka Kamper, Dinald Maatman, Guido Martens, Henk van Plateringen, Geesje Smeenge, Sander Admiraal, Peter Alting Tineke Bos, Esther Bruins, Janneke Dijkstra, Roelien Elema, Bernadette Haamberg, Martijn Hagewoud, Bernadette van Heyningen, Johanna Homan, Anja de Jong, Tineke de Jong, Klaas de Jonge, Annemiek Lechner, Anke Lindeboom-de Vries, Mark Mulder, Susanne Pennings, Marianne Plaa de, Arnold Ritsema, Willeke Scholtens,Roeli Sitepu, Bert Smidt, Hannie Stapersma, Jan Tromp, Alma Vrolijk, Linda de Wit, Jildert van Yperen, Ellen Zwiers, Hein van Assen, Gerard Bakker Rens Bijma, Rene Blok Esther Bouwhuijsen, Sjaak Conijn, Ineke Denekamp, Wim Dieperink Nelleke Dijkman, Nynke Duinstra-De Haan, Marjan Grooters, Ine Heesink, Diet van Houten, Alice de Jong, Jantje Kajuiter, Elske Keizer, Willeke van den Klippe, Rient Koolma, Rob Meurs, Tanja Perdon-Ottens, Margot de Plaa, Mineke Postma, Guido Verhoef, Arnold Verwoerd, Chantal Vos, Bastiaan Dost, Marian Hagenauw -de Vries, Charlotte van Krimpen, Peter Mast, Marielle Nanninga, Kim Peeters, José v.d. Veen, Annelies v.d. Velde-Helmholt, Martine Vos, Fiona de Vries Dank !. En verder, Tjaart van Bruggen, Kamila, Kotarska, Gea Scheeringa, Kawa K al-ali en Nicole Bos.

Eén van de leukste aspecten van promoveren is de mogelijkheid van contact met andere onderzoeksgroepen. Dr Michiel Vaneker en Prof. Dr. Gert Jan Scheffer hebben van de afdeling anesthesiologie in het UMC St. Radboud in Nijmegen mede vorm gegeven aan hoofdstuk 2, waar Francien van de Pol, en Ilona van den Brink hielpen met de praktische uitvoering. Ook met Dr Luc Teppema en Babak Mousavi Gourabi

van de afdeling Anesthesiologie, van het Leids Universitair Medisch Centrum hebben we dierproeven gedaan voor hoofdstuk 2 waar ik ze hartelijk voor bedank. Dr. Jessica Hegeman en Prof Dr. Cobi J Heijnen van het University Medical Center Utrecht hebben de rol van exogeen toegediende rhAng1 in ventilator geassocieerde longschade (VILI) uitgezocht. Ik vond dat een heel leuk en spannende samenwerking en hoop in de toekomst met jullie te kunnen blijven samenwerken. Univ Doz Dr. Med. Philipp Kümpers initiated research on Ang/Tie2 signalling in patients and this was the start of a fruitful cooperation with Hamburg and Muenster. I would like to thank Philipp for the trust that he had to start collaborations with us. This lead to the experiments described in chapter 7. I am confident that we will keep our collaboration in the future.

In 2010 I had the privilege to work 6 months in the lab of Kiichiro Yano in the Center for Vascular Biology, Beth Israel Deaconess Medical Center, Harvard Medical School in Boston. This research center is headed by Prof Dr W.C Aird and is devoted to vascular biology research. Kiichiro, I would like to thank you very much by having me as a research fellow in your lab. Under your direct supervision I was able to master several techniques. Your enormous hands-on experience and your long pre clinical research experience (from male boldness PMID: 11181640, to sepsis research PMID: 16702604 and PMID: 18852292 such a small step) made you an excellent coach and teacher. Not only professionally but also private, yours and mine family get along very well.! First, let's finish chapter 4, where after I hope to keep working on some of your ideas related adiposity and sepsis. I wish you and your family a fortunate start in your new job in Japan. Shulin Lu and Miduri Yano, you were there to coach me into all the practical aspects of the labwork at the CVBR, Shulin thanks for teaching me cell culturing, it is a pleasure to work with you. Dr. Pedro Castro Rebollo , a intensivist from Spain, worked at the lab bench next to me. We, as clinicians, had a lot of fun trying to master some of the techniques, but it worked out very nice in the end. Thank you, Maite and your children for having me in your home for a short period and also for showing me your Spanish ICU. Dr Nate Shapiro, thanks for helping me with the human adiponectin data. I also had the opportunity to meet several other people. Sacha David: thanks for the nice discussions on Ang/Tie2 signalling in sepsis and the combination of research and patient care. Nice to meet you and Claudia in person after publishing an article together

(chapter 7). The Yano Lab had a intense collaboration with the Aird Lab. Dr Bill Aird allowed me to visit his lab meetings together with the Dr. P. Oettgen and Dr. H.F. Dvorak lab. These lab meetings allowed me to absorb so much knowledge on almost everything related to vascular biology. I got all the help, being it professional or private, from Kate Spokes, and Nicolien Wieringa, additional mice from Lauren Janes, and explanation on molecular genetic engineering and tips how to fix all kinds of broken lab equipment from David Beeler.

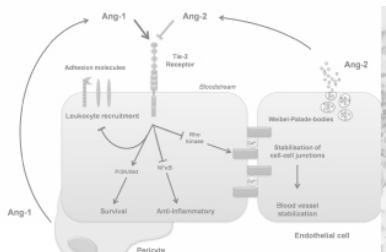
Alle goede vrienden die zich in deze zin herkennen, jullie weten hoe belangrijk jullie zijn! Het leven is oneindig veel leuker met jullie.

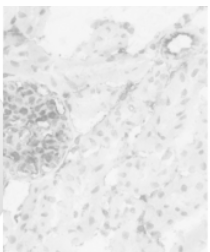
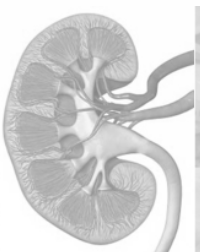
Lieve Wim en Marjan Blomsma, dankzij jullie inzet draait een jong gezin met twee werkende ouders rustig door. Ik kom uit een warm nest, waarin ook tussen de generaties goed voor elkaar gezorgd wordt. Lieve Jasper van Meurs en Tina, Jonas en Mathias, met een broer en een familie in Denemarken is het fijn dat er Skype is. Ik beleef veel plezier aan de ontmoetingen van onze beide families. Lieve John en Marianne van Meurs, dank voor de steun, het vertrouwen en de warmte om me te laten groeien in de dingen die ik leuk en belangrijk vind en de steun in periodes waarin ik dat zelf minder duidelijk voor ogen had.

Lieve Merel Blomsma, Roos en Eva . ‘Ik heb je liever dan geluk’ (H. Finkers). Jullie zijn het belangrijkste in mijn leven. Er verandert niets!

Appendices

Colour figures




COLOUR FIGURES

CHAPTER 1.

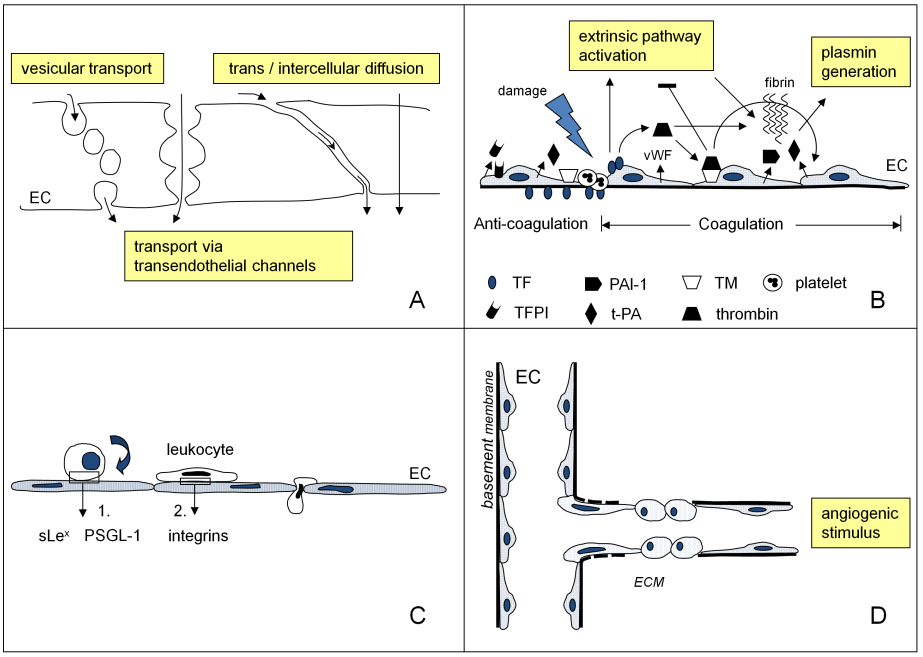


Figure 1.1. Schematic presentation of the major functions of microvascular endothelial cells (page 16). *(A) The endothelium forms a semi-permeable barrier for the transport of substances in the blood to the underlying tissue. (B) The endothelium regulates the expression of pro- and anticoagulative substances. (C) The endothelium expresses a variety of cellular adhesion molecules to tether and activate leukocytes and facilitate leukocyte adhesion and transmigration from the blood into underlying tissue. (D) The endothelium actively engages in angiogenesis in wound healing,tumour growth, as well as in a number of physiological processes. EC, endothelial cells; PAI-1, plasminogen activator inhibitor; PSGL-1: P-selectin glycoprotein ligand; sLex, sialyl Lewis x; TFPI, Tissue Factor pathway inhibitor; TM, thrombomodulin. Modified from Griffioen and Molema, with permission[28].*

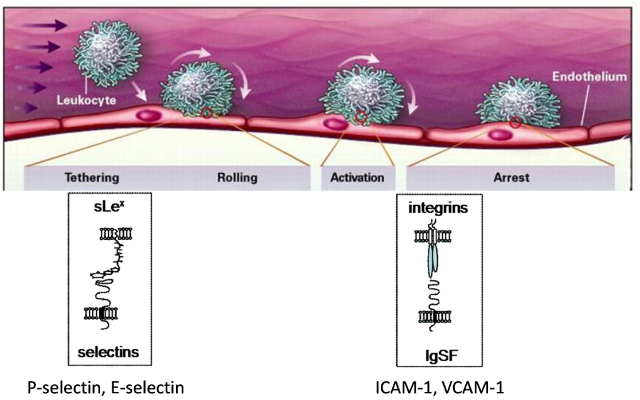


Figure 1.2. Model of endothelial leukocyte interaction in inflammation (enlargement of figure I C) adapted from von Andrian and Mackay[42] and Griffioen and Molema[28] (page 19).
Endothelial cells present adhesion molecules P-selectin and E-selectin, P-selectin binds to P-selectin glycoprotein ligand (PSLG)-1 expressed on leucocytes and E-selectin binds to sialyl-Lewis X expressed on leukocytes, leading to tethering of leukocytes by the endothelium. Thereafter, integrins on leukocytes bind firmly to adhesion molecules of the immunoglobulin superfamily (IgSF) including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on endothelial cells. Thereafter, leukocytes transmigrate towards the subendothelial tissue (not shown).

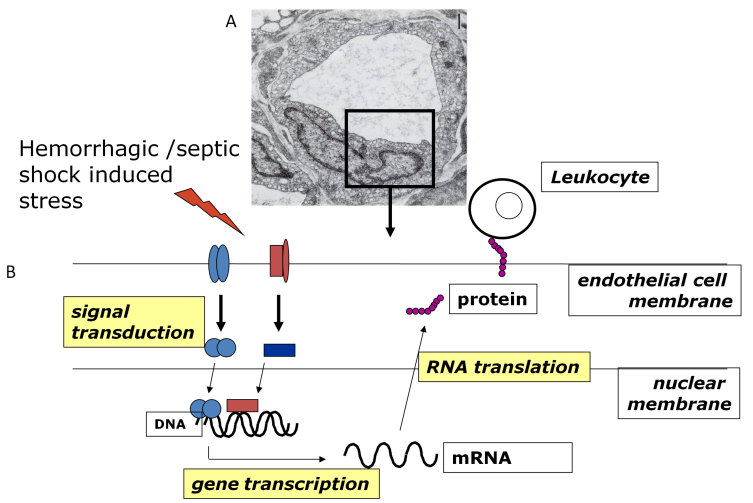


Figure 1.3. Simplified molecular view of pro-inflammatory endothelial activation in shock states (page 21).
(A) Electron microscopy image of an endothelial cell surrounding a capillary. (B) Schematic molecular drawing of the area in the black square denoted in A. Shock induced stress on endothelial cells leads to activation of gene transcription in the endothelial nucleus via (mostly unknown) signal transduction pathways. Proinflammatory genes are transcribed into mRNA, which is transported to the endothelial cytoplasm. In the cytoplasm the mRNA is translated into endothelial adhesion molecule proteins (E-selectin, P-selectin, ICAM-1 and VCAM-1, and others) and cytokines and chemokines which are expressed on the luminal side of the endothelial cell membrane respectively exocytosed. These molecules facilitate leukocyte activation and leukocyte influx into organs.

CHAPTER 2.

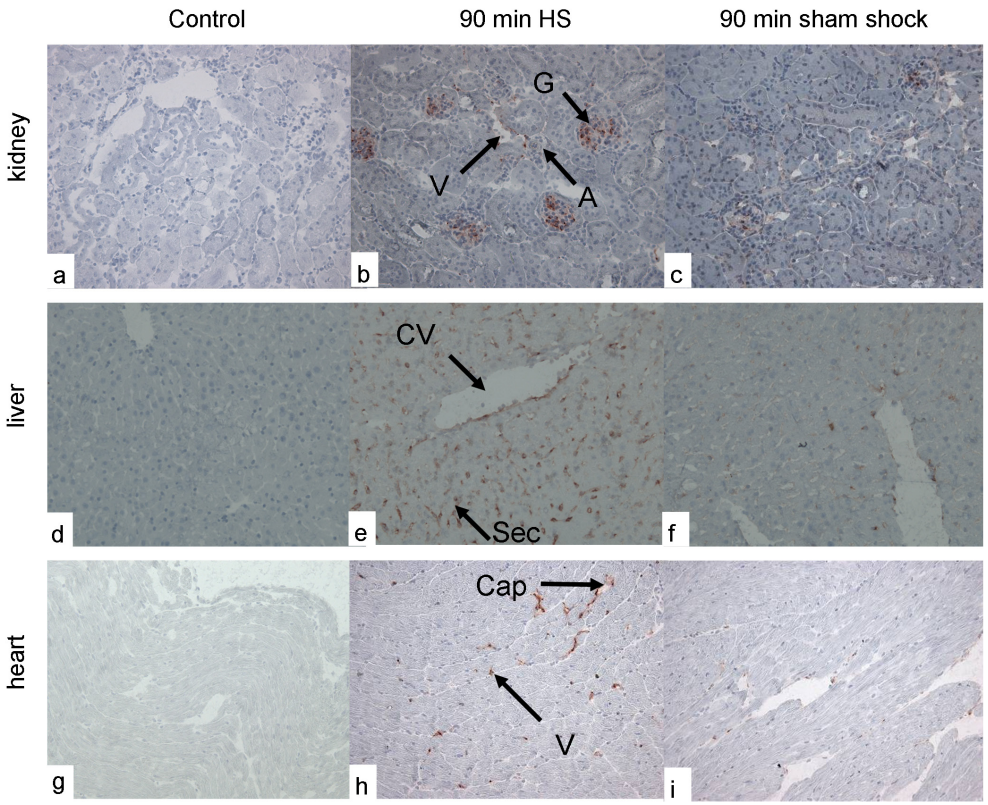


Figure 2.2. Localization of E-selectin expression in kidney, liver and heart during the early phase of HS (page 38).

Immunohistochemical detection of E-selectin in healthy mouse tissue (a,d,g), after 90 minutes of HS (b, e, h), and after 90 minutes of sham shock (c, f, i). Staining was performed respectively on kidney (a-c), liver (d-f), and heart (g-i). Original magnification 200x. E-selectin is stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.

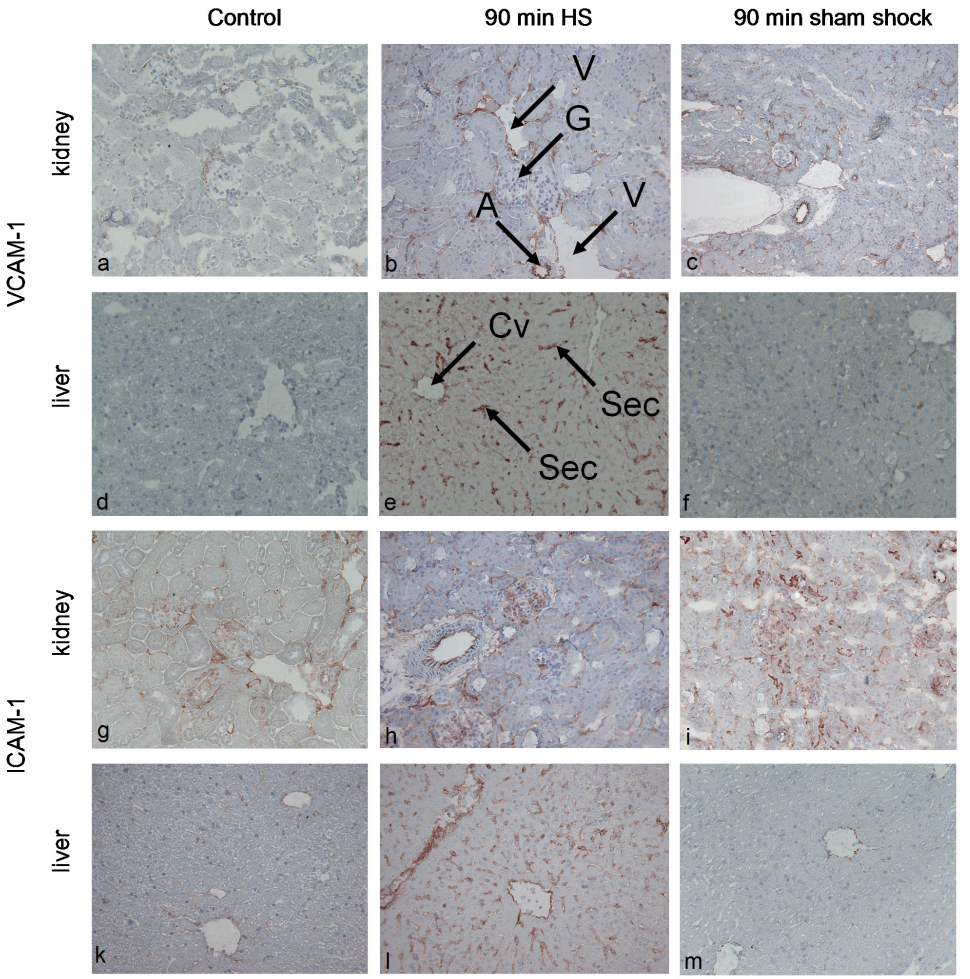


Figure 2.3. Localization of VCAM-1 and ICAM-1 expression in kidney and liver during the early phase of HS (page 39).

Immunohistochemical detection of VCAM-1 and ICAM-1 in healthy mouse tissue (a, d, g, j), after 90 minutes of HS (b, e, h, k), and after 90 minutes of sham shock (c, f, i, l). Staining was performed respectively on kidney (a-c, g-i) and liver (d-f, j-k). Original magnification 200x. VCAM-1 and ICAM-1 and CD31 are stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.

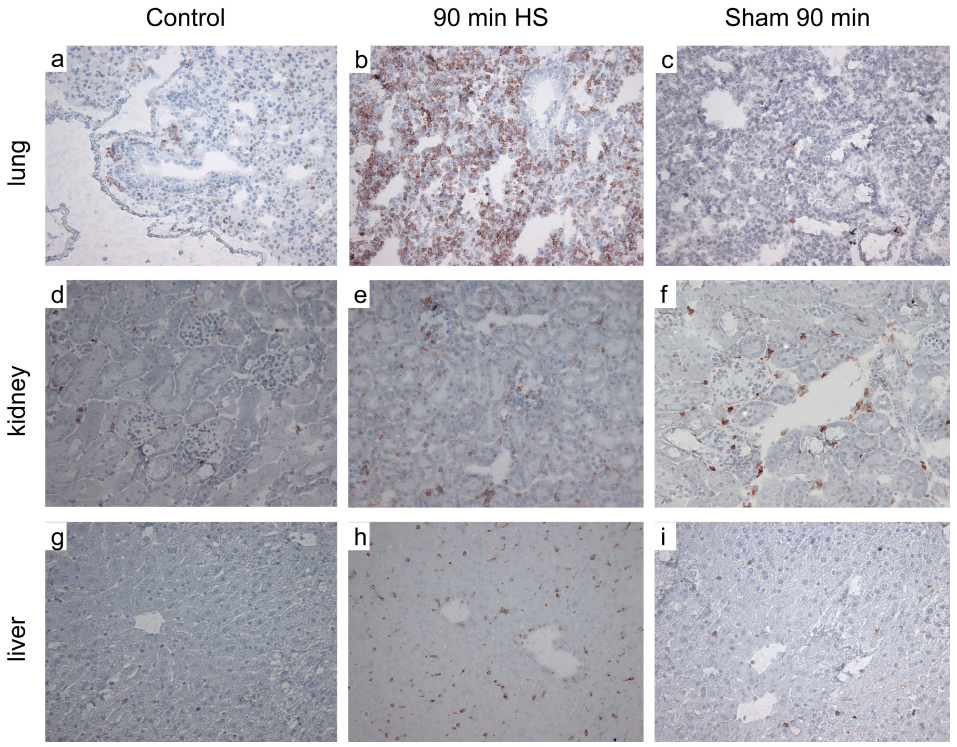


Figure 2.5. Influx of leukocytes in the different organs during HS (page 41).
Immunohistochemical staining with an anti-CD45 pan leukocyte antibody showed influx of leukocytes in lungs (a-c), kidney (d-f), and heart (g-i). Staining was performed respectively on healthy mouse tissue (control) (a, d, g) and 90 minutes HS (b, f, h), and 90 minutes sham shock (c, e, i). Original magnification 200x. Leukocytes are stained red, with increased influx of CD45 positive cells is seen at 90 minutes of shock.

Chapter 3.

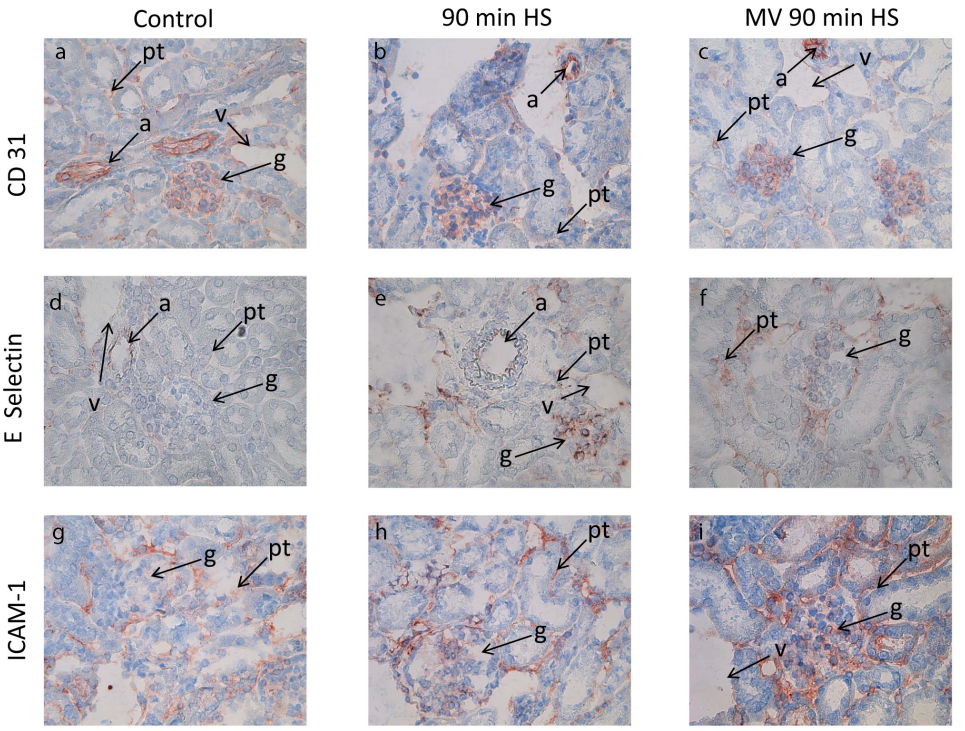


Figure 3.3. Endothelial marker gene CD31 and endothelial cell adhesion molecules E-selectin and ICAM-1 expression during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation (page 58).

Immunohistochemical detection of CD31, E-selectin, and ICAM-1 in mouse kidneys. Staining was performed in healthy mouse tissue (a, d, g), after 90 minutes of HS (b, e, h), and after 90 minutes of Mechanical Ventilation in HS (c, f, i). Original magnification 200x. CD31 (a, b, c), E-selectin (d, e, f), and ICAM-1 (g, h, i) are stained red, while cells are stained blue. Specific renal microvascular beds are indicated by arrows: a = arteriole, g = glomerulus, pt = peritubular vasculature, and v = venule.

CHAPTER 4.

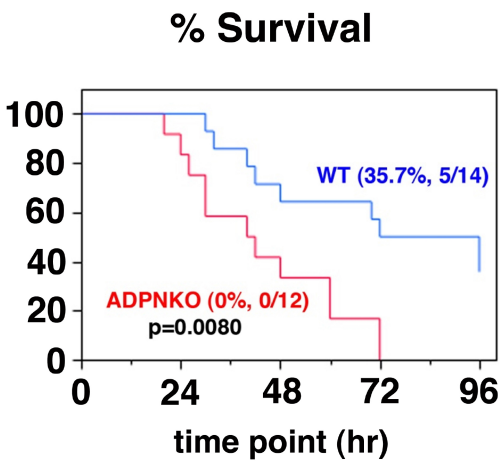


Figure 4.3. Impaired survival in adiponectin deficient mice during polymicrobial sepsis (page 76).
Survival studies on adiponectin KO (n=12) and wildtype (n=14) mice after CLP-induced polymicrobial sepsis.

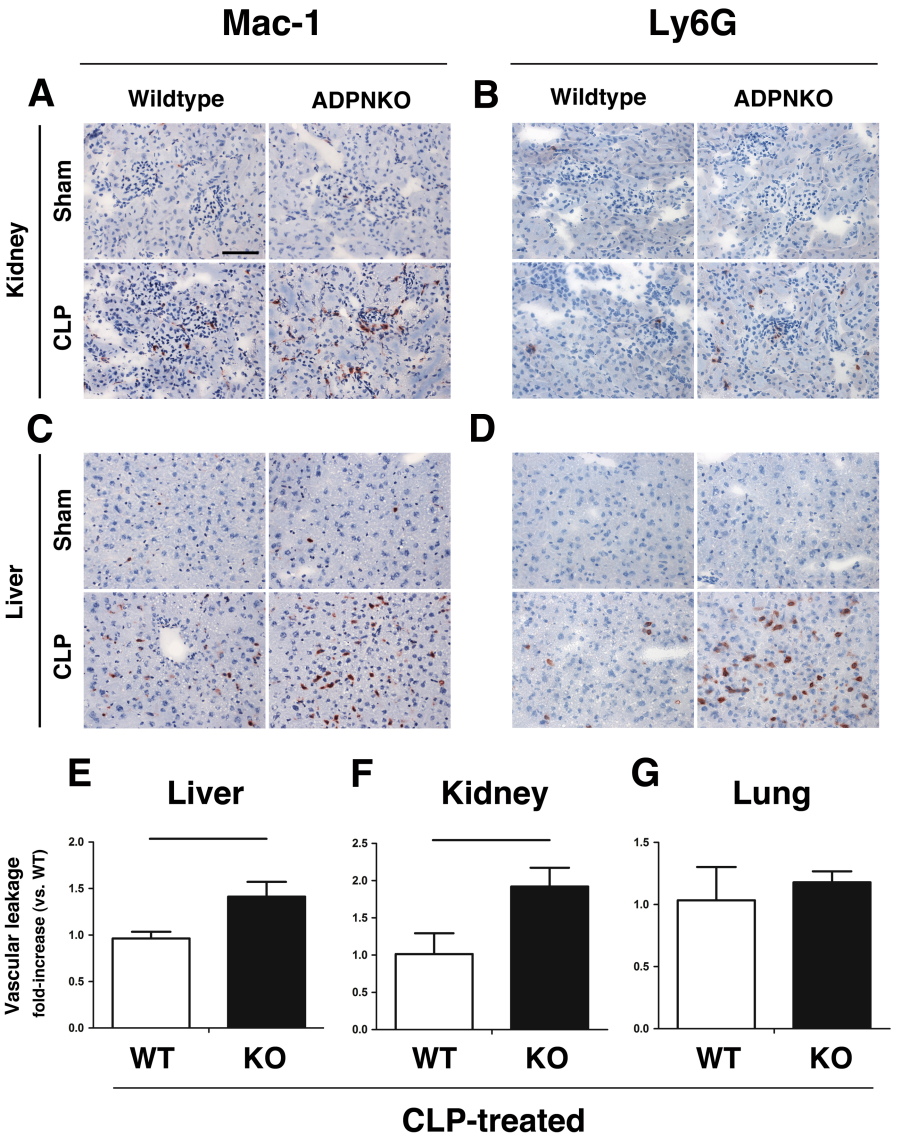


Figure 4.6. Effects of adiponectin deficiency on polymorphonuclear neutrophils and macrophage infiltration, and vascular barrier function in kidney and liver in polymicrobial sepsis (page 80).
Immunohistochemical detection of macrophage influx in mouse kidneys (A) and liver (C), of Sham mouse tissue, respectively CLP exposed mice as assessed by Mac-1 in mouse (Mac-1, red; Hematoxylin blue). Immunohistochemical detection of neutrophil influx as assessed by Ly6G in mouse kidneys (B) and liver (D), of sham-operated mouse tissue, respectively CLP exposed mice (Ly6G: red, Hematoxylin: blue). Twenty-four hours prior to i.v. injection of Evans blue dye, mice were treated with CLP. Quantitative data of Evans blue extravasation in the liver (E), kidney (F) and lung (G) is shown. Data is normalised for the OD 620nm in control organs and expressed as mean ± SEM (n=3) of two independent experiments. Scale bar in panel A applies to other panels B, C and D (scale bar= 50 µm).

CHAPTER 5.

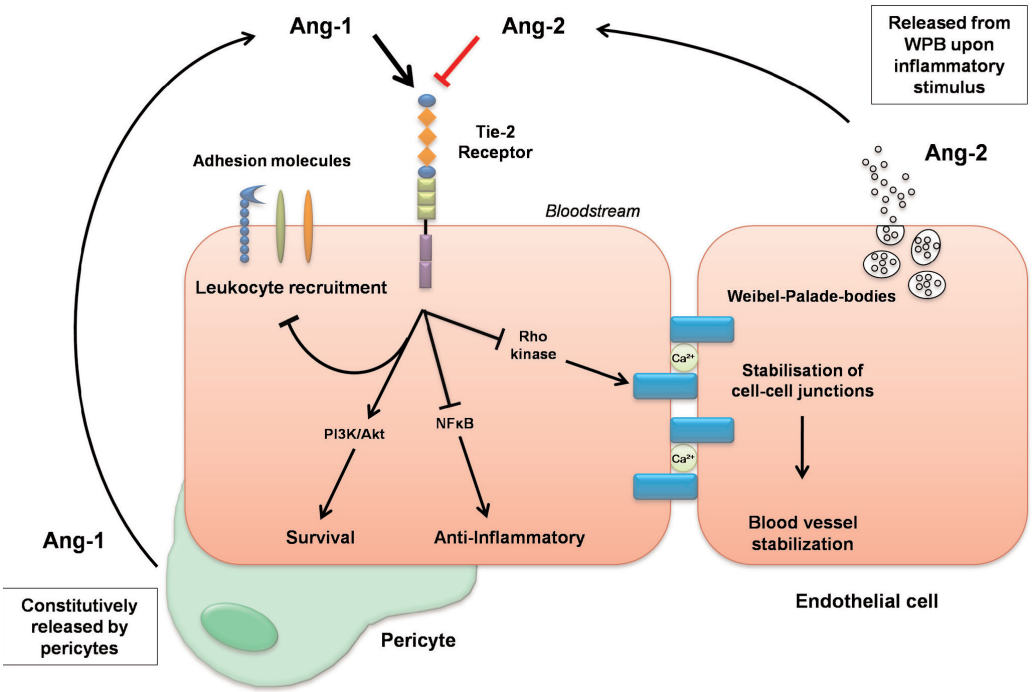


Figure 5.1. A schematic model of the angiopoietin-Tie2 ligand-receptor system (page 94).
Quiescent endothelial cells are attached to pericytes that constitutively produce Ang-1. As a vascular maintenance factor, Ang-1 reacts with the endothelial tyrosine kinase receptor Tie2. Ligand binding to the extracellular domain of Tie2 results in receptor dimerization, autophosphorylation, docking of adaptors and coupling to intracellular signalling pathways. Signal transduction by Tie2 activates the PI3K/Akt cell survival signalling pathway, thereby leading to vascular stabilization. Tie2 activation also inhibits the NF-κB-dependent expression of inflammatory genes, such as those encoding luminal adhesion molecules (for example, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin). Ang-2 is stored and rapidly released from WPBs in an autocrine and paracrine fashion upon stimulation by various inflammatory agents. Ang-2 acts as an antagonist of Ang-1, stops Tie2 signalling, and sensitizes endothelium to inflammatory mediators (for example, tumour necrosis factor-α) or facilitates vascular endothelial growth factor-induced angiogenesis. Ang-2-mediated disruption of protective Ang-1/Tie2 signalling causes disassembly of cell-cell junctions via the Rho kinase pathway. In inflammation, this process causes capillary leakage and facilitates transmigration of leucocytes. In angiogenesis, loss of cell-cell contacts is a prerequisite for endothelial cell migration and new vessel formation. Ang, angiopoietin; NF-κB, nuclear factor-κB; PI3K, phosphoinositide-3 kinase; WPB, Weibel-Palade body.

CHAPTER 6.

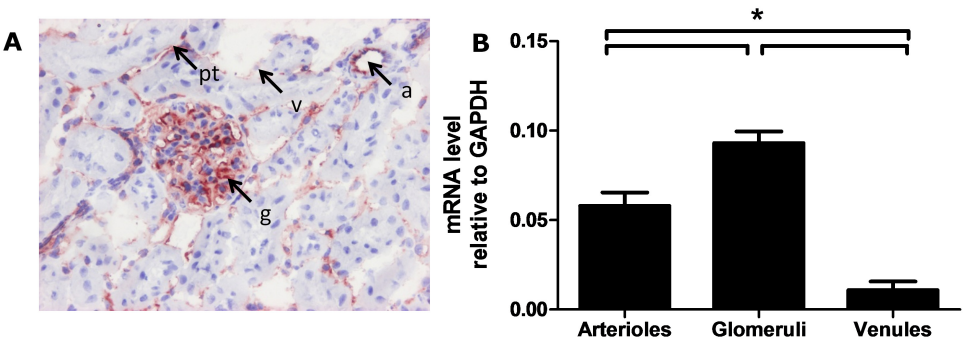


Figure 6.1. Tie2 is expressed in different microvascular beds in healthy mouse kidney (page 124).
*(A) Protein expression detected by immunohistochemical staining. Arrows point at different microvascular beds: arteriole (a), glomerulus (g), peritubular vasculature (pt), and venule (v). (B) Expression of Tie2 mRNA levels by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in three microvascular beds laser microdissected from kidney. Mean values ± SD of 3 mice per group, * p <0.05.*

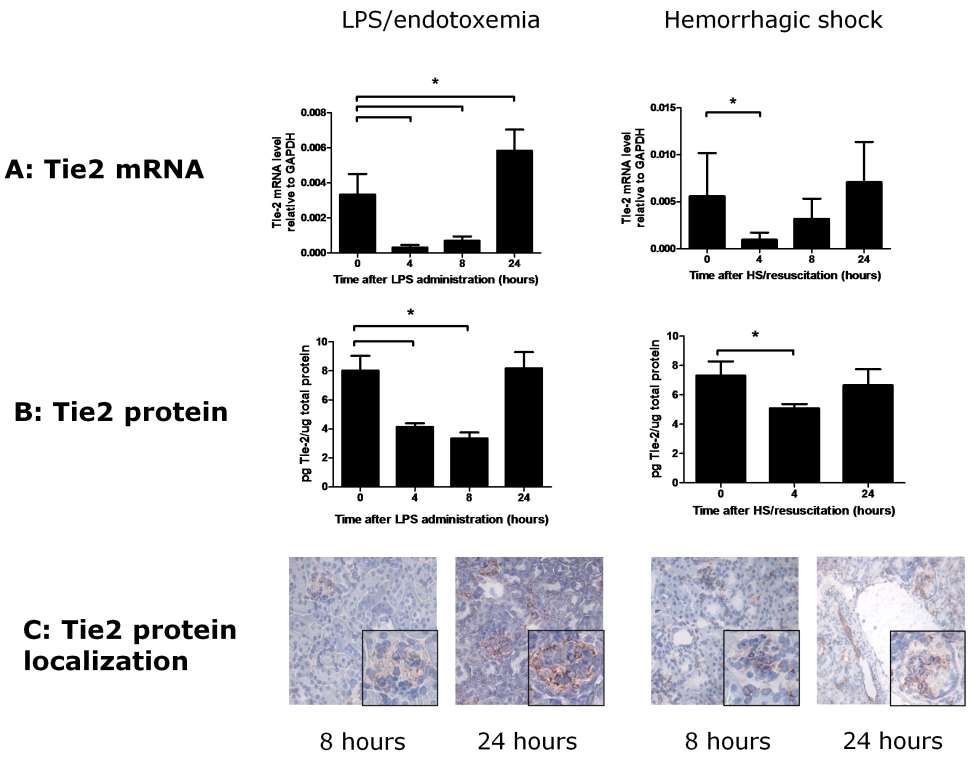


Figure 6.2. Spatiotemporal changes in renal Tie2 mRNA and protein expression in mice subjected to LPS induced shock and hemorrhagic shock followed by resuscitation (page 125).

In the endotoxemia model, LPS was administered at a dose of 0.5 mg/kg mice, while in the hemorrhagic shock model mice were subjected to blood withdrawal to a mean arterial pressure of 30 mm Hg for 90 minutes, after which they were resuscitated with Voluven® as described in Materials and Methods.

*(A) mRNA levels shown are relative to GAPDH as housekeeping gene and determined by quantitative RT-PCR as described in Materials and Methods. Mean values ± SD of at least 5 mice per group , * p<0.05.*

*(B) Protein levels were measured in kidneys homogenates by ELISA as described in Materials and Methods. Mean values ± SD of at least 8 mice per group, * p <0.05.*

*(C) Representative light microscopy pictures of the microvascular localization of Tie2 protein after LPS or hemorrhagic shock respectively and resuscitation 8 hours and 24 hours after induction of shock, assessed by immunohistochemistry. Original magnification 200x, insert 400x * p <0.05.*

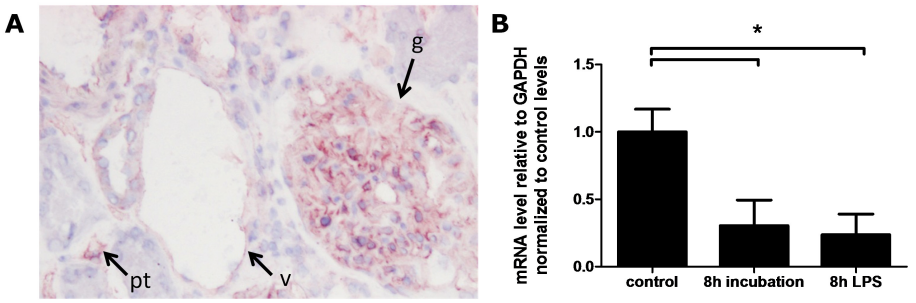


Figure 6.5. Tie2 expression in human kidney slices does not change under the influence of LPS (page 128).
(A) Immunohistochemical detection of Tie2 in human kidney. Tie2 protein expression is visible in glomeruli (g), peritubular (pt), and postcapillary venule (p) endothelial cells.
*(B) Tie2 expression in human kidney tissue in a controlled, ex vivo precision cut tissue slice incubation system. After incubation for 8 hours in medium with and without 50 µg/ml LPS, slices were harvested and processed for mRNA expression analysis. Tie2 mRNA expression decreased significantly upon incubation in medium for 8 hours, yet no additional effect of exposure to LPS on Tie2 mRNA expression levels were observed, * p <0.05.*

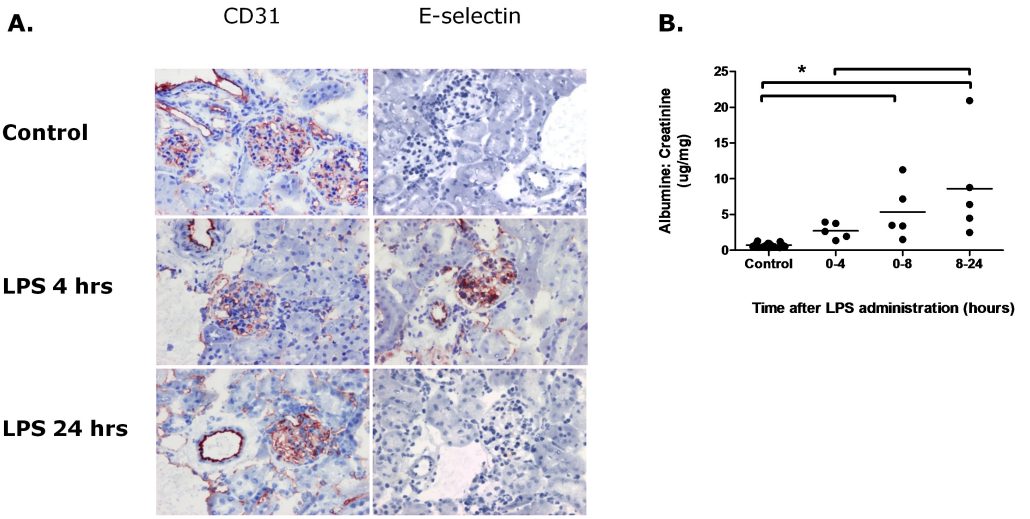


Figure 6.6. LPS administration to mice induced proinflammatory microvascular endothelial cell activation in parallel with proteinuria due to loss of glomerular barrier function (page 129).
*(A) Immunohistochemical staining of CD31 and E-selectin at two different time points after i.p. LPS administration show a minor loss of CD31 mainly from peritubular endothelial cells during the initial stage of shock, while at the same time E-selectin expression was mainly induced in arteriolar, glomerular and peritubular endothelium. Original magnification 200x. Representative sections of biopsies from 5 mice per group are shown, *p <0.05.*
(B) After LPS administration to mice (0.5 mg/kg) loss of glomerular barrier function became visible by an increase in urine albumin/creatinin ratio.

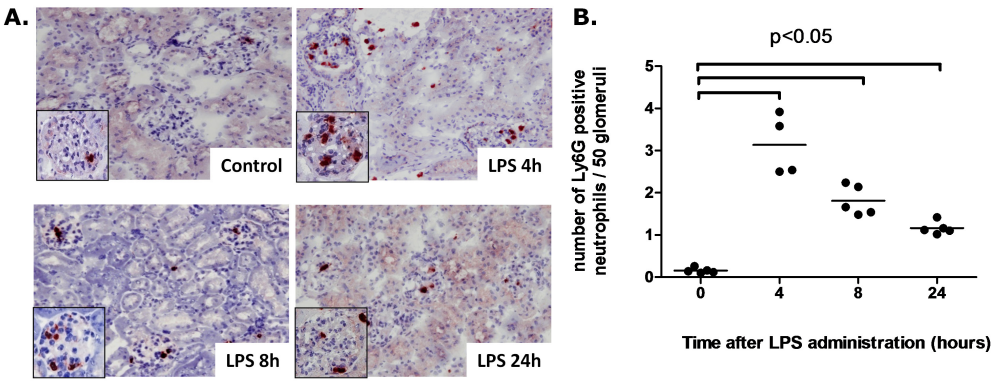


Figure 6.7. Kinetics of neutrophil influx in kidneys of mice subjected to LPS challenge (page 129).
(A) Renal infiltrating neutrophils were detected by Ly6G immunohistochemical staining in mouse kidneys at different time points after LPS administration. Original magnification 200x, inserts show glomeruli at original magnification of 400x.
(B) Quantification of the extent of neutrophil influx was assessed by counting 50 randomly chosen glomeruli per biopsy at 400x magnification p<0.05.*

CHAPTER 8.

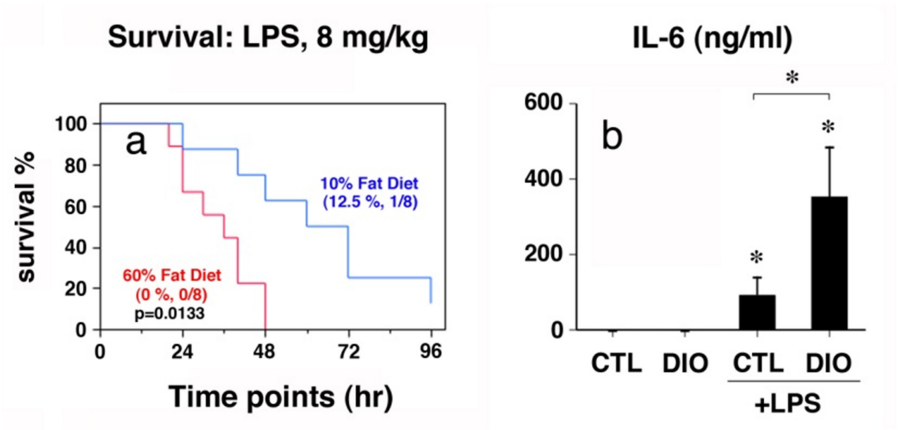


Figure 8.2. Effects of obesity on IL-6 plasma levels and mortality in endotoxemic mice (page 170). *Control mice (CTL) (10% fatty diet fed), DIO (60% fatty diet fed) mice, were injected i.p. with 8 mg/kg LPS or with NaCl 0.9%. (A) Survival was monitored up to 96 hrs. (B) Plasma samples were obtained at 24 hrs after ip injection and IL-6 was analyzed by ELISA. All data are expressed as mean + S.D. of three independent experiments. * : p<0.05 (van Meurs and Yano et al, unpublished).*